Czech Technical University in Prague

Faculty of Electrical Engineering

Department of Physics

Bachelor's thesis

Development of microbial resistance in response to non-lethal nanoparticle exposure: S. aureus study

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Bachelor's thesis supervisor: David Rutherford, Ph.D.

Study program: Medical Electronics and Bioinformatics

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BACHELOR'S THESIS ASSIGNMENT

I. Personal and study details

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Faculty / Institute: **Faculty of Electrical Engineering**

Department / Institute: **Department of Physics**

Study program: **Medical Electronics and Bioinformatics**

II. Bachelor's thesis details

Bachelor's thesis title in English:

Development of microbial resistance in response to non-lethal nanoparticle exposure: S. aureus study

Bachelor's thesis title in Czech:

Vývoj mikrobiální rezistence v reakci na neletální expozici nanočásticům: S. aureus studie

Guidelines:

This experimental-based project will involve investigating the effect on bacteria exposed to sub-lethal concentrations of nanoparticles. Bacteria will be exposed to nanoparticles in microbial growth liquid (broth) for 24 hours, during which the optical density of the incubated bacteria-nanoparticle suspension will be automatically recorded to generate bacteria growth curves, and the concentration of viable cells after exposure will be determined manually at the end of the exposure. Bacteria that remain viable after the first exposure will be re-exposed to the same dose of nanoparticles and the results obtained will be compared with the results from the original (stock) bacteria to ascertain if resistance has developed.

Bibliography / sources:

Rutherford, D., Jíra, J., Mičová, J. et al. Comparison of microbial interactions of zinc oxide nanomaterials in various size and shape. Proceedings 11th International Conference on Nanomaterials, Pages 330-335 (2020). https://doi.org/10.37904%2Fnanocon.2019.8666
Panáček, A., Kvítek, L., Smékalová, M. et al. Bacterial resistance to silver nanoparticles and how to overcome it. Nature Nanotech 13, 65–71 (2018). https://doi.org/10.1038/s41565-017-0013-y

Name and workplace of bachelor's thesis supervisor:

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Date of bachelor's thesis assignment: **08.09.2021** Deadline for bachelor thesis submission: **04.01.2022**

Assignment valid until: **19.02.2023**

David Rutherford, Ph.D. Supervisor's signature	prof. Ing. Ondřej Jiříček, CSc. Head of department's signature	prof. Mgr. Petr Páta, Ph.D. Dean's signature

III. Assignment receipt

The student acknowledges that the bachelor's thesis is an individual work. The student must produce her thesis without the assistance of others, with the exception of provided consultations. Within the bachelor's thesis, the author must state the names of consultants and include a list of references.

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Date of assignment receipt Student's signature

Author statement for undergraduate thesis

I declare that the presented work was developed independently and that I have listed all sources of information used within it in accordance with the methodical instructions for observing the ethical principles in the preparation of university theses.

Prague, date…… ……

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Acknowledgement

I am extremely grateful to David Rutherford, Ph.D., for his guidance, advice and for many discussions we had, which were very beneficial for my thesis.
I really appreciate his support and his help with my English writing.

Abstract: Antimicrobial resistance is a huge problem in modern society and medicine. Nanoparticles can have antibacterial effects so they are now tested if they could be used instead of antibiotics. Zinc oxide nanoparticles were studied to see, if bacteria, specifically *Staphylococcus aureus*, can develop microbial resistance. The bacteria stock was prepared using Mueller-Hinton broth and were cultivated on Mueller-Hinton agar plates. Viable cell concentration was measured at the start and at the end of the experiment and the bacteria growth was studied in bioreactors, which monitored the change in optical density of the solution. After seven re-exposures to the ZnO nanoparticles, there was not seen a significant difference between the ZnO sample and Reference sample. Therefore, under these controlled experimental conditions, after 7 re-exposures and with using a non-lethal ZnO nanoparticles concentration, I did not observe any evidence of developing microbial resistance.

Key words: antimicrobial resistance, zinc oxide, nanoparticles, staphylococcus aureus, bacteria, non-lethal concentration

Abstrakt: Antimikrobiální resistence je považována za velký problém medicíny i celé moderní společnosti. Nanočástice mohou mít antibakteriální efekt, a proto se nyní testují pro případné použití namísto antibiotik. Studie se týkala bakterií *Staphylococcus aureus* a toho, zdali si tyto bakterie dokážou vytvořit mikrobiální resistenci na nanočástice oxidu zinečnatého. Pro výrobu bakteriálního roztoku byl použit Mueller-Hintonův vývar a pro kultivaci byl použit Mueller-Hintonův agar. Koncentrace žijících bakterií se měřila vždy na začátku a na konci experimentu. Zároveň, se v průběhu experimentu v Bioreaktorech měřila aktuální optická hustota obou vzorků, za účelem sledování růstu bakterií a vytvoření růstové křivky. Po sedmi re-expozicích nebyl pozorován žádný statisticky zásadní rozdíl mezi referenčním vzorkem a vzorkem nanočásticemi oxidu zinečnatého. Proto, v těchto kontrolovaných podmínkách, po sedmi re-expozicích a při použití neletální koncentrace nanočástic oxidu zinečnatého, jsem nepozorovala vývoj mikrobiální rezistence.

Klíčová slova: antimikrobiální rezistence, oxid zinečnatý, nanočástice, staphylococcus aureus, bakterie, neletální koncentrace

Content

1. INTRODUCTION

World Health organisation (WHO) stated antimicrobial resistance (AMR) as one of the top ten global public health threats.[1] When microorganisms such as bacteria are developing resistance to drugs like antibiotics, it starts to be difficult to treat infections which can possibly lead to death. And so, antimicrobial resistance is becoming a global danger.[1]

This puts pressure on finding new ways of treating infections. Hope is put on nanoparticles, because studies have proved that nanoparticles can have an antimicrobial effect. [2–4] Since bacteria or viruses have developed resistance to medicines currently used to fight infection, it is important not only to study antimicrobial effect of nanoparticles, but to also study the possibility of development of microbial resistance to nanoparticles.

In this study, non-pathogenic *Staphylococcus aureus* (*S. aureus)* was used, because it is common bacteria, which lives on human's skin or on other surfaces, but pathogenic strains can cause infections and are known to be resistant to certain antibiotics (e.g. methicillin resistant *S. aureus*, M.R.S.A).

The aim of the thesis is to find out if bacteria can develop resistance to Zinc oxide nanoparticles after exposure to non-lethal concentrations and if so, how many re-exposures to nanoparticles were needed for resistance to develop.

2. BACKGROUND

This part provides information about bacteria, their structure, different shapes and types, with information about the specific bacteria that was used in the experiment. The mechanisms of antimicrobial resistance development and its dangers are discussed in this part too. Finally, a short summary of nanoparticles is provided with a focus toward zinc oxide nanoparticles which were used in the experiment.

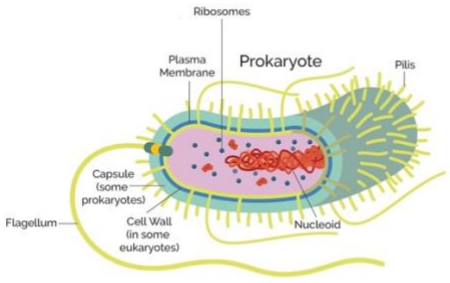
2.1 Bacteria

„Bacteria are microscopic, single-celled organisms that exist in their millions, in every environment, both inside and outside other organisms.“[5]

Bacteria are counted as ‘micro’organisms due to their size in the range of micrometres. Microorganisms include bacteria, fungi, archaea, and protists[6], and sometimes even viruses.[7] They are heterotrophic organisms, so they need organic compounds for their living, and only few can be autotrophic too. Bacteria are mostly aerobe organisms, meaning they need oxygen to survive, however some bacteria can be facultative anaerobe, for example *Staphylococcus aureus*, so they can live without oxygen for a period of time.[5,8]

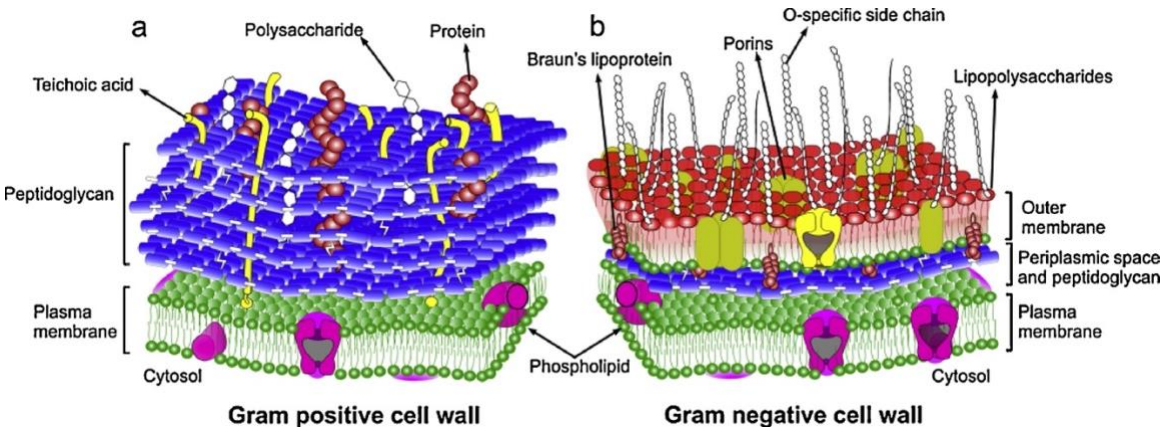
2.1.1 Structure

The bacteria cell structure is specific. „Prokaryotes have a nucleoid (nuclear body) rather than an enveloped nucleus“[9] The term prokaryote comes from Latin, where “pro” means “in favour of”, and “káry(on)” means “nut” or “kernel”.[10] Therefore, prokaryotes are organisms that do not have exactly differentiated nucleus.[11] So bacteria have nucleoid, then they have cell wall, cytoplasmic membrane, cytoplasm, flagella and some bacteria can have pili and or capsule.[12] See *Picture 1*.



Picture 1 Prokaryote cell[13]

Cell wall is tough and inflexible[8] and provides protection. Gram-negative bacteria have thinner layer of peptidoglycans, but they have outer lipoprotein layer, which cause that they can be more resistant to antibiotics. Gram-positive bacteria have thicker layer of peptidoglycans,[8,9] so they are more prone to not be killed as a result of a physical damage, for example cell wall penetration.[14,15] Differences between Gram-positive and Gram-negative bacteria cell wall can be seen in *Picture 2*.



Picture 2 Schematic diagrams of (a) Gram-positive bacteria and (b) Gram negative bacteria cell wall[16]

Water can cross the cell membrane of Gram-positive bacteria more easily than cell membrane of Gram-negative bacteria. This is caused by the missing lipoprotein layer. So, in some cases, Gram-positive bacteria can be killed by antibiotics more easily than Gram negative bacteria, because they are more prone to absorb the antibiotics.[17]

Gram positive bacteria turn blue or purple when Gram staining is used, Gram negative bacteria turn red or pink and can be more easily seen using microscope.[18]

Cytoplasmic membrane of bacteria cell is a semipermeable membrane, which is responsible for transport, energy transduction and many other functions.[8,9] Some bacteria can have a capsule, which serve as another protective shield.[12]

Cytoplasm is a solution which has thicker consistency. [8]„It is mainly composed of water, salts, and proteins.“[19] It consists of nucleoid, plasmids, ribosomes and inclusions.[8] Inclusions are granules of substances, which are distributed randomly in cytoplasm[8,9] and serve as a storage.[20] Nucleoid is made from only one ring shaped double-stranded molecule of deoxyribonucleic acid (DNA).[8]

Plasmids are responsible for pathogenic properties of bacteria. Genetic code is translated from nucleic[12] and proteosynthesis is done in ribosomes.[8]

„There are sufficient differences between bacterial ribosomes and eukaryotic ribosomes that some antibiotics will inhibit the functioning of bacterial ribosomes, but not a eukaryote's, thus killing bacteria but not the eukaryotic organisms which the bacteria are infecting.“[12]

Flagellum is a hairlike structure[12] used for movement.[5] Some bacteria can also have pili, another hairlike structure, that are used for attaching to other surfaces.[12]

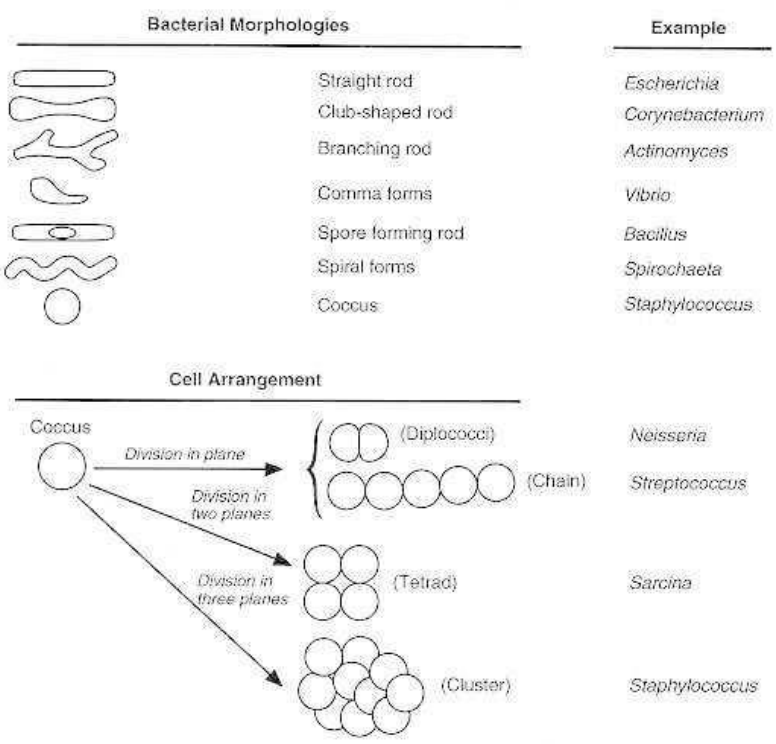
2.1.2 Bacteria types

There are different types of bacteria and there is a number of ways to classify them.

One way to classify them is by their shape. Spherical shaped bacteria, meaning round shaped, are called cocci – plural, coccus – singular. Example of coccus is *Staphylococcus aureus*.[5]

Rod-shaped bacteria are called bacilli – plural, bacillus – singular, example of bacillus is *Bacillus anthracis*[5] that causes the infection ‘anthrax’, or Clostridium species are also rod shaped.[9] Spiral shaped bacteria are known as spirilla – plural, spirillus – singular. Lyme disease or syphilis are caused by spiral shaped bacteria.[5] Comma-shaped bacteria is for example *Vibrio cholerae*.[9]

A summary of the different bacteria shapes can be seen in *Picture 3*.



Picture 3 Bacteria shapes and classification[21]

„Some rods or cocci characteristically grow in chains.“[9] So they can be classified by number of cells that are growing together.

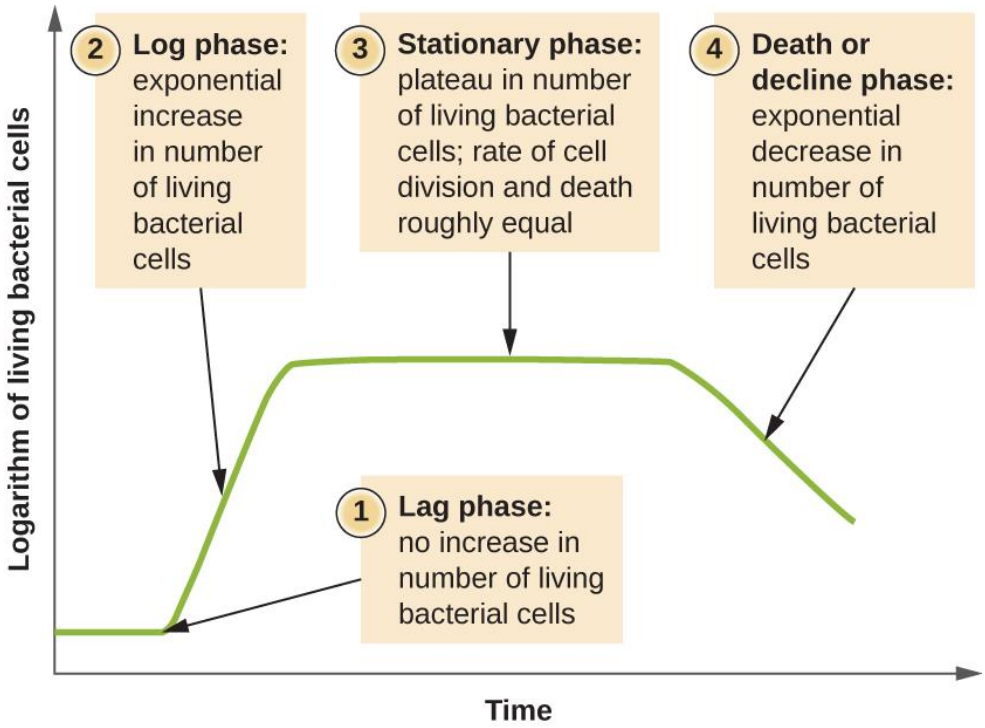
Two round shapes cells joined together are called diplococci, for example *Diplococcus pneumoniae*. If the cells are in cluster they are called staphylococci.[9]

2.1.3 Bacterial growth curve

„When bacteria are introduced into the fresh medium in a closed system, like a test tube, the population of cells always exhibits growth dynamics as follows.“[22]

Bacterial growth curve in a closed system has four phases. Lag phase, Log phase, Stationary phase and Death or Decline phase.[23]

A schematic diagram of a bacterial growth curve can be seen in *Picture 4.*



Picture 4 Bacterial growth curve[24]

First phase is a Lag phase. There is no bacterial growth, the bacteria are getting used to the new environment, the cells can grow in volume, but they are not replicating.[22] Second phase is an exponential growth phase and it is called Log phase. The bacteria have adjusted to the environment and they are replicating exponentially by binary fission.[22,23]

Stationary phase is when the number of new bacteria seems to be the same as the number of dead bacteria due to the lack of nutrients and/or space, since there is not infinite space or infinite amount of nutrients in a closed system.[22]

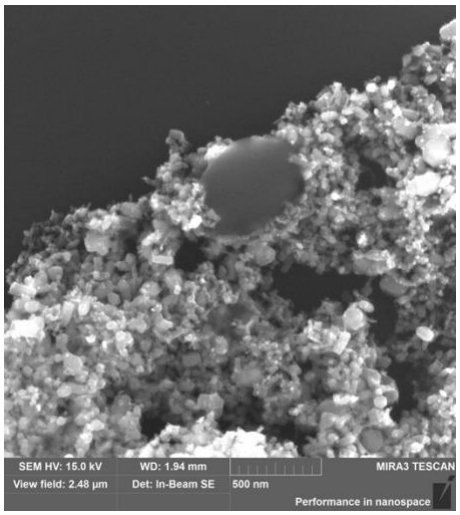
Death phase is when the bacteria have lost the ability to divide, because there are not enough nutrients or space and in the system there are more dead bacteria than live bacteria.[22,23]

Bacteria grow in a liquid called 'broth' which contains all possible nutrients needed for growth. As the number of bacteria cells increase, the optical density of the broth also increases. It is possible to monitor the bacteria growth curve in real time by measuring the change in optical density over time.

2.1.4 *Staphylococcus aureus*

Staphylococcus aureus (SA) is a Gram-positive, facultative anaerobe bacteria, which means that it can live with or without oxygen. It is an immobile coccus, round-shaped bacteria, with 1 μm diameter.[25,26]

SA belongs to the genus Staphylococcus, the family Staphylococcae.[27] One cell of SA can be seen in *Picture 5*.



Picture 5 Staphylococcus aureus[32]

Similar to other bacteria, SA has both pathogenic and non-pathogenic strains.[28,29]

Non-panthogenic SA is naturally living on people's skin[25], as well as nasopharynx and axillae.[30]

„Around 30 to 50% of humans are healthy carriers of *S. aureus* with no detrimental symptoms."[31]

It is part of natural skin flora[31], its habitat are also animals, and it can be found in soil or water.[31]

„S aureus is a major cause of hospital acquired (nosocomial) infection of surgical wounds and, with *S epidermidis*, causes infections associated with indwelling medical devices."[30]

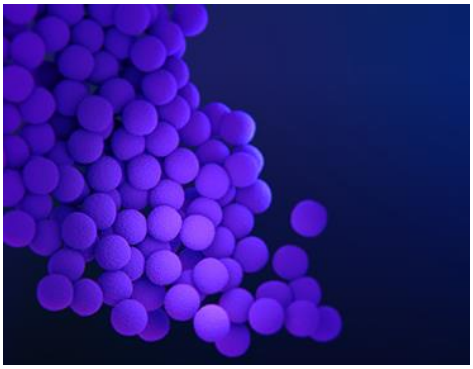
These infections are caused by pathogenic SA.

Infections contracted in hospitals are usually caused by antibiotic resistant bacteria, so they have to be cured by Vancomycin.[30] Vancomycin is a special glycopeptide antibiotic, which is used for treating infections caused by antibiotic resistant bacteria.[32]

SA can cause many diseases such as Osteomyelitis (bone infection), wound infections or ocular infections[25], it also frequently spreads in hospitals, where it can infect patients after surgeries with weak immune system and it can cause problematic healing[1,33]

„Methicillin resistance is indicative of multiple resistance. Methicillin-resistant *S.aureus* (MRSA) causes outbreaks in hospitals and can be epidemic.“[30]

Picture 6 shows an example of a cluster of MRSA cells.



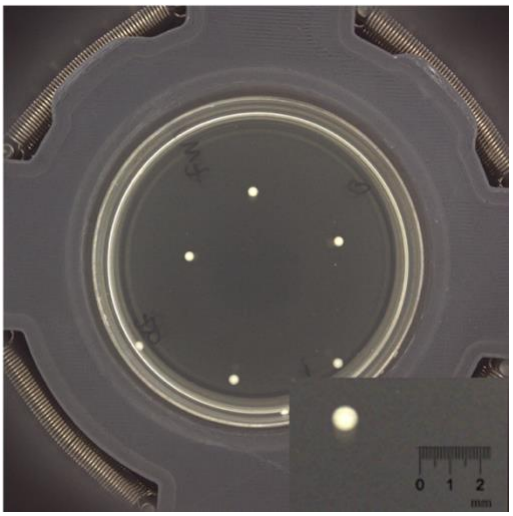
Picture 6 Methicillin-resistant *S. aureus* (MRSA)[34]

S. aureus can also cause animal diseases for example mastitis in cows.[31]

SA produce many enzymes and toxins that later apply in pathogenesis of staphylococci diseases.[25] Some examples, hyaluronidase is an enzyme that hydrolyse hyaluronic acid so it enables the spread of the infection, because it disrupt the integrity of the binder. Leukocidin disrupts semipermeable membrane of monocytes or macrophages[25].

„S. aureus is impressively fast in acquiring antibiotic resistance and multidrug resistant strains are a serious threat to human health.“[35]

Typical *Staphylococcus aureus* cultivated on agar plate has round shape and it is a little bit bulky on top (*Picture 7*). One colony has grown from one bacteria cell. Size of one colony is around 1 mm in diameter.



Picture 7 0.5 mL agar plate with typical SA colonies, dilution factor 10^{-3}

2.2 Antimicrobial resistance

Antimicrobials are medicines used to treat infections caused by microorganisms.

When bacteria, fungi, virus or parasite survive treatment by antimicrobials, changes occur in their genetic code making them less responsive to subsequent treatments of the same antimicrobial, called antimicrobial resistance.[1,36]

„Bacteria have antibiotic resistance when specific antibiotics have lost their ability to kill or stop the growth of the bacteria.“[36] The biggest problem occurs, when bacteria change over time, after the re-exposure to some medicine or treatment and start to be resistant to this medicine, although the medicine was effective previously.[36]

When drugs such as antibiotics or antivirals become ineffective, it starts to be difficult or impossible to treat infections which can lead to patient's death.[1]

„Infections by multidrug-resistant bacteria are estimated to cause 33 000 deaths in the EU every year.“[37]

Bacteria are developing resistance naturally because they are trying to adapt to the environment and become more effective in their growth. However, when antibiotics are over-used or mis-used, the chance of AMR developing increases rapidly.[37]

„Antimicrobial resistance is an ecological problem that is characterized by complex interactions involving diverse microbial populations affecting the health of humans, animals, and the environment.“[38]

Antimicrobial resistance may not only occur in humans, but it can be observed in animals too.[39] It can spread from animals to humans and on the contrary.[37]

AMR is a very complex problem, and it needs to be solved comprehensively. One sector affects the other sector. For example, over-usage of antibiotics can lead to AMR, which then spread from humans to the environment by sewage. From sewage it goes to soil, from soil it can spread to food that people or animals are eating. On the contrary, AMR from animals can transfer to people by meat or dairy products. So, when there is AMR, it is then circulating between people, plants, soil, animals and the environment and new diseases can emerge or re-emerge. Diseases that could be hard to treat due to AMR.[38,40]

The solution is in using antibiotics responsibly,[37] in approaching the problem comprehensively with respect to the environment, because every action has an aftermath[38] and in finding new ways of treating diseases caused by bacteria.

2.2.1 Mechanisms of AMR in bacteria

Firstly, difference between resistance and persistence should be discussed. Daughter cells from resistant bacteria cell will be also resistant. However, persistence is when the bacteria cells are affected by the antimicrobial, but they do not carry resistance genes.[41]

AMR can be divided in three main groups. AMR can be intrinsic, adaptive or acquired.[42]

Intrinsic resistance is based on the specific properties of the bacteria. So, the bacteria are resistant to a specific antibiotic, naturally.[43] For example, some Gram-negative bacteria are naturally resistant to Vancomycin, which is a glycopeptide antibiotic, because their outer membrane is impermeable, thus larger molecules cannot enter the cell.[42,43]

„Adaptive resistance is defined as the resistance to one or more antibiotics induced by a specific environmental signal.“[42] Adaptive resistance is not permanent, when the signal is removed, the bacteria are no longer resistant.

It seems, that Adaptive resistance is a result of epigenetic changes. „Epigenetics is the study of how your behaviors and environment can cause changes that affect the way your genes work.“[44] But, without changing the DNA.[44]

Acquired resistance is when the bacteria develop resistance by mutation or through gain of foreign genetic material.[42,45]

Resistance to antibiotics can happen by more mechanisms. The main mechanisms are antibiotic destruction or modification, reduced antibiotic accumulation, target alterations and active drug efflux.[41,42]

„β-Lactamases are the best example of antibiotic resistance mediated by the destruction of the antibiotic molecule. These enzymes destroy the amide bond of the β-lactam ring essentially rendering the antimicrobial ineffective.“[42]

Antibiotic modification is the most common AMR mechanism of aminoglycoside resistance. Another mechanism is a target replacement, when Penicillin-Binding Proteins are replaced. This is the main mechanism of methicillin resistance in methicillin resistant *Staphylococcus aureus*.[42]

Drug efflux is another AMR mechanism. The initial function of efflux pumps is to clear the bacterial cell from toxic substances.[41]

Other mechanisms are: Target site protection, Target overproduction or Decreased permeability of the bacterial outer membrane. Bacteria can change their porins size, that are the main entry for the antimicrobials, and thus change the permeability of the membrane. [42]

Speaking in particular, bacteria can develop resistance to elevated concentrations of Zn^{2+} ions by changing their DNA.

In 1998 Xiong and Jayaswal[46] published an article about identifying a chromosomal determinant that is responsible for resistance to Zn^{2+} and Co^{2+} ions.[47]

Most of the mechanisms are based on scientific research with lethal drug concentrations.

Lethal drug concentration is a concentration of for example antibiotics that is able to cause the death of the bacteria cells.[48]

However, AMR may develop with non-lethal concentrations too. Non-lethal drug concentrations are more likely to cause high-frequency, but low-cost genetic alterations in bacterial cells.[48]

Bacteria cannot only develop resistance to antibiotics such as usual medication that is used, Panáček et al.[49] proved that bacteria can develop resistance to silver nanoparticles (Ag NPs).[49]

Escherichia coli bacteria cells were re-exposed to subinhibitory concentrations of Ag NPs and gradually developed resistance to the antimicrobial effect of the Ag NPs by producing protein called flagellin after 20 successive culture steps in microwell plates. This protein caused gathering of the silver nanoparticles and so it minimized the antimicrobial effect.[49]

Therefore, it is also important to study development of AMR against other types of nanoparticles too.

2.3 Nanoparticles

Due to increase of antimicrobial resistance, nanoparticles (NP) are tested as a new way to treat infections so that they could be used in the future instead of or in addition to antimicrobials.

It was verified that in some cases nanoparticles can have antibacterial effect.[2,4,20]

"Nanoparticles of different materials and of different sizes vary in their effectiveness"[2]

With decreasing size of nanoparticles, antibacterial effect increases.[51] Antibacterial effect is also greater with higher nanoparticle concentration.[4]

2.3.1 Types of nanoparticles

„Nanoparticles can be classified into different types according to the size, morphology, physical and chemical properties."[52]

There are carbon-based NPs, ceramics NPs, semiconductor NPs, metal NPs and lipid-based NPs.[53]

Fullerenes and carbon nanotubes are classified as carbon-based. Ceramics NPs are non-metallic solid NPs, that are used in catalysis or photocatalysis.[53]

Polymeric nanoparticles are usually organic and are used in diagnostics and sensor technology.[53,54] Lipid-based NPs are effective in biomedical utilization.[53]

„Metal NPs are purely made of the metals precursors."[53] They have uncommon optoelectrical properties. Some examples are Ag NPs, Au NPs, Cu NPs.[53] Silver nanoparticles are having promising antimicrobial properties.[49] Gold nanoparticles are used for coating.[52]

„Semiconductor materials possess properties between metals and nonmetals…"[53] These NPs have found their usage in electronic devices and other applications.[52,53] ZnO NPs, ZnS NPs or CdSe NPs are classified as semiconductor nanoparticles.[52]

2.3.2 ZnO nanoparticles

Zinc oxide nanoparticles were proved by several studies as bio-safe,[3,55] meaning that they are harmless to human body cells.[56] However, they were also proved to have antibacterial effect (AE).[2,4] ZnO nanoparticles at a concentration of 100 μg/mL inhibited the growth of *Staphylococcus aureus* by 55% compared to the reference samples, and the decrease was by 62% at a concentration of 250 μg/mL.[2]

„Zinc oxide nanoparticles are more active against gram-positive bacteria relative to other NPs of the same group of elements.“[56]

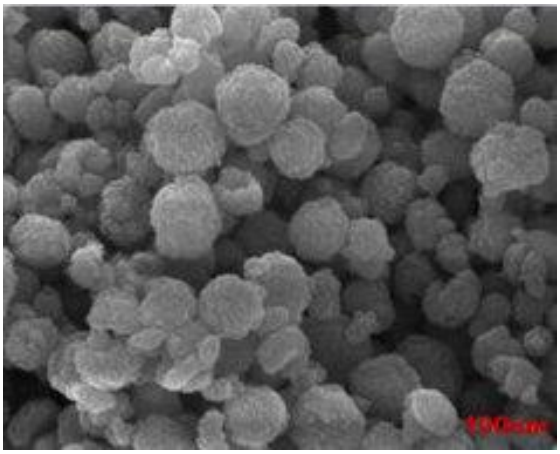
The principle of AE of ZnO NPs is that the nanoparticles first destroy the bacterial cell wall, then disrupt and concentrate in cell membrane. The antibacterial effect depends on the NP size, concentration, morphology and exposure time.[56]

It was found that the antibacterial effect of ZnO nanoparticles can also depend on if the solution is mixed or not. When the solution was mixed in a bioreactor, the nanoparticles did inhibit the growth of the bacteria, because they were able to react with the bacteria.[57] However, when the bacteria were put to agar plates with ZnO NPs solution the antibacterial effect was not observed. The AE also depends on the reaction capability of ZnO NPs.[57]

It is not needed to use that advanced instruments to prepare ZnO nanoparticles[4], therefore ZnO NPs are frequently used in studies.

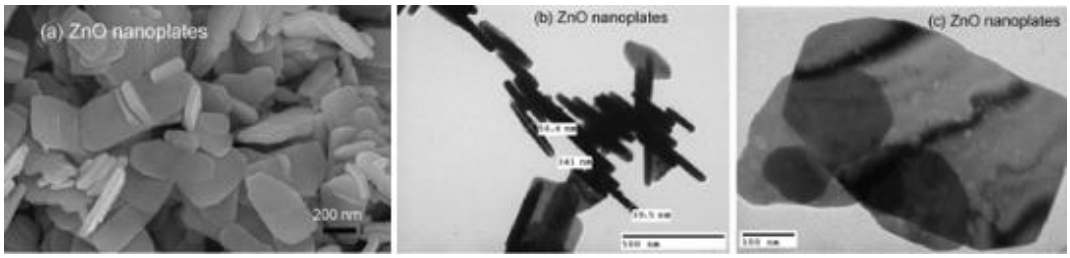
ZnO nanoparticles can have different shapes, depending on how they were synthesised. There are hedgehog-like nanoparticles, nanorods, nanoplates, nanospheres, nanotubes, nanorings, hexagonal-shaped nanoparticles, nanocages or nanoflowers.[56]

In this experiment round-shaped nanoparticles were used, because they are more commercially available. Round-shaped ZnO NPs can be seen in *Picture 8.*

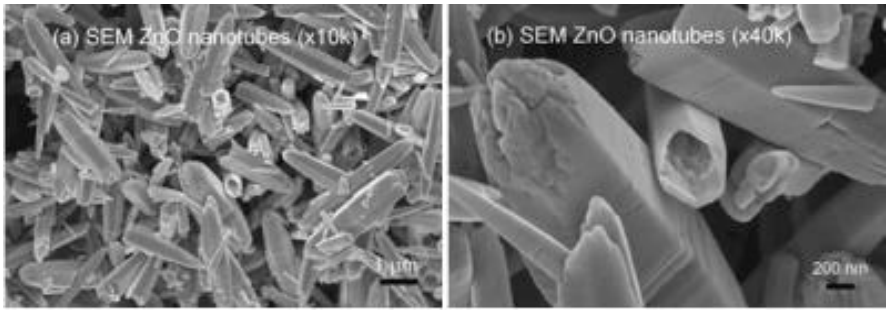


Picture 8 Round-shaped ZnO NPs[58]

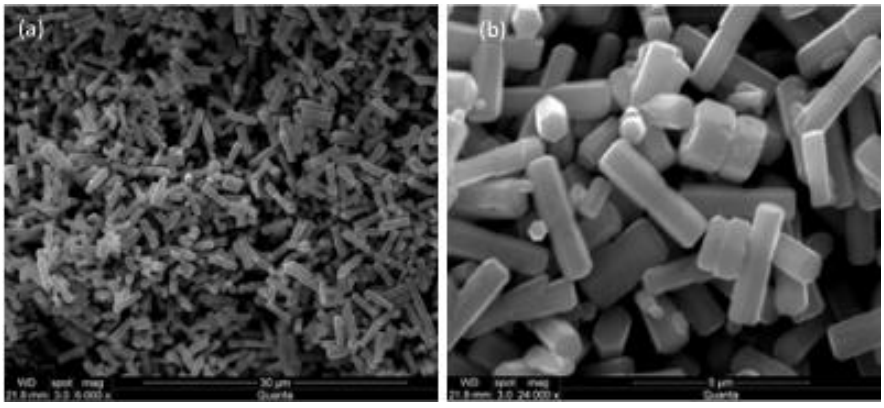
Some examples of the different morphologies of ZnO NPs can be seen in *Pictures 9-12.*



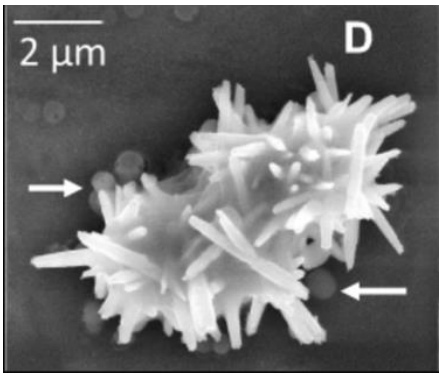
Picture 9 ZnO nanoplates[59]



Picture 10 ZnO nanotubes[60]



Picture 11 ZnO microrods[61]



Picture 12 ZnO Hedgehog-like nanoparticle[62]

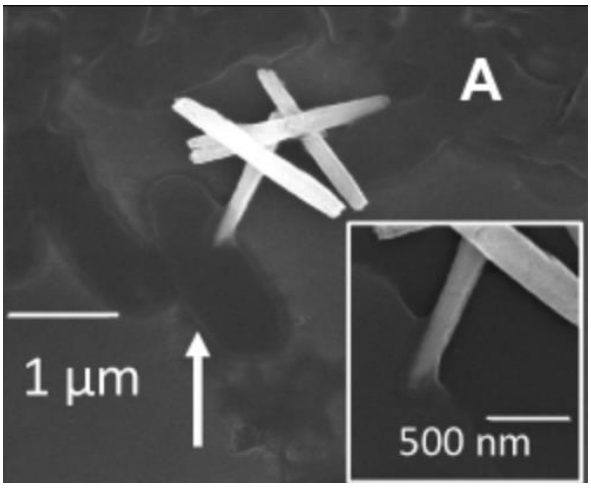
2.3.3 ZnO NPs antibacterial mechanisms

Antibacterial mechanism of the ZnO nanoparticles depends on their size, morphology, concentration and other characteristics.[3]

„In this regard, the shape of ZnO nanostructures can influence their mechanism of internalization such as rods and wires penetrating into cell walls of bacteria more easily than spherical ZnO-NPs“[3]

The morphology of hedgehog-like nanoparticles enable them to pierce the bacteria cell wall and therefore the bacteria will probably die.[62]

In *Picture 13* is captured how hedgehog-like ZnO NP was able to pierce an *E. coli* bacteria cell.



Picture 13 Escherichia coli pierced by hedgehog-like ZnO nanoparticle[62]

There are more ways how ZnO NPs can kill the bacteria. The mechanisms are loss of the cellular integrity after contact between the bacteria cell wall and ZnO NP, production of Reactive Oxygen Species (ROS), ZnO NPs internalisation and the release of Zn^{2+} ions.[15]

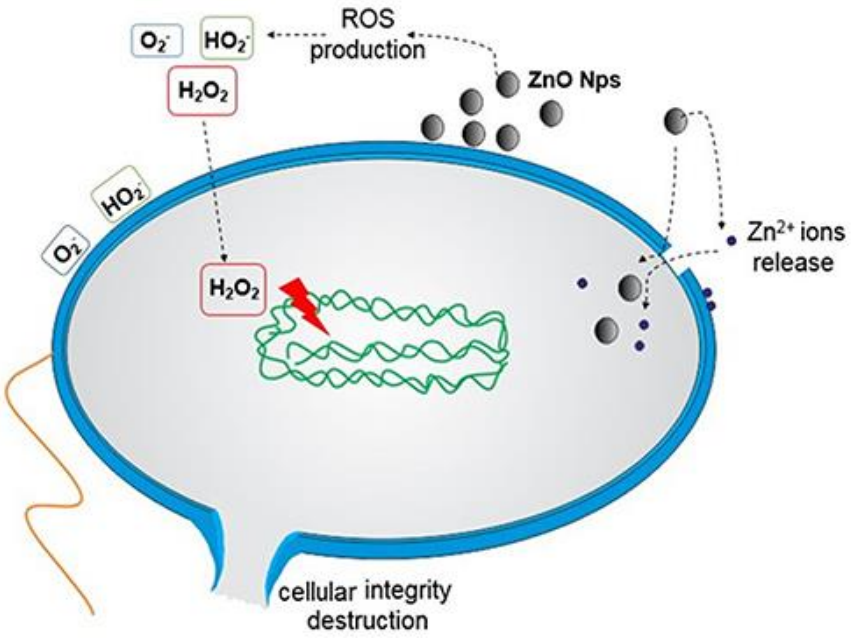
ZnO NPs produce wide range of ions and of ROS when interacting with bacteria.[63]

„ZnO-NPs with defects can be activated by UV and visible light, creating electron hole pairs resulting in the splitting of suspended H_2O molecules into OH^- and H^+. The dissolved molecules eventually react to form H_2O_2, a ROS that is long-lasting and able to penetrate the cell membrane and kill bacteria.“[64] OH^- and H^+ are also ROS that can cause damage to bacteria cells, but are short-lived.[15]

D'agua et al.[65] observed, that Gram-positive bacteria were more sensitive to H_2O_2 than Gram-negative bacteria.[65]

ROS can also cause damage inside the bacteria cells. ROS can damage structural proteins, organelles, DNA or enzymes and therefore, again, kill the bacteria.[63]

Different antibacterial mechanisms of ZnO NPs can be seen in *Picture 14*.



Picture 14 Different mechanisms how ZnO NPs can destroy bacteria cell[15]

Release of Zn^{2+} ions happen when ZnO NPs are in solution and, therefore partial dissolution results in increase of Zn^{2+} ions. These ions have antimicrobial effect by decreasing amino acid metabolism of the bacteria. The ZnO NPs dissolution can happen only under some conditions. For example, ZnO NPs dissolution is possible in bacteria cell's lysosomes, and therefore it leads to the inhibition of bacterial growth. Zn^{2+} ions have lower antibacterial effect than ROS.[15]

Bacteria cells have developed special transport proteins, which they use for import and export of Zn^{2+}, so they can control the intra-cellular Zn^{2+} ion concentration.[47]

As mentioned previously, the ZnO NPs can kill bacteria aby damaging its cell wall, which then results in the loss of membrane integrity.[15] Lallo da Silva et al.[66] observed holes in *S. aureus* cell membrane after exposing it to the ZnO NPs.[66]

Nanoparticle size plays another important role of the antibacterial effect. „It was found that ZnO-NPs antibacterial activity toward *S. aureus* and *E. coli* increases with decreasing the size.“[3]

The smaller size NPs are accumulating in the bacteria cell, until they reach the cytoplasmatic zone and therefore are able to kill the bacteria.[3] Smaller size NPs have larger interfacial area and therefore are more able to pass through the bacteria cell wall.[3]

It was further proved that the AE depends on the concentration. With higher concentration of ZnO NPs the antibacterial effect is also higher. But for low concentration there is not that significant decrease in number of bacteria cells that are alive after the exposure to the ZnO NPs solution. So lower concentrations of ZnO NPs do not have antibacterial effect.[62]

3. EXPERIMENT

In this section, the bacteria preparation and the zinc oxide preparation will be described in detail.

The following section will also provide information about the bioreactor experiment.

3.1 Preparation of Bacteria cultures

For this experiment Gram positive *Staphylococcus aureus* (CCM 3953) bacteria were used. Mueller-Hinton (MH) broth (*CM0405, Oxoid*), prepared in ratio 2.1g of broth to 100 mL of warm purified water, and Mueller-Hinton agar (*X926.1, Carl Roth*), prepared in ratio 3.8g of agar to 100 mL of warm purified water, were used for bacteria cultivation. MH broth and MH agar were sterilized at 121 °C for 15 minutes in an autoclave (*Classic Model, Prestige Medical*) before using.

For the first experiment, bacteria stock was removed from -20 °C freezer and allowed to thaw. Then, dilution series was done with eight Eppendorf tubes. First, 900 µL of 0.9% NaCl was put to all Eppendorf tubes and then 100 µL was taken from the original bacteria stock and put to the first Eppendorf tube and mixed (1:10 dilution). Then 100 µL was taken from the first Eppendorf tube and was put to the second Eppendorf tube. This was done for all eight Eppendorf tubes.

So, the last Eppendorf tube had a maximal dilution factor 10^{-8}. When the dilution series was done, 500 µL from each Eppendorf tube was pipetted from the last three Eppendorf tubes and was added to three labelled MH agar plates. The plates are usually labelled with the bacteria abbreviation, in this experiment SA, then the dilution factor, so for the three MH agar plates it was 10^{-8}, 10^{-7} and 10^{-6}. On the following day, only one bacteria colony was taken from the agar plate (it was a plate with a dilution factor 10^{-8}, because there were only few colonies, so it was possible to take only one colony) using a sterile loop and added to 100 mL of MH broth in 250 mL bottle. For bacteria cultivation in broth, bigger bottle is needed, so there is enough air and space for the bacteria growth.

The bottle was then put in orbital shaker *(PSU-10i, BioSan)* where the speed was 150 rpm, meaning revolutions per minute, and incubated at 37ºC overnight.

All bacteria in the culture should be as similar as possible which was why the cultivation was done from one colony only.

The next day, the bacteria cultivated in broth were pipetted to sterile test tube and then diluted using MH broth, to get McFarland's density (MF) of 1.0 using a densitometer (*Den 1-B, BioSan*), which is approximately 1×10^9 cfu/mL (*colony forming units per millilitre*). For the experiment, the stock of MF 1.0 was diluted to 1:1000 using MH broth.

For the first dilution and second 1:10 dilutions, 0.5 mL of MF 1.0 bacteria stock was added to 4.5 mL of MH broth. For the third 1:10 dilution (i.e. 1:1000), 36 mL of MH broth and 4 mL of the 1:100 diluted stock was used and put to the third test tube. For the experiment in the bioreactor 18 mL of 1:1000 bacteria stock was needed for both, reference (R) sample and ZnO (Z) sample.

In the following weeks of the experiment, the bacteria stock was prepared differently than in the first week.

Bacteria from the previous week that survived exposure to ZnO were used. Right after the end of the bioreactor experiment, dilution series with 0.9% NaCl (*Penta*) was done for Z and R samples, and then 0.5 mL of diluted bacteria stock was put to MH agar plate and cultivated in 37 °C overnight.

The cultivated bacteria from previous week, meaning the bacteria that survived the exposure from the previous experiment, were removed from MH agar plates with a sterile loop and added to 10 mL of MH broth and diluted using MH broth to MF density of 1.0 for both Z and R. It usually took five to ten colonies, based on their size, to get the MF density of 1.0.

For Z and R samples, the same dilution series was done separately for each sample Z and R, and the stock of 1.0 MF density was diluted to 1:1000 using MH broth.

This time, for the third dilution, 1:1000, only 18 mL of MH broth was put to two 50 mL Falcon tubes and 2 mL of 1:100 diluted stock was pipetted to the Falcon tube for each sample, Z and R.

Because, for the experiment only 18 mL in each Falcon tube were needed, 2 mL of the 1:1000 stock was removed from each Falcon tube, so there was space for 2 mL of purified water (R sample) or 2 mL of ZnO NP solution (Z sample).

Every week the cultivated bacteria from previous week were used, so the bacteria were re-exposed to ZnO, but without increase of ZnO in the stock.

3.2 ZnO preparation

There are more types of zinc oxide nanoparticles, although, for this experiment spherical zinc oxide nanoparticles were used (Sigma Aldrich). In order to study the resistance, only low concentrations, non-lethal concentrations, were used. It was needed to study the bacterial growth curve and with high concentrations of the ZnO NPs solution the curve would be only flat (i.e. no increase of optical density), therefore it would not be possible to study the difference between R growth curve and Z growth curve.

Non-lethal concentration was also used, because the bacteria cells that survived the exposure to ZnO NPs were used the next week and therefore they could have passed on the resistant genes, if they would have any.

Firstly, 10 mL of purified water were mixed with 20 mg of zinc oxide nanoparticles and then sterilized, which resulted in concentration of 2 mg per 1 mL (2 mg/mL) ZnO solution. After the sterilization was done, the ZnO solution was put to ultrasonic bath (*Bandelin Sonorex digitec, Maneko, with fixed settings: 35 kHz, 160 W*) for 30 minutes. After sonication, the ZnO solution was diluted. For the first dilution 1:10, 4.5 mL of purified water was mixed with 0.5 mL of sonicated ZnO solution and for the second dilution 2 mL of purified water was mixed with 2 mL of 1:10 diluted ZnO solution, which resulted in concentration of 100 μg ZnO NP per 1 mL of purified water.

Fresh zinc oxide solutions were prepared before the bioreactor experiment each week.

3.3 Bioreactor experiment

The bioreactor experiment was the main part of the project. Bacteria growth and potential bacteria resistance was studied there.

Two samples, Z and R, were used for the bioreactor experiment. For the Z sample, prepared 50 mL Falcon tube with 18 mL of 1:1000 bacteria stock was used and then 2 mL of ZnO NP solution in a concentration of 100 μg/mL was used. Which means that in the Falcon tube, the ZnO NP concentration was 10 μg/mL of purified water.

For the R sample prepared Falcon tube with 18 mL of bacteria stock was used, however, instead of ZnO NP solution, 2 mL of purified water was added to the Falcon tube.

Both Falcon tubes were labelled so at the end of the experiment they were recognizable.

Before starting the bioreactor experiment, dilution series was done for both Z and R samples in a sterile space, in order to count the bacteria before the experiment at the time 0 hours (t0). This was done to verify that both, Z and R samples were similar at the beginning of the experiment and so the results were not affected by e.g. contamination.

The Falcon tubes were then inserted to bioreactors (*RTS-1, BioSan*) for 24 hours. The settings were 37°C, 2000 RPM (*rotations per minute*), 1 second reverse spin, measurement wavelength 850 nm.

Optical density (OD) of the solution in the Falcon tube was measured every 15 minutes generating growth curves, which represent the change in an optical density of the solutions over time. With bacteria growing, the solutions were getting less clear and the optical density was higher. After the end of the bioreactor experiment, a second dilution series was done, so it was possible to count the bacteria at the time 24 hours (t24), and therefore possible to observe the differences between R and Z samples after the experiment, to prove or deny the bacteria resistance. Also, to examine more the bacteria growth by comparing the number of viable bacteria cells at the time 0 hours and at the time 24 hours.

Both dilution series were done with 1.5 mL Eppendorf tubes.

First dilution series, before the bioreactor experiment, had the maximal dilution factor 10^{-4} , so four Eppendorf tubes for Z sample and four Eppendorf tubes for R sample were placed to a rack and 1350 µL of 0.9% NaCl were pipetted to each Eppendorf tube.

Then, for the Z and R samples, 150 µL of the MH Broth with bacteria and with the NP solution (Z sample) or with purified water (R sample) were taken from the Falcon tubes and added to the first Eppendorf tubes, the solution in the first Eppendorf tube was mixed by hand and again 150 µL were taken from the first Eppendorf tube and added to the second Eppendorf tube. This procedure was done for all four Z and all four R Eppendorf tubes.

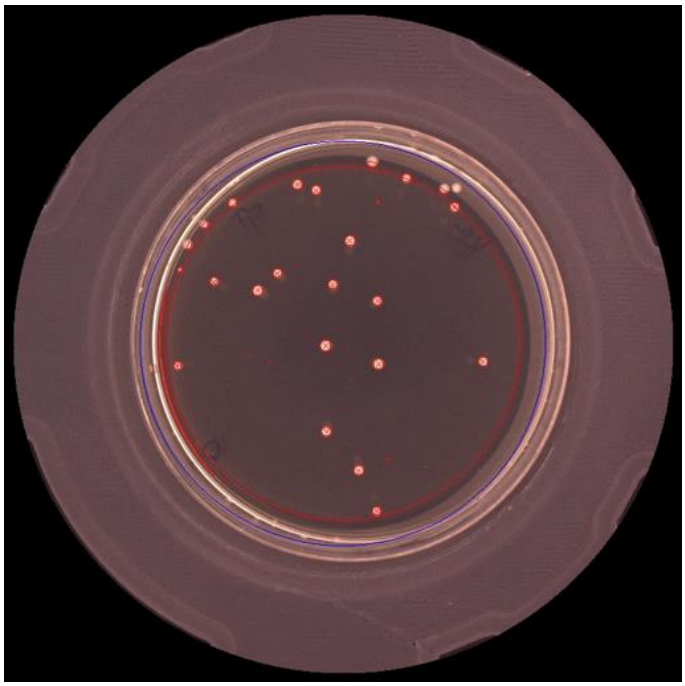
Thereafter, 500 µL were taken from the Eppendorf tubes with dilution factors 10^{-4}, 10^{-3} and 10^{-2} and added to 0.5 mL MH agar plates in duplicate. All the plates were labelled with Z or R, time 0 and the dilution factor, let dry in a sterile space and then incubated at 37°C overnight.

The next day the colonies formed on the MH agar surface were counted using an automatic counter (*SphereFlash, IUL Instruments*), so it was possible to count cfu/mL at the time 0 hours. Machine settings that can be optimized depending on the colony type and size. Illumination type and plate diameter. The sharp illumination method was selected, which

is suitable for colonies with sharply defined edges, such as SA. The diameter of the Petri dish used was 60 mm, therefore the plate diameter setting was 55 mm.

The diameter is smaller than the actual size of the plate. That is because at the edges the counter can interfere with the plate and it can lead to wrong number of colonies.

Also, near the edges of the plate, there is a bigger chance of contamination. The contamination could influence the concentration, and that is not wanted. After the picture has been taken, it is possible to change the absolute minimum diameter setting in order to only count SA colonies and not bubbles or contamination.



Picture 15 Processed picture from automatic counter *Sphere Flash*

An example of a processed picture from the automatic counter looks like can be seen in *Picture 15.* Red line which is around the plate is the set diameter, where the counter is counting colonies. Every colony is then circumscribed with a red line and in the center is a red cross which represents the center point of the colony, and the automatic counter is counting the colonies based on the marking.

The second dilution series, after 24 hours, meaning at the end of the bioreactor experiment, was done the same way as the first dilution series, but the second dilution series had the maximal dilution factor 10^{-8}. Eight Eppendorf tubes for both Z and R samples were placed to a rack and filled with 1350 µL of 0.9% NaCl. Right after the Falcon tubes were removed from the bioreactor, 150 µL was taken from the tube and added to the first Eppendorf tube, mixed in hand and then again 150 µL was taken from the first Eppendorf tube and pipetted to the second Eppendorf tube and this procedure was done for all the nine Eppendorf tubes for both Z and R samples.

Then, 500 µL were taken from the Eppendorf tubes with dilution factors 10^{-8}, 10^{-7} and 10^{-6} and added to 0.5 mL MH agar plates in duplicate. All the plates were labelled with Z

or R, time 24 and the dilution factor, let dry in a sterile space and then incubated at 37°C overnight.

After another 24 hours, the incubated colonies on the MH agar surface were counted using the automatic counter (*SphereFlash, IUL Instruments*) again, so it was possible to count cfu/mL at the time 24 hours.

The automatic counter is not counting the right concentration, because it assumes 1 mL was added to each plate. However, in this experiment 0.5 mL in duplicate were added, so recalculation was needed, and the number of counted colonies (from automatic counter) had to be multiplied by two.

For the recalculation, it was necessary to have dilution factor of the certain sample and then information from the automatic counter: counted colonies on the certain MH agar surface and counted volume of the sample.

The formula for recalculating the cfu/mL was:

$$recalculated\ concentration = \frac{counted\ colonies \times 2 \times dilution\ factor}{counted\ volume} \quad (1)$$

If the dilution factor on the plate was for example 10^{-8}, the number of colonies was multiplied by 2 and then by 10^{8} after that the number was divided by volume counted by the automatic colony counter.

For every sample, recalculation was needed using this formula, to get the right concentration in cfu/mL (colony forming units per millilitre). From all plates was then done mean, for Z sample at time 0 and 24 hours and for R sample at time 0 and 24 hours.

These values were then used for charts and for comparison.

4. RESULTS AND DISCUSSION

In this part results from the bioreactors and cfu/mL results from each week are showed and discussed. Also, more specific results like length of lag phase and growth rate are provided and discussed. Weeks 1 to 7 are discussed separately from week 8.

4.1 Weeks 1 to 7 – OD and cfu/mL

Table 1 shows mean of cfu/mL for weeks 1 to 7 for Z sample at time 0 and at time 24 hours. In *Table 2* are results of mean of cfu/mL for weeks 1 to 7 for R sample, again at time 0 and at time 24 hours. For the cfu/mL were only used plates with colonies, so empty plates were not used for counting.

The results from the bioreactors needed to be blank corrected. The first measurement, 15 minutes after the start of the experiment, the value is OD of the broth, it is not measuring the bacterial growth. In order to measure only bacterial growth, the first value (at time 15 minutes) was subtracted from all the values, so the OD at the beginning of the experiment starts at zero.

Table 1 cfu/mL weeks 1 to 7, Z sample

cfu/mL							
ZnO	week 1	week 2	week 3	week 4	week 5	week 6	week 7
t0	$1{,}16*10^{5}$	$6{,}70*10^{4}$	$5{,}35*10^{4}$	$6{,}16*10^{4}$	$5{,}36*10^{4}$	$6{,}95*10^{4}$	$1{,}16*10^{5}$
t24	$2{,}09*10^{9}$	$2{,}00*10^{9}$	$2{,}30*10^{9}$	$1{,}59*10^{9}$	$7{,}99*10^{8}$	$1{,}25*10^{9}$	$2{,}00*10^{9}$

Table 2 cfu/mL weeks 1 to 7, R sample

cfu/mL							
Ref	week 1	week 2	week 3	week 4	week 5	week 6	week 7
t0	$1{,}04*10^{5}$	$5{,}77*10^{4}$	$5{,}16*10^{4}$	$4{,}66*10^{4}$	$6{,}50*10^{4}$	$1{,}13*10^{5}$	$1{,}04*10^{5}$
t24	$2{,}74*10^{9}$	$2{,}02*10^{9}$	$1{,}65*10^{9}$	$1{,}39*10^{9}$	$1{,}12*10^{9}$	$1{,}25*10^{9}$	$1{,}70*10^{9}$

From *Table 1* and *Table 2* can be seen that at the beginning of the experiment, time 0, the cfu/mL was around 10^4 or 10^5 for both samples. After 24 hours in the bioreactor, time 24, the concentration is higher, and the cfu/mL was around 10^8 or 10^9 for both samples. For each week, I made a bar chart with mean of cfu/mL at time 0 and at time 24 hours for Z and R samples. These bar charts can be seen in *Figure 1 a) – g).*

In *Figure 2 a) – g)* is OD measured from bioreactors for weeks 1 to 7 for Z samples and R samples.

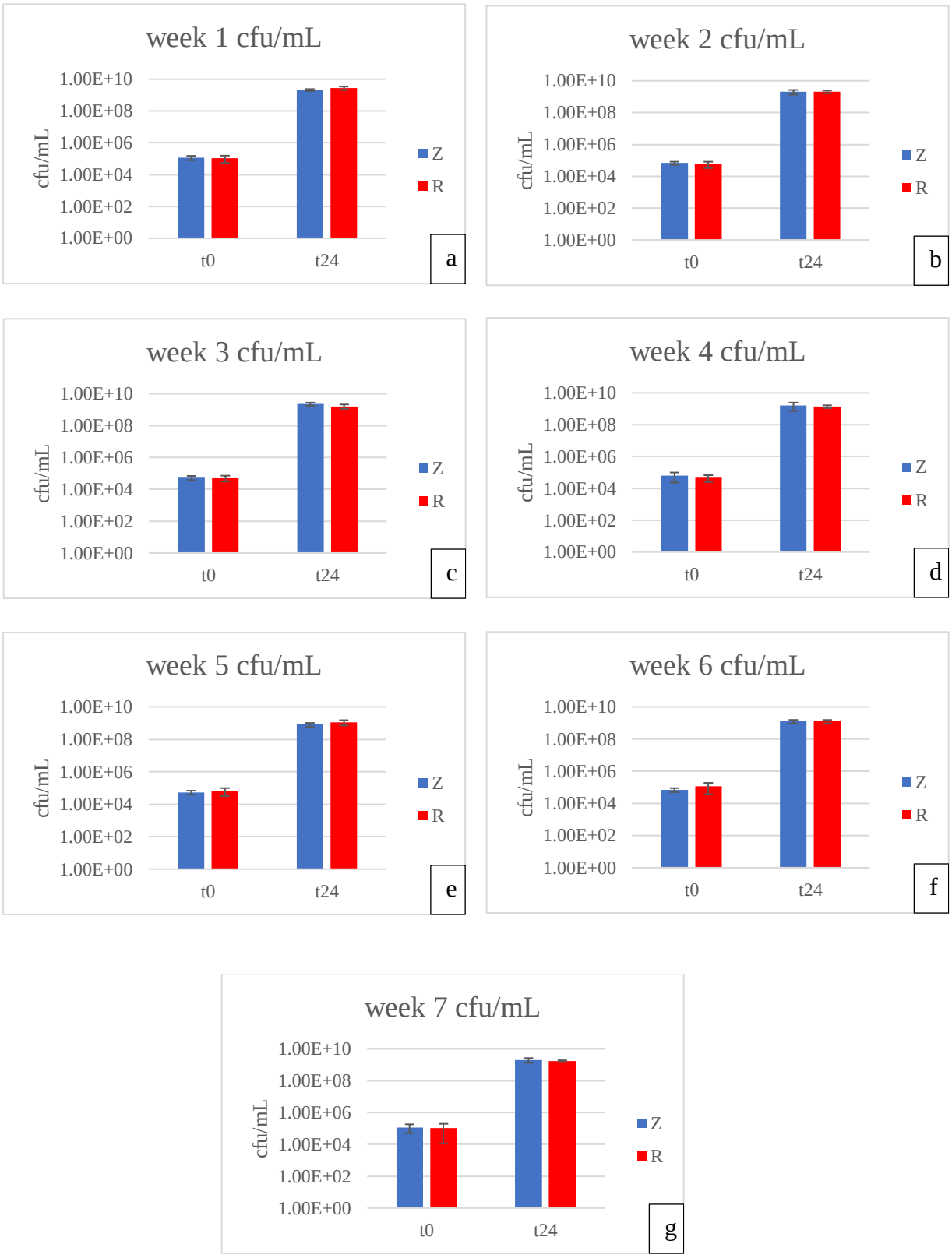


Figure 1 a) - g) cfu/mL weeks 1 to 7

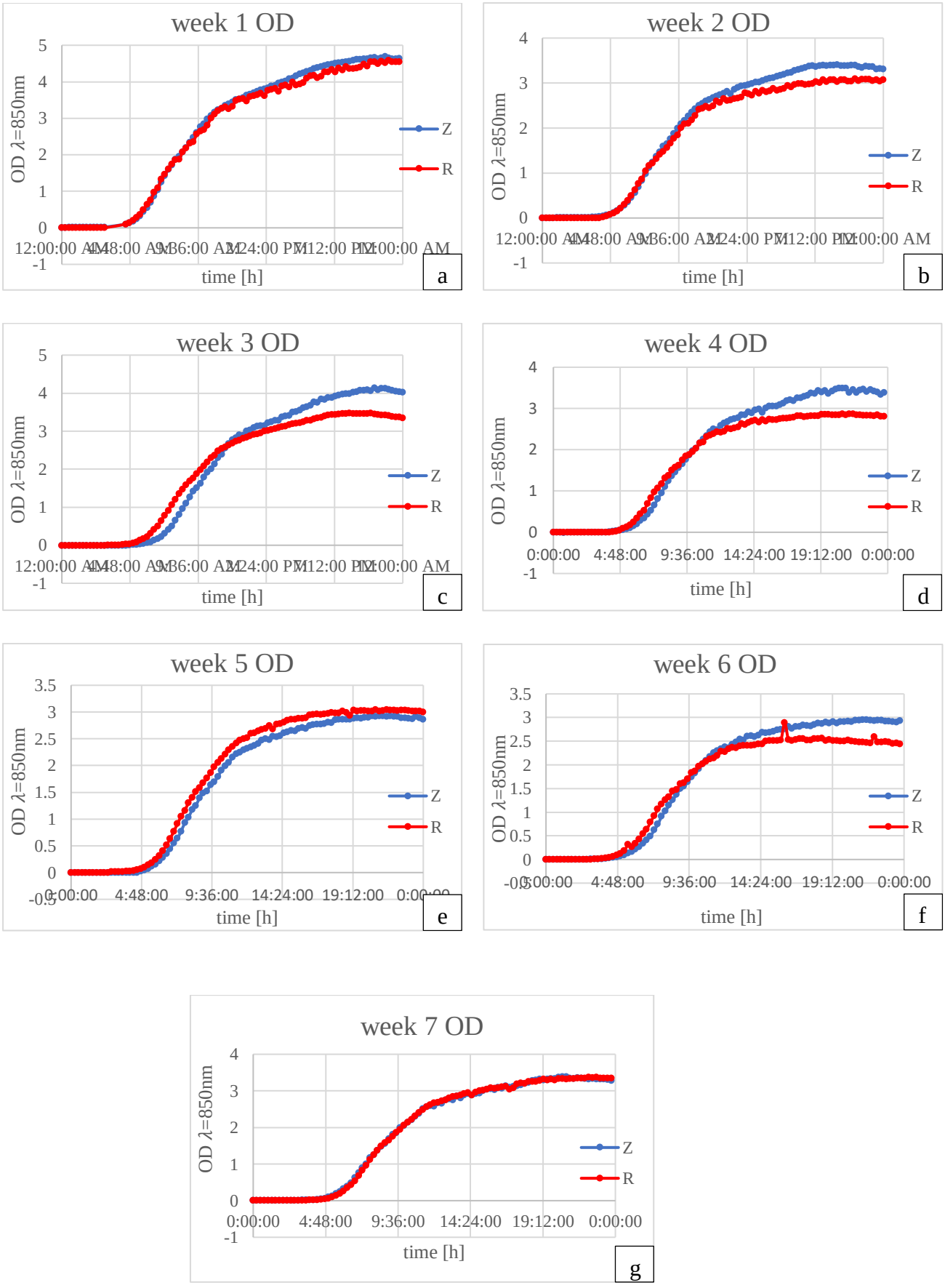


Figure 2 a) - g) OD week 1 to 7

In *Figure 1 a) – g)* can be seen standard deviation for each sample at time 0 and at time 24 hours. Standard deviation was counted using function *Standard Deviation* in *MS Excel* from all agar plates.

As can be seen in *Figure 2 a*) Z sample and R sample grew pretty much the same, the OD was similar for both samples and also the cfu/mL, which can be seen in *Figure 1 a)*, was similar for both samples at time 0 and at time 24 hours.

In *Figure 2 a)* is a line that starts around time 3 hours and ends around 4:30 hours. In the laboratory, where the measurement was happening, the power was accidentally down for circa 1 and a half hour and therefore there were no measurements at this time. This is why there is only a line and no points, which are signifying each measurement.

This incident does not have influence on the experiment and therefore it was not needed to repeat the week 1.

In week 2 Z sample grew more than R sample, based on the OD – *Figure 2 b)*. But cfu/mL proved that the concentrations of both samples were similar at time 24 hours, see *Figure 1 b).*

In week 3 it took longer time for Z sample to start growing, see *Figure 2 c)*, this might be caused by the ZnO nanoparticles. Because the environment is different, it may take longer time for the bacteria to get used to the environment and start growing. After that the Z sample grew higher, but again the cfu/mL was similar for both samples at time 24 hours – *Figure 1 c).*

In week 4, *Figure 1 d)* and *Figure 2 d)*, the results were similar as in week 3, but the standard deviation was higher in cfu/mL for Z sample, so even though it looks that the Z sample has higher cfu/mL at time 24 hours, that it grew more, there was not a statistically significant difference between Z and R.

In week 5, the OD was higher at time 24 hours for Reference sample than for ZnO sample, see *Figure 2 e)* and from the bar chart *Figure 1 e)* it seemed that there might be a difference between Z and R sample, which then might be a proof that the bacteria are interacting with the ZnO nanoparticles.

But statistics showed that there was no statistically significant difference between Z and R sample in week 5.

Statistics was done in MS Excel, using data analysis *t-Test: Two-Sample Assuming Equal Variances*, alpha was set to 0,05 and concentrations on agar plates at time zero for both samples were analysed and also the same data analysis was done for concentrations on agar plates from time 24 hours.

The t-Test analysis counts a *P(T<=t) two-tail* value. If the value is lower than 0,05 then there is a statistically significant difference. The statistics at time 0 is a check that the preparation was done correctly and to confirm, that there was not a difference between the two samples at the beginning of the experiment. Statistics done at the end of the experiment shows if there is a statistically significant difference between the two samples after 24 hours in the bioreactor.

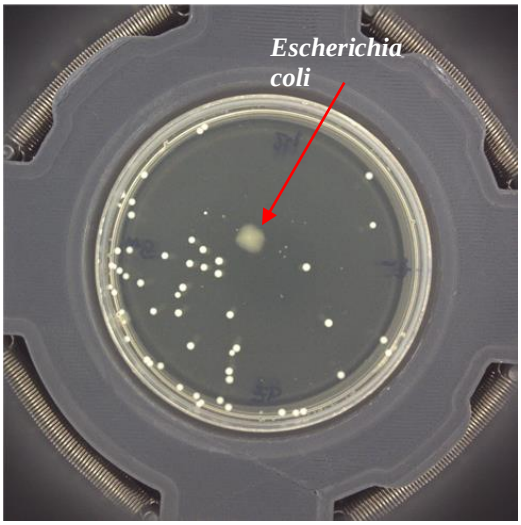
In week 6, *Figure 2 f)*, the growth curve was similar to the one from week 3 – *Figure 2 c)* and week 4 – *Figure 2 d)*, but the results of cfu/mL – *Figure 1 f)* were the same as in week 3 and week 4. There was not a difference between the two samples at time 24 hours. This was again proved by statistics done in *MS Excel* because the *P(T<=t) two-tail* value was not lower than 0,05.

In week 7, both samples grew similarly – *Figure 2 g)* and the cfu/mL was similar – *Figure 1 g)*.

One week needed to be repeated, because in the 50 mL Falcon tube, which is used for the bioreactor experiment was alcohol, that is used for sterilization. The alcohol in the test tube killed the bacteria, so there was only flat line from the OD measurement instead of growth curve and the experiment was repeated next week, this time focusing on the test tubes to be clean and without alcohol.

Also, week 4 was repeated, because the experiment needs to run for 24 hours, but the computer shut down at night due to actualisation.

Another problem that occurred during the experiment was that in some agar plates was a contamination. In microbiology laboratory contamination can happen and it is not something rare.



Picture 16 MH agar plate with *E. coli* contamination

In week 5, on one 24 hours plate, one colony of bacteria *Escherichia coli* (*E. coli*) was observed. In the laboratory it is also working with *E. coli* bacteria, so the contamination of *E. coli* is possible. But because the *E. coli* has different shape and size – the colony is bigger than SA and not round, it is fuzzier, it was still possible to count the concentration of the SA on these plates and the contamination did not have influence on the experiment results. Plates with *E. coli* contamination can be seen in *Picture 16.*

Table 3 P(T<=t) two-tail value for weeks 1 to 7

P(T<=t) two-tail value				
	t0	lower than 0,05	t24	lower than 0,05
week 1	0,714	FALSE	0,053	FALSE
week 2	0,912	FALSE	0,960	FALSE
week 3	0,864	FALSE	0,118	FALSE
week 4	0,422	FALSE	0,607	FALSE
week 5	0,468	FALSE	0,119	FALSE
week 6	0,202	FALSE	0,998	FALSE
week 7	0,797	FALSE	0,311	FALSE

Statistics made in MS Excel proved that there was no statistically significant difference at the beginning of the experiment, so the preparation was done correctly, and it is repeatable. The *P(T<=t) two-tail* value was not lower than 0,05.

Also, for all the 7 weeks, there was not a statistically significant difference between the Z and R sample after 24 hours in the bioreactor, again the *P(T<=t) two-tail* value was not lower than 0,05. The *P(T<=t) two-tail* value for all 7 weeks at time 0 and at time 24 hours can be seen in *Table 3*.

From the statistics and from the OD and cfu/mL I did not observe developing resistance after 6 re-exposures to ZnO nanoparticles, using this technique and this particular concentration.

4.2 Week 8 – OD and cfu/mL

For week 8 the OD results were blank corrected too the same way as it was done for other weeks.

In week 8, the Z sample grew differently than R sample based on the OD, this can be seen in *Figure 3*.

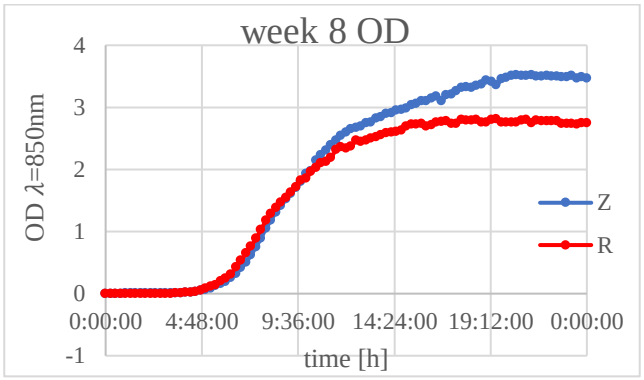


Figure 3 OD week 8

Based on the OD the Z sample started growing later than R sample, but after that, it grew higher than R sample and this was also proved by cfu/mL.

Bar chart of cfu/mL of week 8 is in *Figure 4*. Only plates with dilution factor 10^{-7} and 10^{-6} were used for counting cfu/mL, because on plates with dilution factor 10^{-8} was more than 400 colonies so it was not possible to count them properly.

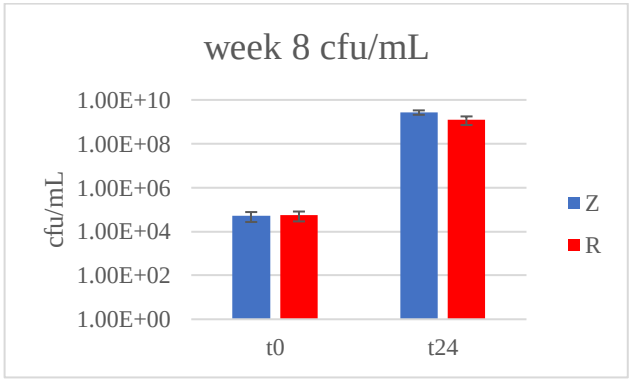


Figure 4 cfu/mL week 8

Statistics proved that there was a statistically significant difference between the Z and R sample. The *P(T<=t) two-tail* value was 0.01. Also, there was not a difference at time 0 hours, so the results are not misleading and influenced by difference between samples at the beginning. The *P(T<=t) two-tail* value at the beginning of the experiment was 0.84, so there was no statistically significant difference at the start.

Since there was a difference at t24, I decided to do the dilution series again, 24 hours after the end of the experiment, and the results are in *Table 4*.

Table 4 Repeated dilution series, week 8

Repeated dilution series, 24 hours after the end of the experiment		
	Z	R
cfu/mL	$2{,}10*10^9$	$2{,}10*10^9$
	$1{,}58*10^9$	$1{,}58*10^9$
	$1{,}50*10^9$	$2{,}50*10^9$
	$1{,}29*10^9$	$2{,}92*10^9$
mean	**$1{,}62*10^9$**	**$2{,}27*10^9$**

Unfortunately, there was no statistically significant difference at time 24 hours after the end of the experiment. So, the dilution series is not reproducible, and the dilution series has to be done right after the experiment. Because when the dilution series is done later, the results can be different and cannot be used.

Therefore, I decided to repeat the whole experiment to investigate more the difference between the two samples. To do week 8 again to prove or deny the difference between the Z and R sample and therefore prove or deny the possible developing resistance.

Next week the experiment was repeated using 4 bioreactors, so both Z and R samples were done in duplicates. OD from repeated week 8 can be seen in *Figure 5*.

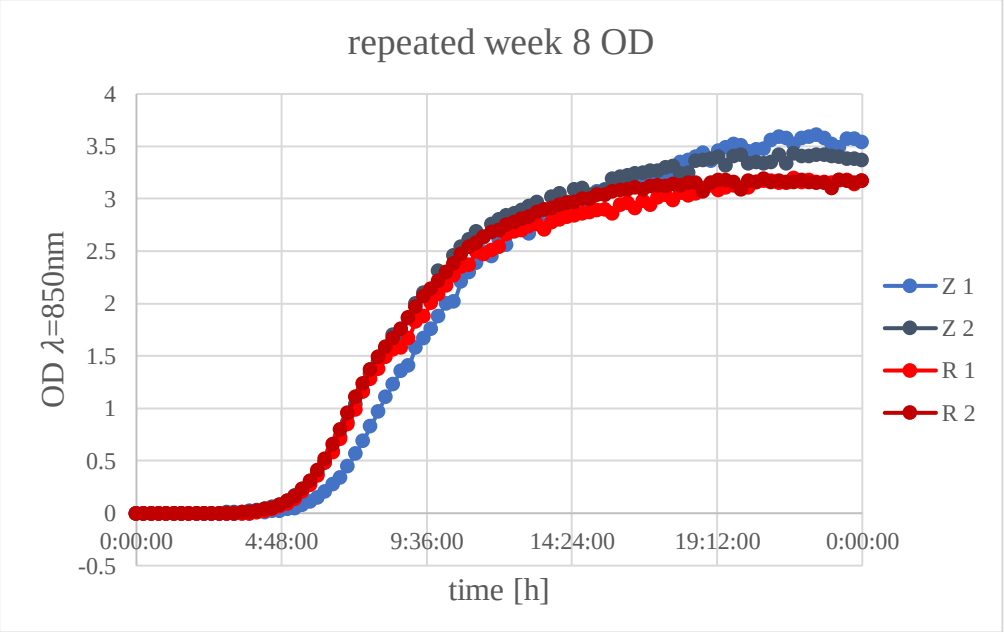


Figure 5 OD repeated week 8

One Z sample started to grow later than other samples (Z 1), but then both Z samples grew more than R samples.

Dilution series was again done before and after the experiment. The dilution series at time 0 hours showed that there was not a statistically significant difference at the beginning of the experiment, the *P(T<=t) two-tail* value 0.44, therefore the preparation was done correctly, because the samples were similar at the start of the experiment.

But the growth on agar plates at time 24 hours was strange.

Only eight from twelve plates for R sample were used and only two from twelve samples were used for Z sample. On R agar plates with dilution factor 10^{-6} were too many colonies, from 500 to 800 hundred, so it was not possible to count the number of colonies correctly and therefore the plates could not be used.

For the Z sample only two agar plates with dilution factor 10^{-7} were used because on other agar plates were too many colonies, even on plates with dilution factor 10^{-8}, where should be only few colonies. Probably because of contamination.

The counted cfu/mL for repeated week 8 can be seen in *Figure 6*. The Z sample has higher value than R sample, but it is a result from only 2 plates so the result can be misleading. Another dilution series was done, but I was not able to use the results because there were too many colonies even on plates with high dilution factor which again leads to a possibility of contamination or another experiment error.

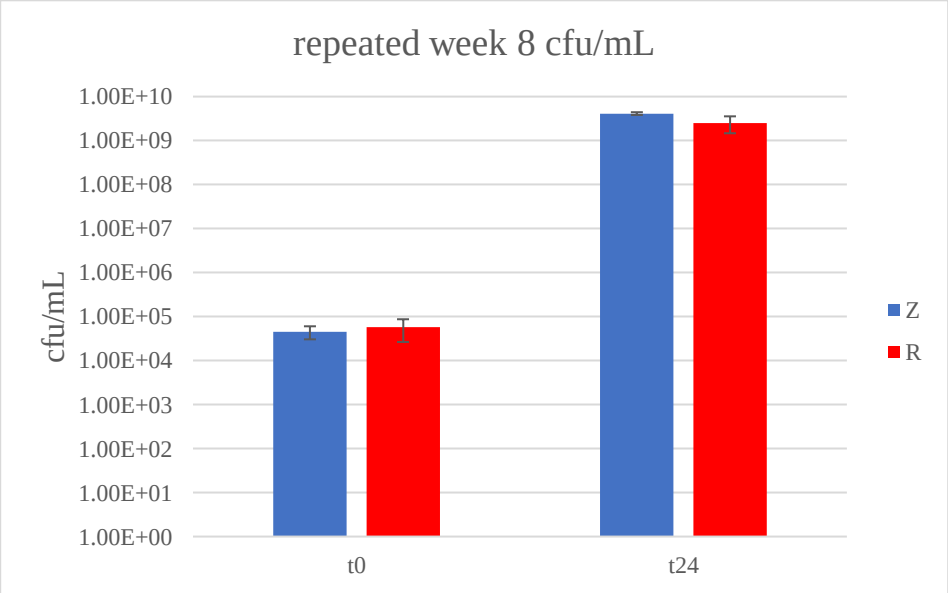


Figure 6 cfu/mL repeated week 8

The *P(T<=t) two-tail* value from *MS Excel* statistics was 0.07. So, in the repeated (rpt) week 8, there was not statistically significant difference between Z and R sample and the possible development of resistance was not proved.

In *Figure 7* is a comparison of cfu/mL from week 1 and week 8.

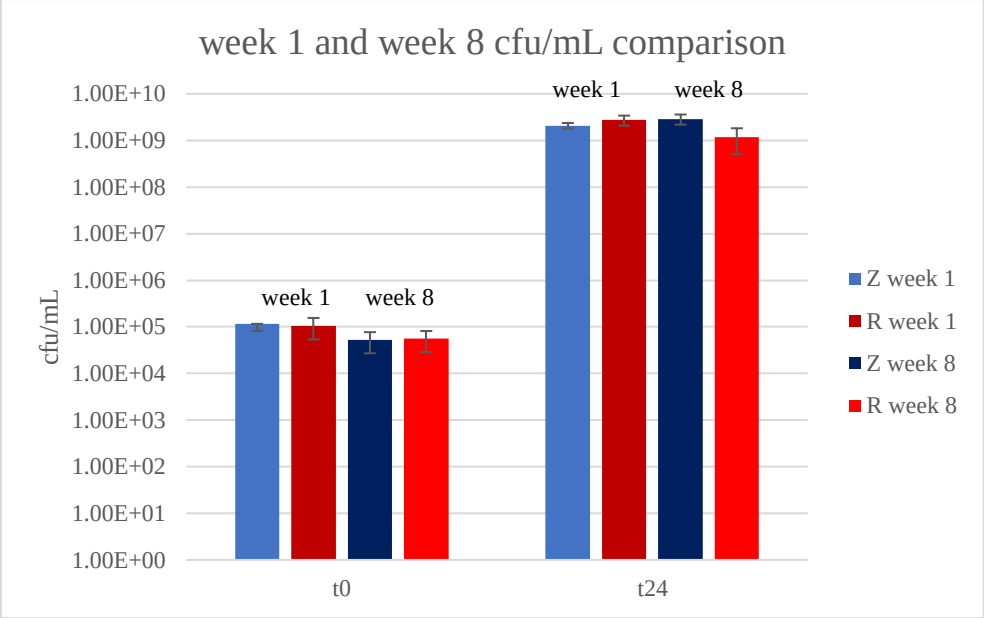


Figure 7 cfu/mL week 1 and week 8

From the *Figure 7* can be seen that the cfu/mL did not changed drastically for the Z sample after seven re-exposures to ZnO nanoparticles.

4.3 Growth rate and Lag phase analysis

I also analysed growth rate of the samples, Lag phase length and the *P(T<=t) two-tail* value.

„Growth rates refer to the percentage change of a specific variable within a specific time period.“[67]

The bioreactor is providing information about the growth rate is a percentage change of the OD in time. I was analysing at what time of the experiment was the growth rate maximal and if there was a difference between the Z and R maximal growth rate.

The growth rate results are in *Table 5*.

Table 5 Maximal growth rate and the time, when the maximum was observed

Z	time [h]	μ (h^{-1}) max
week 1	7,3	0,61
week 2	8,12	0,48
week 3	9,38	0,55
week 4	8,12	0,53
week 5	8,87	0,45
week 6	8,44	0,45
week 7	7,86	0,49
week 8	8,62	0,48
	8,88	0,48
rpt week 8	7,35	0,48

R	time [h]	μ (h^{-1}) max
week 1	7,02	0,61
week 2	7,61	0,47
week 3	8,37	0,51
week 4	10,66	0,45
week 5	8,11	0,47
week 6	7,67	0,45
week 7	7,86	0,49
week 8	8,12	0,48
	8,12	0,47
rpt week 8	8,12	0,49

From *Table 5* can be seen that maximal difference between the Z sample growth rate and R sample growth rate was in week 4, which is the week where Z grew higher, but there was not a significant difference in cfu/mL. In the other weeks, the growth rates were similar, and the maximal growth rate was observed usually between 7 to 9 hours, from the beginning of the experiment.

Only in week 4, the R sample achieved the maximal growth rate at time 10.66 hours. It might be caused by some experiment error, but it is only one strange result from nine experiments.

Then, I analysed the length of Lag phase. The length of the Lag phase could be counted as a time from beginning to a point when the OD achieves higher value than 0.2. From this time starts Log phase.

In *Figure 8* is a graph of length of Lag phase duration for all 8 weeks.

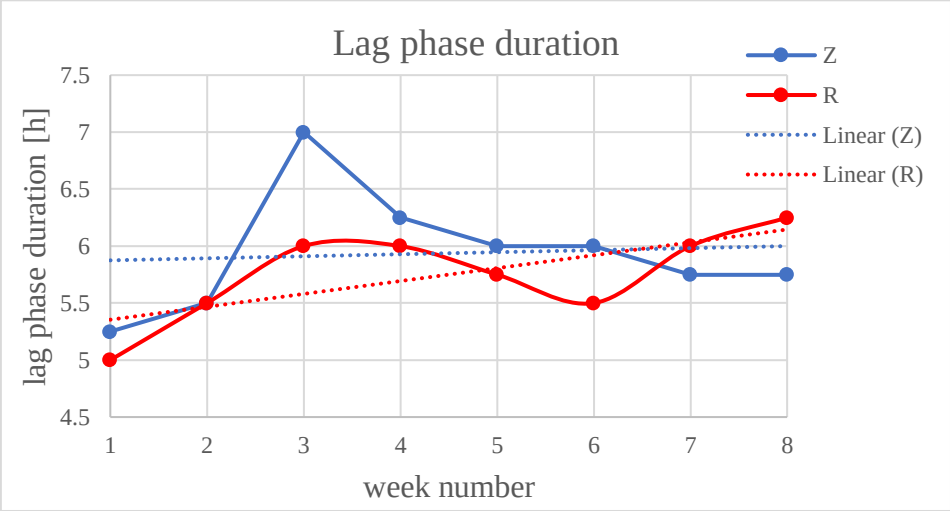


Figure 8 Lag phase length

I analysed the Lag phase duration to see if there is any trend with more weeks, but from the *Figure 8* is obvious, that there is not any trend. The Lag phase length was usually between five and seven hours.

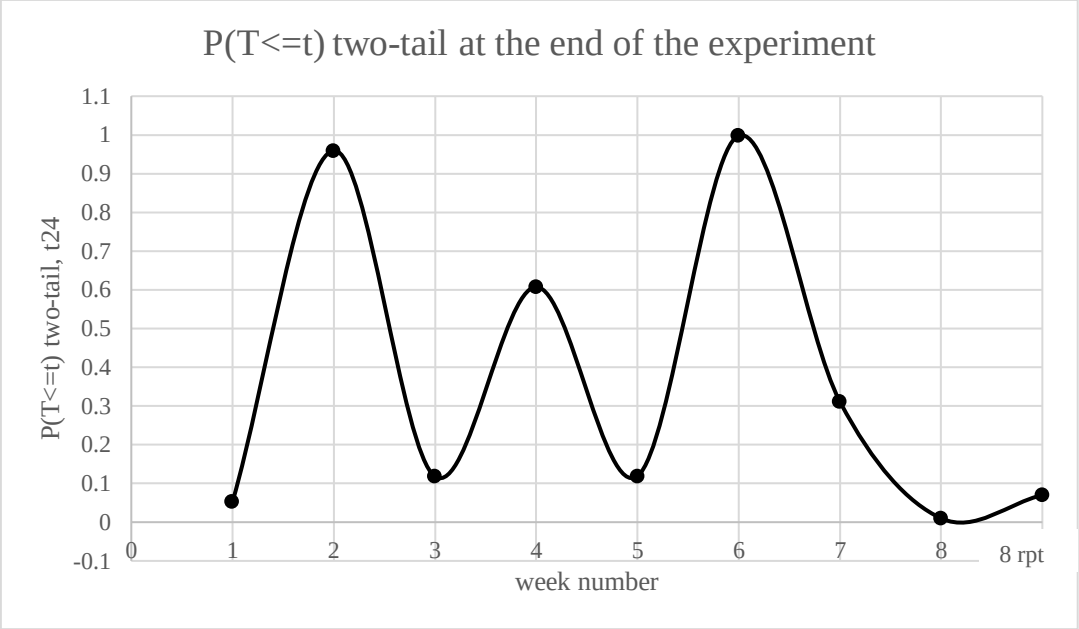


Figure 9 P(T<=t) two-tail value at time 24 hours

Figure 9 shows *P(T<=t) two-tail* value at t24 within the weeks. From *Figure 9*, the *P(T<=t) two-tail* was getting near the value 0.05 with the last re-exposures.
More re-exposures might show a difference between the two samples.

5. CONCLUSION

In this experiment, non-pathogenic bacteria *Staphylococcus aureus* was exposed to non-lethal concentration of ZnO nanoparticles in bioreactors.

Development of microbial resistance was studied using two different methods. Measurement of change in optical density over time and therefore growth curve of the bacteria in the bioreactors. And colony forming units, which were counted at the beginning and at the end of the experiment.

After seven re-exposures, using these methods and with the particular ZnO nanoparticle concentration (10 ug/mL), I did not observe any development of microbial resistance.

Also, analysis of growth rate and Lag phase duration did not show any trend.

However, deeper study is needed, because the microbial resistance might be observed after more re-exposures or a higher non-lethal concentration of ZnO nanoparticles are used.

It is important to study the antimicrobial effect of nanoparticles, but also the possible development of resistance to nanoparticles in order to use them in medicine in the future.

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REFERENCES

(1) Antimicrobial resistance https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance (accessed 2021 -05 -05).
(2) Seil, J. T.; Webster, T. J. Antibacterial Effect of Zinc Oxide Nanoparticles Combined with Ultrasound. *Nanotechnology* **2012**, *23* (49), 495101. https://doi.org/10.1088/0957-4484/23/49/495101.
(3) Sirelkhatim, A.; Mahmud, S.; Seeni, A.; Kaus, N. H. M.; Ann, L. C.; Bakhori, S. K. M.; Hasan, H.; Mohamad, D. Review on Zinc Oxide Nanoparticles: Antibacterial Activity and Toxicity Mechanism. *Nano-Micro Lett.* **2015**, *7* (3), 219–242. https://doi.org/10.1007/s40820-015-0040-x.
(4) Rajendar, V.; Shilpa Chakra, C. H.; Rajitha, B.; Venkateswara Rao, K.; Chandra Sekhar, M.; Purusottam Reddy, B.; Park, S.-H. Effect of TWEEN 80 on the Morphology and Antibacterial Properties of ZnO Nanoparticles. *J. Mater. Sci. Mater. Electron.* **2017**, *28* (4), 3272–3277. https://doi.org/10.1007/s10854-016-5919-x.
(5) Bacteria: Types, characteristics, where they live, hazards, and more https://www.medicalnewstoday.com/articles/157973 (accessed 2021 -09 -15).
(6) What are microorganisms? https://www.uib.no/en/geobio/56846/what-are-microorganisms (accessed 2021 -09 -15).
(7) What Counts as a Microbe? https://asm.org/Articles/2021/April/What-Counts-as-a-Microbe (accessed 2021 -09 -15).
(8) Repetitorium http://web2.mendelu.cz/af_291_projekty2/vseo/print.php?page=130&typ=html (accessed 2021 -09 -15).
(9) Salton, M. R. J.; Kim, K.-S. Structure. In *Medical Microbiology*; Baron, S., Ed.; University of Texas Medical Branch at Galveston: Galveston (TX), 1996.
(10) Prokaryote https://www.biologyonline.com/dictionary/prokaryote (accessed 2021 -12 -13).
(11) prokaryote | Definition, Example, & Facts | Britannica https://www.britannica.com/science/prokaryote (accessed 2021 -12 -13).
(12) Molecular Expressions Cell Biology: Bacteria Cell Structure https://micro.magnet.fsu.edu/cells/bacteriacell.html (accessed 2021 -09 -25).
(13) Gleichmann, N. Prokaryotes https://cdn.technologynetworks.com/tn/images/body/prokaryotes1592215565409.jpg (accessed 2021 -09 -25).
(14) Rutherford, D.; Jíra, J.; Mičová, J.; Remeš, Z.; Shu Hsu, H.; Rezek, B. COMPARISON OF MICROBIAL INTERACTIONS OF ZINC OXIDE NANOMATERIALS IN VARIOUS SIZE AND SHAPE; 2020; pp 330–335. https://doi.org/10.37904/nanocon.2019.8666.
(15) Silva, B. L. da; Abuçafy, M. P.; Manaia, E. B.; Junior, J. A. O.; Chiari-Andréo, B. G.; Pietro, R. C. R.; Chiavacci, L. A. <p>Relationship Between Structure And Antimicrobial Activity Of Zinc Oxide Nanoparticles: An Overview</P>. *Int. J. Nanomedicine* **2019**, *14*, 9395–9410. https://doi.org/10.2147/IJN.S216204.
(16) Tripathi, P.; Beaussart, A.; Andre, G.; Rolain, T.; Lebeer, S.; Vanderleyden, J.; Hols, P.; Dufrêne, Y. F. Towards a Nanoscale View of Lactic Acid Bacteria. *Micron* **2012**, *43* (12), 1323–1330. https://doi.org/10.1016/j.micron.2012.01.001.
(17) Difference Between Gram-Positive and Gram-Negative Bacillus https://www.webmd.com/a-to-z-guides/difference-between-gram-positive-bacillus-gram-negative-bacillus (accessed 2021 -11 -04).
(18) Aryal, S. Differences between Gram Positive and Gram Negative Bacteria.

Microbiology Info.com, 2015.
(19) cytoplasm | Learn Science at Scitable https://www.nature.com/scitable/definition/cytoplasm-280/ (accessed 2021 -09 -24).
(20) bacteria - Genetic content https://www.britannica.com/science/bacteria (accessed 2021 -10 -25).
(21) Salton, M. R. J.; Kim, K.-S. *Typical Shapes and Arrangements of Bacterial Cells*; University of Texas Medical Branch at Galveston, 1996.
(22) Wang, L.; Fan, D.; Chen, W.; Terentjev, E. M. Bacterial Growth, Detachment and Cell Size Control on Polyethylene Terephthalate Surfaces. *Sci. Rep.* **2015**, *5* (1), 15159. https://doi.org/10.1038/srep15159.
(23) B. A., B.; A. S., N.; B. A., B.; A. S., N. The Bacterial Growth Curve and the Factors Affecting Microbial Growth https://www.thoughtco.com/bacterial-growth-curve-phases-4172692 (accessed 2021 -09 -25).
(24) OpenStax Microbiology. Microbial growth curve https://s3-us-west-2.amazonaws.com/courses-images/wp-content/uploads/sites/1094/2016/11/08222450/OSC_Microbio_09_01_growthcurv1.jpg (accessed 2021 -09 -25).
(25) Staphylococcus aureus http://mikrobiologie.lf3.cuni.cz/rep/stau.htm (accessed 2021 -05 -05).
(26) AO Research Institute, Clavadelerstrasse, CH 7270 Davos, Switzerland; Harris, L.; Foster, S.; Richards, R. An Introduction to Staphylococcus Aureus, and Techniques for Identifying and Quantifying s. Aureus Adhesins in Relation to Adhesion to Biomaterials: Review. *Eur. Cell. Mater.* **2002**, *4*, 39–60. https://doi.org/10.22203/eCM.v004a04.
(27) *Staphylococcus Aureus: Microbiology, Pathology, Immunology, Therapy and Prophylaxis*; Bagnoli, F., Rappuoli, R., Grandi, G., Eds.; Current Topics in Microbiology and Immunology; Springer International Publishing: Cham, 2017; Vol. 409. https://doi.org/10.1007/978-3-319-72063-0.
(28) Rosenstein, R.; Götz, F. What Distinguishes Highly Pathogenic Staphylococci from Medium- and Non-Pathogenic? *Curr. Top. Microbiol. Immunol.* **2013**, *358*, 33–89. https://doi.org/10.1007/82_2012_286.
(29) Lorenz, B.; Ali, N.; Bocklitz, T.; Rösch, P.; Popp, J. Discrimination between Pathogenic and Non-Pathogenic E. Coli Strains by Means of Raman Microspectroscopy. *Anal. Bioanal. Chem.* **2020**, *412* (30), 8241–8247. https://doi.org/10.1007/s00216-020-02957-2.
(30) Foster, T. Staphylococcus. In *Medical Microbiology*; Baron, S., Ed.; University of Texas Medical Branch at Galveston: Galveston (TX), 1996.
(31) Staphylococci https://www.pasteur.fr/en/medical-center/disease-sheets/staphylococci (accessed 2021 -09 -25).
(32) PŘÍBALOVÁ INFORMACE: INFORMACE PRO UŽIVATELE. 11.
(33) Hurtado, M. P.; de la Parte, M. A.; Brito, A. Staphylococcus Aureus: Revisión de Los Mecanismos de Patogenicidad y La Fisiopatología de La Infección Estafilocócica. *Rev. Soc. Venez. Microbiol.* **2002**, *22* (2), 112–118.
(34) CDC. Antibiotic-resistant Germs: New Threats https://www.cdc.gov/drugresistance/biggest-threats.html (accessed 2021 -10 -25).
(35) Pozzi, C.; Olaniyi, R.; Liljeroos, L.; Galgani, I.; Rappuoli, R.; Bagnoli, F. Vaccines for Staphylococcus Aureus and Target Populations. *Curr. Top. Microbiol. Immunol.* **2017**, *409*, 491–528. https://doi.org/10.1007/82_2016_54.
(36) Antimicrobial resistance https://www.ecdc.europa.eu/en/antimicrobial-resistance (accessed 2021 -09 -26).

(37) Anonymous. Antimicrobial resistance https://www.ema.europa.eu/en/human-regulatory/overview/public-health-threats/antimicrobial-resistance (accessed 2021 -09 -26).
(38) Antimicrobial Resistance: a One Health Perspective https://journals.asm.org/doi/abs/10.1128/microbiolspec.ARBA-0009-2017 (accessed 2021 -09 -26).
(39) Antimicrobial Resistance. *OIE - World Organisation for Animal Health*.
(40) Woolhouse, M. E. J.; Gowtage-Sequeria, S. Host Range and Emerging and Reemerging Pathogens - Volume 11, Number 12—December 2005 - Emerging Infectious Diseases Journal - CDC. https://doi.org/10.3201/eid1112.050997.
(41) Reygaert, W. C. An Overview of the Antimicrobial Resistance Mechanisms of Bacteria. *AIMS Microbiol.* **2018**, *4* (3), 482–501. https://doi.org/10.3934/microbiol.2018.3.482.
(42) Christaki, E.; Marcou, M.; Tofarides, A. Antimicrobial Resistance in Bacteria: Mechanisms, Evolution, and Persistence. *J. Mol. Evol.* **2020**, *88* (1), 26–40. https://doi.org/10.1007/s00239-019-09914-3.
(43) Intrinsic resistance https://www.futurelearn.com/info/blog (accessed 2021 -11 -22).
(44) CDC. What is Epigenetics? | CDC https://www.cdc.gov/genomics/disease/epigenetics.htm (accessed 2021 -11 -22).
(45) Hollenbeck, B. L.; Rice, L. B. Intrinsic and Acquired Resistance Mechanisms in Enterococcus. *Virulence* **2012**, *3* (5), 421–569. https://doi.org/10.4161/viru.21282.
(46) Xiong, A.; Jayaswal, R. K. Molecular Characterization of a Chromosomal Determinant Conferring Resistance to Zinc and Cobalt Ions InStaphylococcus Aureus. *J. Bacteriol.* **1998**, *180* (16), 4024–4029. https://doi.org/10.1128/JB.180.16.4024-4029.1998.
(47) Blencowe, D. K.; Morby, A. P. Zn(II) Metabolism in Prokaryotes. *FEMS Microbiol. Rev.* **2003**, *27* (2–3), 291–311. https://doi.org/10.1016/S0168-6445(03)00041-X.
(48) Hughes, D.; Andersson, D. I. Selection of Resistance at Lethal and Non-Lethal Antibiotic Concentrations. *Curr. Opin. Microbiol.* **2012**, *15* (5), 555–560. https://doi.org/10.1016/j.mib.2012.07.005.
(49) Panáček, A.; Kvítek, L.; Smékalová, M.; Večeřová, R.; Kolář, M.; Röderová, M.; Dyčka, F.; Šebela, M.; Prucek, R.; Tomanec, O.; Zbořil, R. Bacterial Resistance to Silver Nanoparticles and How to Overcome It. *Nat. Nanotechnol.* **2018**, *13* (1), 65–71. https://doi.org/10.1038/s41565-017-0013-y.
(50) Gabrielyan, L.; Hovhannisyan, A.; Gevorgyan, V.; Ananyan, M.; Trchounian, A. Antibacterial Effects of Iron Oxide (Fe3O4) Nanoparticles: Distinguishing Concentration-Dependent Effects with Different Bacterial Cells Growth and Membrane-Associated Mechanisms. *Appl. Microbiol. Biotechnol.* **2019**, *103* (6), 2773–2782. https://doi.org/10.1007/s00253-019-09653-x.
(51) Padmavathy, N.; Vijayaraghavan, R. Enhanced Bioactivity of ZnO Nanoparticles—an Antimicrobial Study. *Sci. Technol. Adv. Mater.* **2008**, *9* (3), 035004. https://doi.org/10.1088/1468-6996/9/3/035004.
(52) What are the Different Types of Nanoparticles? https://www.azonano.com/article.aspx?ArticleID=4938 (accessed 2021 -11 -04).
(53) Khan, I.; Saeed, K.; Khan, I. Nanoparticles: Properties, Applications and Toxicities. *Arab. J. Chem.* **2019**, *12* (7), 908–931. https://doi.org/10.1016/j.arabjc.2017.05.011.
(54) Nanoparticles types, properties and uses

https://www.nanowerk.com/what_are_synthetic_nanoparticles.php (accessed 2021 -11 -04).
(55) Colon, G.; Ward, B. C.; Webster, T. J. Increased Osteoblast and Decreased Staphylococcus Epidermidis Functions on Nanophase ZnO and TiO2. *J. Biomed. Mater. Res. A* **2006**, *78A* (3), 595–604. https://doi.org/10.1002/jbm.a.30789.
(56) Siddiqi, K. S.; ur Rahman, A.; Tajuddin; Husen, A. Properties of Zinc Oxide Nanoparticles and Their Activity Against Microbes. *Nanoscale Res. Lett.* **2018**, *13* (1), 141. https://doi.org/10.1186/s11671-018-2532-3.
(57) Rutherford, D.; Jíra, J.; Kolářová, K.; Remeš, Z.; Rezek, B. Bactericidal Effect of Zinc Oxide Nanoparticles on Grampositive and Gram-Negative Strains in Reverse Spin Bioreactor. *IOP Conf. Ser. Mater. Sci. Eng.* **2021**, *1050*, 012013. https://doi.org/10.1088/1757-899X/1050/1/012013.
(58) Rupa, E. J.; Arunkumar, L.; Han, Y.; Kang, J. P.; Ahn, J. C.; Jung, S.-K.; Kim, M.; Kim, J. Y.; Yang, D.-C.; Lee, G. J. Dendropanax Morbifera Extract-Mediated ZnO Nanoparticles Loaded with Indole-3-Carbinol for Enhancement of Anticancer Efficacy in the A549 Human Lung Carcinoma Cell Line. *Materials* **2020**, *13* (14), 3197. https://doi.org/10.3390/ma13143197.
(59) Nguyen, D. D. ZnO, NP nanoplates https://www.oatext.com/img/AMS-4-147-g002.gif (accessed 2021 -09 -26).
(60) Nguyen, D. D. ZnO NP, nanotubes https://www.oatext.com/img/AMS-4-147-g005.gif (accessed 2021 -09 -26).
(61) Nguyen, D. D. ZnO NP, microrods https://www.oatext.com/img/AMS-4-147-g003.gif (accessed 2021 -09 -26).
(62) Rutherford, D.; Jíra, J.; Kolářová, K.; Matolínová, I.; Mičová, J.; Remeš, Z.; Rezek, B. Growth Inhibition of Gram-Positive and Gram-Negative Bacteria by Zinc Oxide Hedgehog Particles. *Int. J. Nanomedicine* **2021**, *Volume 16*, 3541–3554. https://doi.org/10.2147/IJN.S300428.
(63) Niño-Martínez, N.; Salas Orozco, M. F.; Martínez-Castañón, G.-A.; Torres Méndez, F.; Ruiz, F. Molecular Mechanisms of Bacterial Resistance to Metal and Metal Oxide Nanoparticles. *Int. J. Mol. Sci.* **2019**, *20* (11), 2808. https://doi.org/10.3390/ijms20112808.
(64) Slavin, Y. N.; Asnis, J.; Häfeli, U. O.; Bach, H. Metal Nanoparticles: Understanding the Mechanisms behind Antibacterial Activity. *J. Nanobiotechnology* **2017**, *15* (1), 65. https://doi.org/10.1186/s12951-017-0308-z.
(65) Água, R. B. d'; Branquinho, R.; Duarte, M. P.; Maurício, E.; Fernando, A. L.; Martins, R.; Fortunato, E. Efficient Coverage of ZnO Nanoparticles on Cotton Fibres for Antibacterial Finishing Using a Rapid and Low Cost in Situ Synthesis. *New J. Chem.* **2018**, *42* (2), 1052–1060. https://doi.org/10.1039/C7NJ03418K.
(66) Lallo da Silva, B.; Caetano, B. L.; Chiari-Andréo, B. G.; Pietro, R. C. L. R.; Chiavacci, L. A. Increased Antibacterial Activity of ZnO Nanoparticles: Influence of Size and Surface Modification. *Colloids Surf. B Biointerfaces* **2019**, *177*, 440–447. https://doi.org/10.1016/j.colsurfb.2019.02.013.
(67) Growth Rates https://www.investopedia.com/terms/g/growthrates.asp (accessed 2021 -12 -15).