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Trabajo Fin de Grado

Study of Multidrug Resistance in Environmental Microorganisms

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1. INTRODUCTION:

ABSTRACT: Prokaryotic kingdom constitutes the most abundant and omnipresent form of life. The constant contact of environmental bacteria with human beings, has allowed them to develop numerous mechanisms to protect themselves from the products generated by mankind. In this way, common and non-pathogenic bacteria, normally found in products of daily use, can present multiresistance to antibiotics. During our investigation, we will focus on bacteria obtained from different environmental samples, which have in common being innocuous and Gram-negative. It is suggested that all of them show resistance to antibiotics, the type of resistance that gives the presence of efflux pumps in the cell envelope. These pumps are responsible for expelling pathogenic compounds, from inside to outside the cell, before they can damage the bacteria. To test our hypotheses, it will be studied the minimal inhibitory concentration for the tetracycline and chloramphenicol antibiotics, as well as the bacterial membrane potential against the polymyxin B antibiotic. For the last procedure, the TPP^+ marker will be used.

The development of these experiments has allowed the obtainment of a clear result: a large part of the bacteria studied have the expected antibiotic resistance. It has also been proven that some of them are capable of resisting the presence of the three antibiotics used, proving to be multiresistant bacteria. However, a small number of experiments have been carried out, without taking into account all the bacteria obtained. The lack of comparative results prevents obtaining a broader view of the matter. Further studies, more detailed and taking into account the information obtained in this present, will be necessary to obtain more solid conclusions.

1.1 Objectives.

a) Test resistance to generic antibiotics of the strains obtained in environmental samples by the MIC method.

b) Test resistance to generic antibiotics of the strains obtained in environmental samples by electrochemical experiments.

c) Comparison of the results obtained in the two different procedures.

2. LITERATURE:

2.1 Bacteria kingdom.

In this research we will focus on bacterial strains found in environmental samples of different nature. All the bacteria studied here are non-pathogenic, mostly Gram-negative, having in common their multidrug resistance using efflux pumps.

Bacteria are prokaryotic microorganisms that have a micrometric size and various forms, including filaments, cocci, bacilli, vibrios and spirals. Bacteria are prokaryotic cells, so, unlike eukaryotes, they do not have a defined nucleus or, in general, any internal membranous organelle. They usually have a cell wall composed of peptidoglycan. Many bacteria are mobile, having flagella or other displacement systems.[1]
Bacteria are the most abundant organisms on our planet. They are ubiquitous, found in all terrestrial and aquatic habitats. Some bacteria can even survive in the extreme conditions of outer space.[2] In our case, we are going to study bacterial strains found in environmental samples. These are non-pathogenic bacteria that can be found in products and utensils that we use in our daily lives.

One of the most important features of bacteria is their surface. The complex conformation of its cell wall has allowed them to become the most efficient forms of life. The cell wall is considered to be the principal stress-bearing structure and surrounds the bacterial cytoplasmic membrane.[3]

2.2 Gram-negative bacteria.

Gram stain is a staining method used to distinguish and classify bacterial species into two large groups according to their reaction to various dyes: Gram-negative and Gram-positive bacteria. It differentiates bacteria by the chemical and physical properties of their cell walls. The Gram stain is usually the first step in the preliminary identification of a bacterial organism.[4]

In microbiology, Gram-negative bacteria are those that do not stain violet by the Gram stain dye called crystal violet, and they do so in a light pink color: hence their name, negative for Gram dye. This characteristic is intimately linked to the dimeric structure of their distinctive cellular envelope, since it has a double cellular membrane. Between their two bilipid membranes a thin cell wall of peptidoglycan is placed, whereas Gram-positive bacteria have only one lipid membrane and a much thicker peptidoglycan wall. Because of having a thin wall, the Gram-negative bacteria do not retain the dye during Gram's stain.[5]

The cellular surface of Gram-negative bacteria is composed of a cytoplasmic membrane (inner membrane), a thin cell wall of peptidoglycan and an outer membrane that lines the cell wall of these bacteria. Between the internal cytoplasmic membrane and the outer membrane is located the termed periplasmic space, filled with a substance called periplasm, which contains crucial enzymes for their nutrition. [5][6]

The outer membrane contains various proteins; among them, porins or channel proteins that allow the passage of certain substances. It also has a component called lipopolysaccharide (LPS), which is formed by three regions: the polysaccharide O (antigen), a central polysaccharide structure

(core polysaccharide) and the lipid A (endotoxin). The lipopolysaccharide contains a thermostable toxin that is released when the Gram-negative bacteria breaks.

Gram-negative bacteria may have a S layer that rests directly on the outer membrane and not on the peptidoglycan wall, as in the Gram-positive ones. If they have flagella, they have four support rings instead of the two from Gram-positive bacteria. They do not present teichoic acids or lipoteichoic acids, typical of Gram-positive ones. The lipoproteins are bound to the nucleus of polysaccharides, while the Gram-positive bacteria do not present that type of proteins. [6]

The mentioned outer membrane protects bacteria from various antibiotics, dyes and detergents that would normally damage the inner membrane or cell wall of peptidoglycan. The outer membrane gives these bacteria resistance to lysozyme and penicillin.[7]

2.3 Antibiotics.

Throughout this investigation, several antibiotics will be used, they will be explained in this section. An antibiotic is a chemical produced by a living being or synthetic derivative, that either kills or prevents the growth of mainly pathogenic microorganisms. The principal target are pathogenic bacteria so they are usually known as "antibacterial".[8]

2.3.1 Chloramphenicol.

Chloramphenicol (CHPC, D (-) *threo*-2-dichloroacetamido-1-*p*-nitrophenyl-1,3-propanediol) is part of the protein synthesis inhibitors. This kind of antibiotics are able to inhibit some steps or the whole protein synthesis process.[9] This drug is a broad-spectrum antibiotic that acts stopping the production of bacterial proteins. It is a bacteriostatic agent since it does not directly produce the death of the bacteria but prevents its growth.[10] This drug, thanks to its extremely lipid-solubility is able to penetrate into the cell by facilitated diffusion. Afterwards it binds to the 50S fraction of the bacterial ribosome preventing the transpeptidation between the amino acids of the peptide chain, thus preventing elongation of the growing chain. Chloramphenicol is capable of inhibiting the activity of the enzyme peptidyl transferase, preventing the binding of the substrate in the ribosome. In this way this antibiotic inhibits the total protein synthesis.[11]

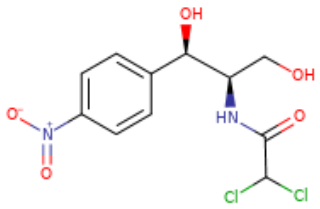

Figure 1: Chloramphenicol structure. National Center for Biotechnology Information. PubChem Database.

Studying its structure, it is considered that this compound has two asymmetric carbons, giving rise to four possible stereoisomers. The natural isomer (D (-) *threo*), the L (+) *threo* isomer with a very reduced activity and finally two isomers are biologically inactive.

Some experiments have shown that the stereochemical configuration on carbon 1 is essential for any biological activity. It is also known that the structure of the propanediol half is critical for the

antibiotic activity being its side chain considered the specific pharmacodynamic portion of the molecule, the hydrogen atoms on carbon 2 and 3 and the amide nitrogen are the attachment points with the enzyme. In the acetamide side chain, if the free hydrogen on the nitrogen atom is replaced, all antibiotic activity is lost because of being needed for the interaction with the polar groups of the proteins. Consequently, certain features of the chloramphenicol molecule seem important for its antibacterial function.[12]

2.3.2 Tetracycline.

The tetracyclines are also part of the protein synthesis inhibitors and consist of eight related broad-spectrum antibiotics. They contain a naphthalene ring of four atoms and are chemical derivatives of the polycyclic naphthalenecarboxamide, tetracyclic nucleus, from which the name of the group derives.[13] They act mainly as bacteriostats at usual doses, although they are bactericidal at high doses, generally toxic ones. They use several mechanisms: decoupling oxidative phosphorylation, inhibiting protein synthesis and even, in some cases, altering the cytoplasmic membrane.[10]

Nevertheless, these antibiotics are mostly known for their action against protein synthesis. To carry out this job, they act at the bacterial ribosome level, but for having access to it, their passive diffusion through the outer cell membrane through the hydrophilic pores is required, needing a second energy-dependent process that actively transports all the tetracyclines through the internal cytoplasmic membrane.[14] Once they are inside the bacteria, tetracyclines inhibit protein synthesis by binding to the A-site of the ribosomal 30S subunit, they prevent access of the aminoacyl-tRNA to the A-site of the mRNA-ribosome complex, and this results in the non-addition of amino acids to the growing peptide chain. Thus without the sequential attachment of the tRNA at the A-site, protein biosynthesis cannot occur.[15]

Considering their chemical structure, tetracyclines include a linear fused tetracyclic nucleus (rings designated A, B, C, and D) with various functional groups attached to it. The simplest tetracycline to display detectable antibacterial activity is 6-deoxy-6-demethyltetracycline (Figure 2).

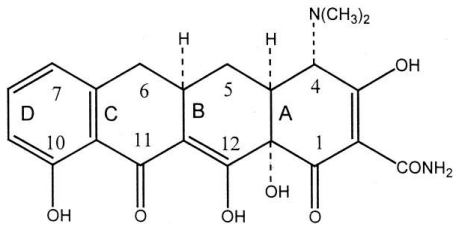

Figure 2: The simplest tetracycline molecule[15]

Firstly, this group of antibiotics is characterized by a strong chelating action and both their antimicrobial and pharmacokinetic properties depend on this property. Important antibacterial features among the tetracyclines are mainly maintenance of the linear fused tetracycle, and conservation of the keto-enol system (positions 11, 12, and 12a) in proximity to the phenolic D ring. It is proven that any kind of substitution at positions 1, 3, 4a, 10, 11, or 12 affects negatively their antibacterial activity, a considerable number of other substitutions at different positions on the B, C, and D rings are, however, tolerated.[16]

2.3.3 Polymyxin.

Polymyxin is included in the cell membrane inhibition activity antibiotics. These antibiotics disorganize the structure or inhibit the normal function of bacterial membranes. There are 5 different types of polymyxins: A, B, C, D and E, but only types B and E are used as chemotherapeutic drugs. The B type is formed by two different molecules, B1 and B2. They are all part of a broader class of molecules called nonribosomal peptides.[17]

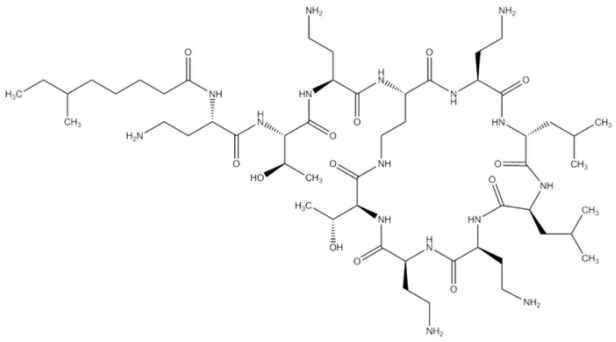

Figure 3: Chemical structure of Polymyxin B[18]

The chemical structure of polymyxin consists of basic cyclic decapeptides, with a high content of diaminobutyric acid, with dextrorotatory and levorotatory amino acids, with a fatty acid, usually methyloctanoic acid. Polymyxins have a spectrum of activity limited almost exclusively to Gram-negative bacilli.[18]

Polymyxins are all cationic detergent-type antibiotics. They are amphipathic molecules with surface activity. Interacting in a potent way with phospholipids, they break the structure of cell membranes. The permeability of the bacterial membrane changes immediately upon contact with this drug.[19]

The chemical composition of polymyxin is crucial for its antibacterial activity. The different polymyxin molecules have in common two hydrophobic domains separated by polar and cationic residues. The three-dimensional configuration gives it its amphipathic character. Its hydrophobic domains are responsible to interact with the lipopolysaccharides of the outer membrane of Gram-negative bacteria and have an important role in causing the membrane damage.[20]

When it is in solution, part of the molecule is ionized, presenting a positive partial charge, an important characteristic for its interaction with the negatively charged phosphate groups of lipid A of the LPS of the Gram-negative outer membrane. The electrostatic interaction between the positively charged residues and the negatively charged lipid A phosphates causes the displacement of divalent cations (Ca^{2+} and Mg^{2+}) that normally function to bridge and stabilize the LPS outer membrane monolayer.[21]

Due to the hydrophobic and electrostatic interactions, the outer membrane of the bacteria is de-sized. Polymyxin is able to replace the formation of LPS by the formation of channels and pores, permeating the outer membrane it allows the passage of higher concentrations of polymyxin. In this way, this antibiotic breaks the physical integrity of the bacterial membrane leading to its death.[22]

2.4 Antibiotic resistance: Efflux pumps.

Due to their continuous contact with humans, the strains studied in this research have been able to develop mechanisms to protect themselves against compounds that we constantly utilize, such as antibiotics.

Antibiotic resistance is the ability of a microorganism to resist the effects of a certain antibiotic. It is a subset of AMR, antimicrobial resistance, the ability of a microbe to resist the effects of drugs that once were able to kill them.[20] Resistance occurs naturally by natural selection through mutations produced by chance. The antibiotic, when it contacts with a bacterial population, allows only the proliferation of those bacteria that have that natural mutation that cancels the action of the antibiotic. Once this genetic information is created, the bacteria can transmit the new resistance genes through horizontal transfer by exchange of plasmids. If a bacteria carries several resistance genes, it is called multi-resistant as those studied in this research.[23]

Gram-negative bacteria have a broad arsenal of antibiotic resistance mechanisms. These resistance mechanisms could be summarized in four categories: enzymatic modification, changes in the permeability of the outer membrane, alterations in the site of action and efflux pumps.[24] Efflux systems are energy-dependent mechanisms that act by pushing out harmful substances through specific efflux pumps.[25] Some efflux systems are drug-specific so they only excrete one drug or one class of drugs; whereas others may accommodate multiple drugs, pumping out a wide range of compounds. Specifically, efflux pumps are efflux systems formed by protean active transporters that are found in the cytoplasmic membrane.[26]

Active efflux systems have been seen to cause drug resistance in Gram-negative bacteria. The majority of Gram-negative bacterial multidrug efflux pumps are completely different in their construction in comparison to the traditional ones. They cross both the cytoplasmic (inner) and outer membranes by using three protein components which facilitates direct passage of the substrate into the external medium.[27] This tripartite efflux system is composed by a transporter located in the inner membrane, an outer membrane channel and a periplasmic accessory protein. It is considered that the outer membrane barriers and the multicomponent efflux systems act synergistically to lower the cytoplasmic and even the periplasmic concentrations of antibiotics.[28]

2.4.1 ABC transporters.

The ATP-Binding Cassette (ABC) transporters are conserved from bacteria to humans and are able to pump out a wide range of substrate using the energy produced by ATP hydrolysis.[29] In Gram-negative organisms, ABC transporters can mediate secretion through both membranes simultaneously, bypassing the periplasmic space. This secretion pathway involves two additional accessory proteins as was explained.

Functional ABC pumps consist of two transmembrane domains (TMDs) responsible for substrate recognition and its transport, and two cytosolic nucleotide binding domains (NBDs), also known as ATP-binding cassettes, where ATP is hydrolyzed.[30] Despite the large diversity of the transport substrates, the sequences of the ABC components are remarkably conserved. Being the most conserved structure the one that corresponds to NBD due to its common function. [31]

Each NBD can be divided into two domains: a larger RecA-like subdomain consisting of two β-sheets and six α-helices which corresponds to the catalytic core and a smaller helical subdomain

formed by three to four α-helices. Several conserved sequence motifs, such as the Walker A and Walker B motifs that are found in many ATPases, can be identified here. In spite of the fact that the helical subdomain is specific to the ABC. [32] ATP binds to the catalytic subdomain, and its γ-phosphate is positioned close to the edge of one of the β-sheets where it interacts with several residues. The Walker A motif, also known as the P loop, forms a loop that binds to the phosphates of ATP or ADP. The main responsible for the hydrolysis of the ATP is the Walker B motif.[33] Inside the helical subdomain we can find a unique structure, the called signature motif, also known as the LSGGQ motif, linker peptide, or C motif. It has been used as the "signature" to identify ABC transporters and is the only major conserved motif that does not contact nucleotide in the monomer structure. [34]

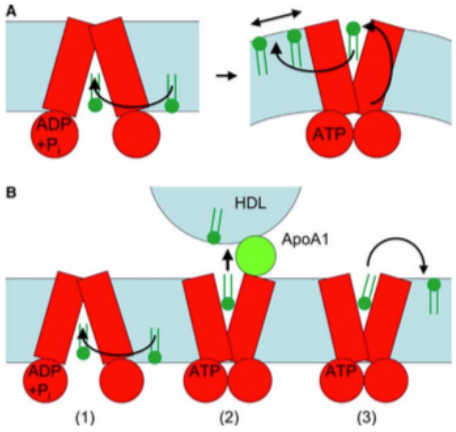

Figure 4: Mechanistic models for ABC transporters.[35]

ATP binding and hydrolysis are coupled to conformational changes in the MSDs that mediate the transit of substrates across the membrane. This ATP switch mechanism for ABC transporters takes place when a nucleotide-driven interaction of the NBDs causes reorientation of the TMDs. [35] This process is mediated by two different sets of transmembrane helix interactions. The outward-facing conformation is caused by ATP binding and reflects the ATP-bound state, with the two transmembrane domains forming a central cavity that is thought to be the drug translocation pathway. The inward-facing conformation is promoted by dissociation of the hydrolysis products (ADP and phosphate) and shows the substrate-binding site accessible again from the cell interior. [36]

2.4.2 MATE pumps.

The Multi antimicrobial extrusion protein family is the most recently described efflux pump within the five multidrug efflux transporter families. [37] The common structure within the members of the MATE family es a 12 transmembrane hydrophobic helix topology with an internal twofold sequence similarity reflected in the tertiary disposition. [38] Structures also display an external-facing cavity, extending approximately halfway across the membrane. This family confers resistance to multiple cationic toxic agents acting as H^+- or Na^+-antiporters. Almost all MATE-family transporters can recognize fluoroquinolones as transport substrates, such as norfloxacin. Although it presents a smaller amount of possible substrates than the RND transporters this family is still considered a multidrug efflux transporter. [39]

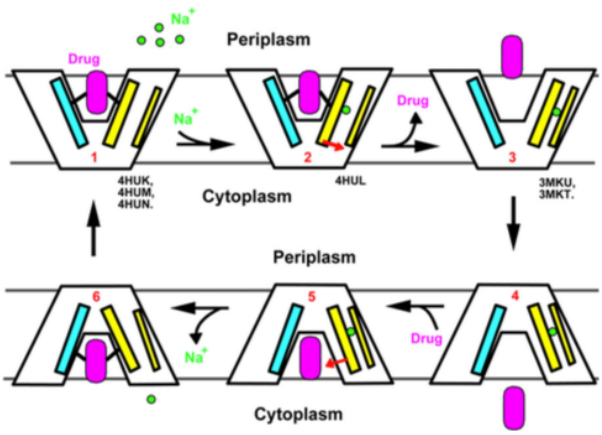

Figure 5: Proposed antiport mechanism.[39]

2.4.3 MFS pumps.

The major facilitator superfamily forms the largest multidrug efflux transporters family. This family is composed of antiporter transporters with tripartite structure (as explained) in Gram-negative bacteria kind. Uniporters and symporters can also be found within this family. They are responsible of the transport of a wide range of structurally diverse low molecular weight substrates, being lipophilic cations the most remarkable ones.[33]

These pumps work using the potential produced by some electrochemical process. Most of them use the energy generated by the proton gradient. Their stoichiometry is 1:1, a substance expelled by each proton used. In this way, MFS pumps are the most energy efficient pumps that use protons. Structurally, these transporters are constituted by twelve or fourteen transmembrane helices of variable conformation that constitute a compact structure with four of them facing away from the interior. The remaining transmembrane helices form a central cavity with hydrophobic conserved residues.[40]

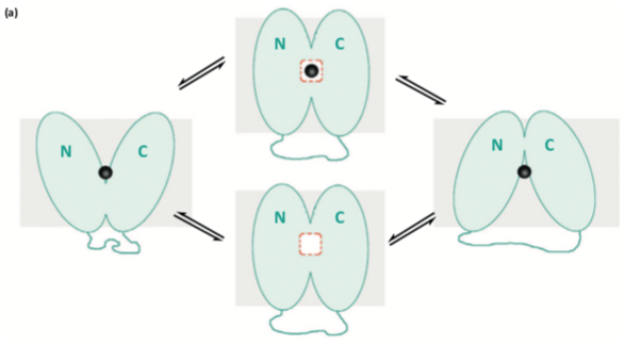

Figure 6:Schematic diagram to illustrate the alternating access mechanism for MFS transporters.[41]

It is proposed that the substrate binding site faces one side of the membrane when it is resting and it orients itself via a conformational change once the substrate appears, the binding site is able to face the other side of the membrane in order to facilitate the transport.[41]

2.4.4 SMR transporters.

The small multidrug resistance family are the smallest in terms of length at just 100–120 amino acids can transport many quaternary ammonium compounds (QAC) in addition to other lipophilic cations.[29] It functions as an antiparallel dimer, with a fixed stoichiometry of two protons exchanged per substrate molecule, meaning that transport of monovalent cations results in net charge movement (electrogenic), whereas transport of divalent cations does not (electroneutral). These proteins are believed to span the cytoplasmic membrane as four transmembrane α-helices with short hydrophilic loops making them very hydrophobic, a characteristic that permits their solubilization in organic solvents Structural plasticity and flexibility is decisive in its multidrug recognition and transport.[42]

2.4.5 RND transporters.

The resistance-nodulation-cell division superfamily in Gram-negative bacteria are found in the inner membrane and work together with two other proteins forming a tripartite complex that extends through both bacterial membranes through the periplasmic space. These transporters have membrane-spanning domains and a very large periplasmic domain.[33]

The most commonly studied members of this family are AcrB (*E. coli*) and MexB (*Pseudomonas aeruginosa*). This pumps work like molecular machines, where their inner membrane protein associates with a periplasmic adaptor protein and a long helical tunnel protein to cross the double membrane of the Gram-negative bacteria and effect the efflux of toxic compounds directly to the extracellular environment.[28] RND transporters are able to capture even those substrates that cannot permeate across the cytoplasmic membrane suggesting that the capture can occur from the periplasm. It was also suggested that the capture occurs from the cytoplasmic membrane/periplasm interface, because most substrates contain a sizable hydrophobic domain. Because of that it is proven that much of the substrate specificity is determined by their periplasmic domains. This family presents a fairly broad spectrum of substrates including antibacterial and chemotherapeutic agents.[43]

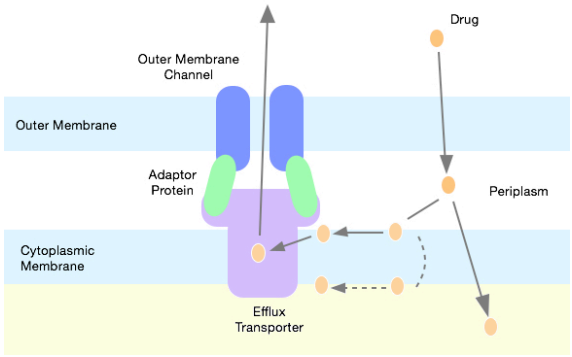

Figure 7: Tripartite structure of RND transporters.

The transport through these pumps takes place thanks to the protonic force since they are treated as proton/drug antiporters. The transfer of protons is carried out by three charged amino acids present in the fourth and tenth transmembrane domain. The transmembrane domains have conserved structures and practically have the same residues, including the triplet involved in proton translocation.[44]

3. MATERIALS AND METHODS:

3.1. Materials.

3.1.1 Strains.

Bacteria used in this study were found in diverse environmental samples and are listed in table 1. It is worth mentioning that those discovered in cosmetic products were obtained in previously unsealed and used products. As control groups, there will be used *Pseudomonas aeruginosa* wild-TYPE bacteria (PA01) and MUTANT *Pseudomonas aeruginosa* bacteria (PT629).

Table 1 A: Bacteria strains.[45]

Nº	**Code name**	**Bacterial type**	**Sample location**
1.	F2	*Pseudomonas rhodesiae*	Livestock Farm
2.	F3	*Pseudomonas* spp.	Livestock Farm
3.	F4	*Pseudomonas* spp.	Livestock Farm
4.	F5	*Pseudomonas* spp.	Livestock Farm
5.	F6	*Pseudomonas* spp.	Livestock Farm
6.	F7	*Pseudomonas* spp.	Livestock Farm
7.	F8	*Pseudomonas koreensis*	Livestock Farm
8.	F9	*Pseudomonas rhodesiae*	Livestock Farm
9.	PT629	*Pseudomonas aeruginosa*	University's collection
10.	F10	*Pseudomonas koreensis*	Livestock Farm

Table 1 B: Bacteria strains.[45]

1.	K3	*Bacilus* spp.	Essence Eyebrow Shade
2.	K5	*Escherichia coli*	Essence Eyebrow Shade
3.	K6	*Escherichia coli*	Loreal Foundation
4.	K7	*Escherichia coli*	Loreal Foundation
5.	K8	*Escherichia coli*	Essence Lip Pencil
6.	Q10	*Pseudomonas* spp.	Essence Lip Pencil
7.	B3	*Escherichia coli*	Deodorant "Old Spice Champion" cover
8.	PA01	*Pseudomonas aeruginosa*	University's collection
9.	K1	*Bacilus* spp.	Essence Eyebrow Shade
10.	B2	*Eschericia coli*	"Sensodyne" toothpaste

3.1.2 Culture media.

The culture media used are sterilized in an autoclave for 15 minutes at 121 º C and prepared with distilled water. The detailed preparation will be explained in section 3.4.1. The composition of the media used is presented in table 2.

Table 2: Luria-Bertani (LB) Broth and Agar composition (Lennox).

	LB Agar	LB Broth
Tryptone	10 g/L	10 g/L
Yeast extract	5 g/L	5 g/L
Sodium chloride (NaCl)	5 g/L	5 g/L
Agar	15 g/L	-

3.1.3 Reagents. Antibiotics.

- Chemical reagents:
  - TRIS Buffer. ("ROTH") 100mM at pH 8
  - EDTA, ethylenediaminetetraacetic acid. ("ROTH") 100 mg/mL
  - $Na_2S_2O_4$, sodium dithionite. ("ROTH")
- Antibiotics:
  - Chloramphenicol. ("Fluka") 40 mg/mL
  - Tetracycline. ("ROTH") 50 mg/mL
  - Polymyxin B. ("Sigma-Aldrich") 50 mg/mL

3.1.4 Laboratory equipment.

- Centrifuge ("Allegra 64R Centrifuge")
- Shaking incubator ("Environmental Shaker-Incubator ES-20")
- Autoclave ("LABOKLAV 25")
- Laminar flow cabinet ("Bioair")
- Spectrophotometer ("Amersham")
- Microplate Reader ("Tecan Machine")

3.2 Methods.

3.2.1 Preparations prior to the experiments.

a) Agar plates preparation:

First of all, the dehydrated components of the medium have to be dissolved in distilled water, following the instructions of the manufacturer. While the medium contains a solidifying agent (agar-agar) it is necessary to heat the preparation until boiling it, stirring from time to time. Once it is dissolved, the medium must be sterilized to prevent the growth of contaminants. For solid media in plate we have to cover the flask with aluminum foil and carry out sterilization to the autoclave (121 ºC) for 15-20 minutes. Once sterile, we have to distribute in sterile petri dishes inside of a laminar flow cabinet in order to prevent possible contamination and leave to rest to solidify. It is also advisable to use the UV-G germicidal lamp of the cabinet for the same purpose. We will culture our strains from the frozen stocks using the "streaking" technique.

b) LB broth preparation:

A similar procedure will be followed to dissolve the LB broth. According to the manufacturer's instructions, the volume needed will be prepared with the dehydrated components and distilled water. Then, the solution will be sterilized by autoclavation.

c) Over-night culture:

Grown colonies will be taken using a sterile plastic loop and will be mixed in a tube with 5 mL of also sterile LB broth previously prepared. This solution will be kept in a shaking incubator (30 ºC and 220 rpm) for at least 16 hours.

d) Day culture:

A certain volume of the overnight culture will be taken and passed to 50 mL LB broth placed in an Erlenmeyer flask. The absorbance of the resulting culture will be 0.1. This new culture will be stored in a shaking incubator (220 rpm at 30ºC) until its absorbance reaches the required value for our experiment, that is to say, 1.2.

3.2.2. MIC Method: Dilution method to determine the MIC of antimicrobial substances using 96-well microplates.

The minimal inhibitory concentration (MIC), in microbiology, is the lowest concentration of an antimicrobial agent that inhibits the growth of a microorganism after its incubation. The minimum inhibitory concentrations can be determined by broth microdilution methods, as it is in our case.[46]
A transparent polystyrene microplate with 96 wells, 12 columns (1-12) and 8 rows (A-G) is used. Our final volume will be 200 µL/well, using row G as positive control (bacterial culture is added but not antibiotic) and H as negative control (only sterile LB medium).
This procedure can be divided in the following steps:[47]

a) Preparation of inoculum:
   1) Preparation of overnight cultures of the strains of study as explained at 3.2.1 c section.
   2) After approximately 16 hours, check of the OD_{600} with a spectrophotometer. Knowing that 1 OD_{600} is equal to $8x10^8$ CFU/mL, our bacterial concentration will be calculated.
   3) Dilution of the bacterial solution with LB Broth to obtain a $5x10^5$ CFU/mL suspension.

b) Inoculation, incubation and reading:
   1) Addition of 180 µL of LB Broth in every well of the microplate using a multichannel pipette.
   2) Addition of 20 µL of antibiotic (tetracycline or chloramphenicol) in the A row.
   3) Addition of 200 µL of LB Broth in the first row and carry out a serial dilution. Mixture and transference of the volume to the next line. The procedure has to be repeated until the F row. The 200 µL added has to be removed after the last mixture.
   4) Addition of the bacterial volume prepared at a section from first row to G row.
   5) Incubation in a 30ºC incubator for 24-30 hours.
   6) Spectrophotometer reading on a microplate reader at 612 nm.

3.2.3 TPP^+ measurement: Electrochemical method.

For the study of the action of antimicrobial agents, the measurement of the bacterial membrane permeability and potential are crucial. It is a way to evaluate the ability of these antimicrobial agents to generate channels or, more generally, to increase permeability and to abolish membrane potential in bacterial cytoplasmic membranes in situ.[48] Lipophilic cation TPP^+ or tetraphenylphosphonium is a membrane potential probe as it passes through the cytoplasmic membrane and accumulates in the cytosol depending on the potential (inside negative) formed in the membrane. Therefore, if an antibiotic has the ability to induce membrane depolarization, it will cause an efflux out of the accumulated TPP^+.[49]
Preparation of overnight culture of the strains (3.2.1 c section) with a half hour interval between each other.[50]

1. Preparation of day culture as explained in 3.2.1 d section.
2. Preparation and calibration of the equipment: Immersion of the reference electrode and the TPP^+ electrode in 5 mL of TRIS buffer. The solution is contained in termasized glass buckets, aerated using magnetic stirrers and maintained at a constant temperature of 30ºC.
3. The LabChart program from a computer is connected to the equipment and prepared to measure the ion concentrations during the experiment.
4. The bacterial suspension from the day culture will be centrifugated (10 min, 4ºC and 3000xG) once the OD is 1.2 and the supernatant will be decanted. The cells are resuspended in 100 µL of TRIS buffer.
5. Measurement of the sample absorbance in order to calculate the volume of suspension needed to obtain an OD of 3 in the bucket.
6. The experiment can get started according to the following schedule:
    - Minute 2: 5 µL of TPP^+ dissolved in distilled water are added.
    - Minute 4: Another 10 µL are added.
    - Minute 7: The volume of cell culture calculated above is added.
    - Minute 9: 4 µL of EDTA are added.
    - Minute 12: 5 µL of polymyxin (25 mg/mL) are added.
    - Minute 14: A small amount of a reducing agent ($Na_2S_2O_4$) is added to bind to the rest of the oxygen in the medium and thus end the test.
7. The results are obtained in txt format and, using the SigmaPlot program, they can be studied and converted into graphs.

3.2.4 Statistical methods and analysis of results.

All computationally obtained results are processed and refined using the SigmaPlot program (electrochemical experiments) and the MS Excel software (MIC experiments). Both programs permit the obtainment of graphic results, in order to facilitate the study of the antibiotic effect over time. Each experiment has been repeated three times, thus being able to standardize the results. Basic statistical tools such as arithmetic mean or standard deviation have been used.
Being, respectively:

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \bar{x})^2}$$

$$\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i = \frac{x_1 + x_2 + \cdots + x_n}{n}$$

4. RESULTS:

4.1 MIC experiment.

This experiment is developed to study the effect of different concentrations of tetracycline and chloramphenicol antibiotics on the growth of our strains under study. A range of concentrations of 0 to 2.5 µg / mL will be used in both antibiotics and the spectrophotometric results obtained in the form of an excel document will be refined and studied graphically:

a) Tetracycline resistance:

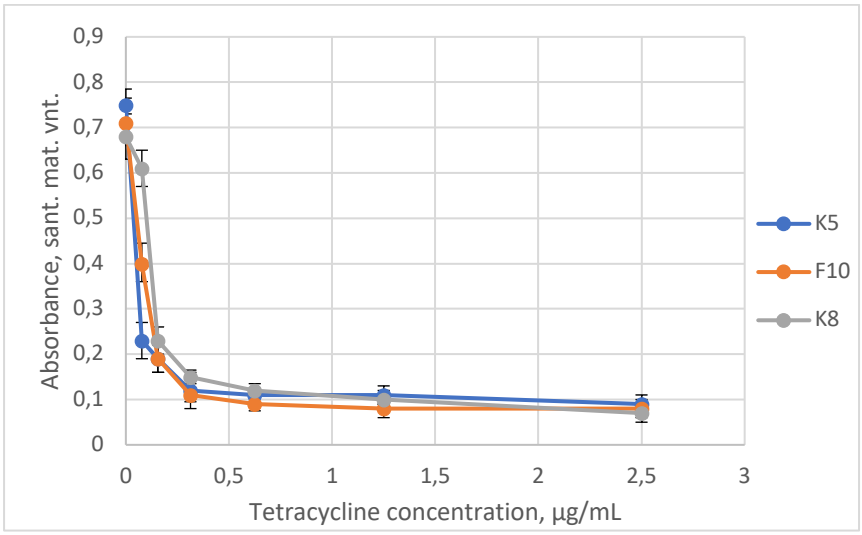

Figure 8: MIC method of K5, F10 and K8 strains using tetracycline.

According to the Figure 8, the strains studied together in this assay show a fairly similar behavior of tetracycline sensitivity. The three of them suffer the completely growth suppression pretty soon, once the antibiotic reaches the value 0.313 µg/mL value in the case of K5 and F10, and 0.625 µg/mL for K8.

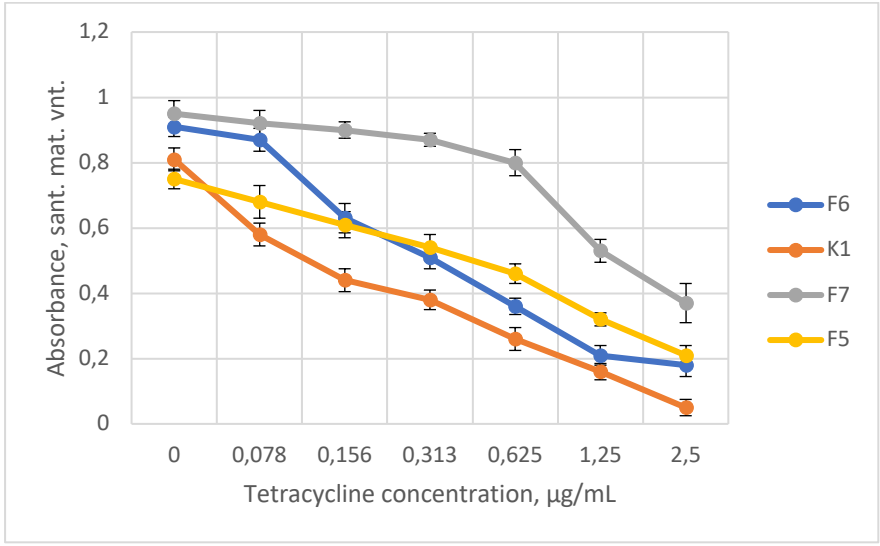

Figure 9: MIC method of F6, K1, F7 and F5 strains using tetracycline.

In the case of the graph of Figure 9, it can be observed that the four strains suffer the increase in antibiotic concentration in a not homogeneous or similar way. The growth of bacteria F6 and K1 is suppressed when the tetracycline concentration is 2.5 µg/mL. In the case of bacteria F5 and F7, their growth slows down partially but they manage to survive even at the maximum concentration of 2.5 µg/mL.

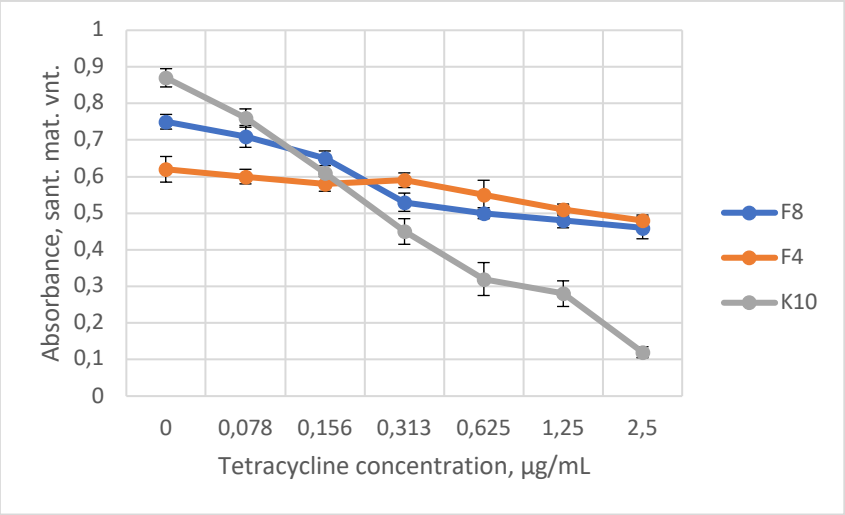

Figure 10: MIC method of F8, F4 and K10 strains using tetracycline.

In Figure 10, two different responses can be observed. F8 and F4 have a clear resistance to the antibiotic, and may even increase the population at higher concentrations, while the K10 strain is affected, stopping reproducing at the maximum concentration (2.5 µg / mL).

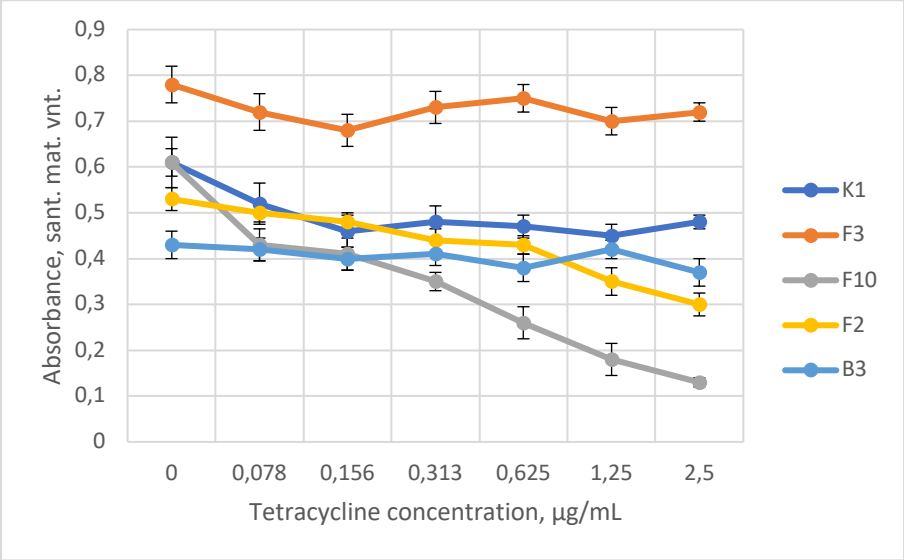

Figure 11: MIC method of K1, F3, F10, F2 and B3 strains using tetracycline.

In Figure 11, four strains of similar behavior and one strain that differs are distinguished. Only a clear decrease in the bacterial population can be observed in the case of strain F10 whose growth is completely suppressed when the concentration is 2.5 µg/mL. For the rest of strains, K1, F2, F3 and B3, no clear antibacterial effect is observed, so it can be said that they are resistant bacteria.

b) Chloranphenicol resistance:

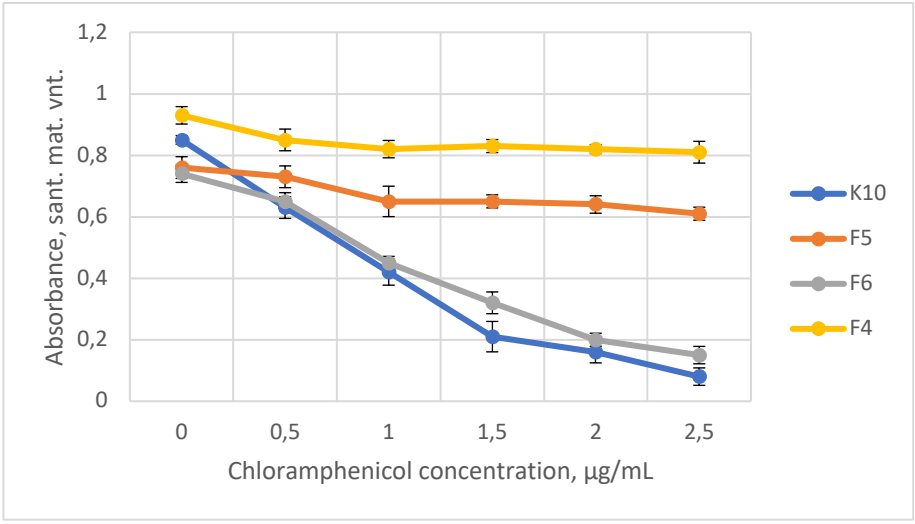

Figure 12: MIC method of K10, F6, F5 and F4 strains using chloramphenicol.

A clear difference can be observed between strains F6 and K10 versus F5 and F4 in Figure 12. The first two, show a sensitivity to the antibiotic, inhibiting their growth once the concentration of chloramphenicol reaches the maximum used (2.5 µg / mL). On the other hand, strains F5 and F4 show resistance to this antibiotic, maintaining a mainly constant growth despite the increasing concentration of antibiotic.

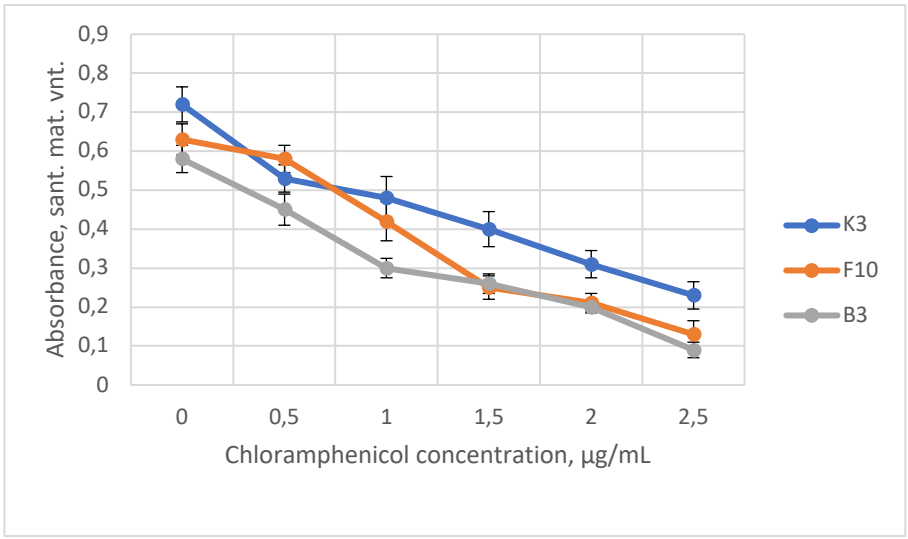

Figure 13: MIC method of K3, F10 and B3 strains using chloramphenicol.

In the case of the strains treated in the test of Figure 13, we can defend that they are bacteria sensitive to the antibiotic but with a higher MIC. The inhibition of these bacteria (K3, F10, B3) occurs when the concentration of chloramphenicol is 2.5 µg/mL. A greater sensitivity can be observed with F10 and B3 because their inhibition is more drastic than in the case of K3.

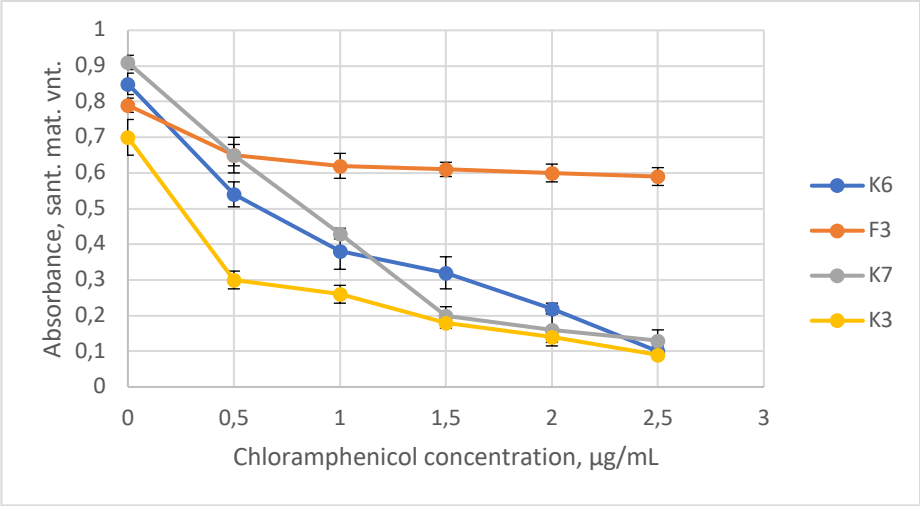

Figure 14: MIC method of K6, F3, K7 and K3 strains using chloramphenicol.

According to Figure 14, there are again two distinguishable behaviors. The inhibition for K6, K7 and K3 bacteria occurs when the concentration of antibiotics reaches the maximum of 2.5 µg/mL, the growth of bacteria in this concentration of chloramphenicol is suppressed. On the other hand, the growth of F3 bacteria is not affected by the presence of this antibiotic. A small initial decrease can be observed but after it, the bacterial population remains constant even at 2.5 µg/mL of chloramphenicol.

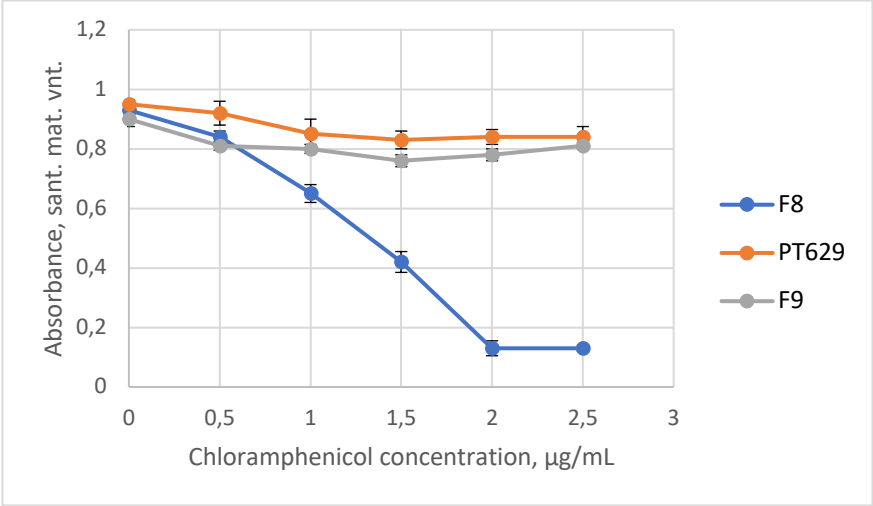

Figure 15: MIC method of F8, PT629 and F9 strains using chloramphenicol.

In this experiment we used the stock strain PT629 (check table 1A), corresponding to *Pseudomonas aeruginosa*. PT629 together with F9 present a clear resistance to this antibiotic, being able to use it as carbon source. For the F8 strain, the growth inhibition is gradual but constant, completely suppressed at 2 µg/mL of chloramphenicol.

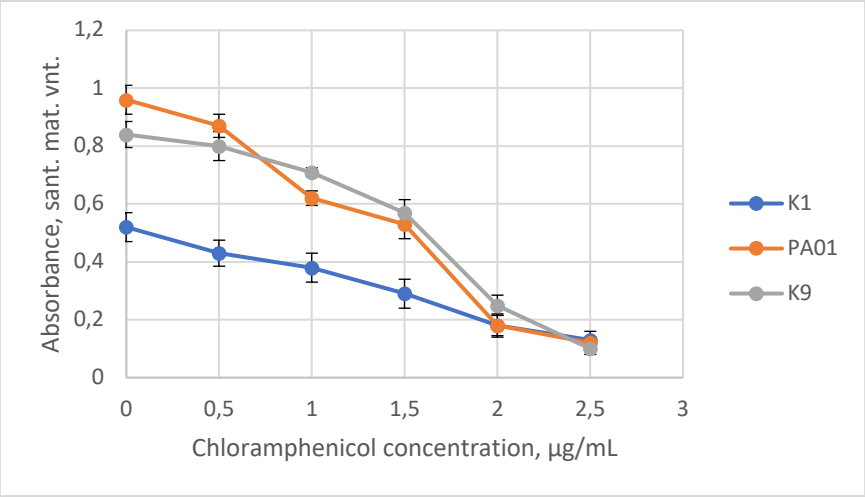

Figure 16: MIC method of K1 PA01 and K9 strains using tetracycline.

In this assay, although the K1 strain has a much lower initial population, the three bacteria studied behave similarly. In this case, stock strain PA01 (*Pseudomonas aeruginosa* wildtype) was used. PA01 and K1 are inhibited once the antibiotic concentration reaches the value of 2 µg/mL. Strain K9 is more clearly suppressed when the concentration reaches the maximum used, 2.5 µg/mL, even though it is already affected at the concentration of 2. However, for K9 and PA01 we can observe a more pronounced decrease than for K1.

After this resistance testing of several of our bacterial strains (listed in table 1A and 1B) it has been possible to prove, as a rule, a greater resistance to the chloramphenicol and tetracycline antibiotics in those bacteria from the farm samples; requiring the maximum concentration to suppress its growth and, sometimes, not even at such concentration is inhibited. It is also remarkable that when studying the strains from University's collection, the mutant strain shows resistance while the wildtype is clearly antibiotic sensitive.

4.2. TPP^+ measurement.

These experiments have been performed using the antibiotic Polymyxin B (prepared at a concentration of 2.5 mg/mL) and buffer TRIS at 100 mM and pH 8; at a constant temperature of 30 ° C. Due to the fact that we are working with Gram-negative bacteria, it is necessary to treat them previously with the EDTA agent to allow the TPP^+ entrance. The concentration of the ion in the medium will allow us to elucidate the state of the membrane potential of our bacteria throughout the process.

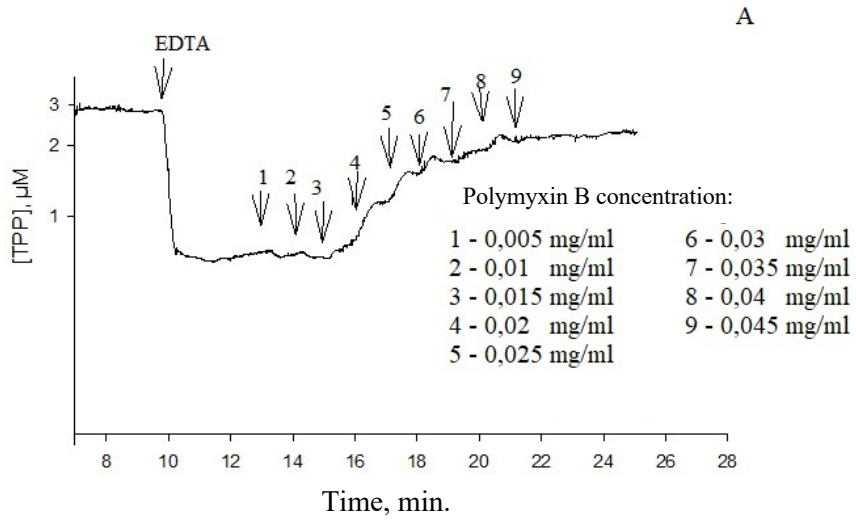

Figure 17: Bacterial membrane potential assay using TPP^+ in F3 strain.

Graph A (Figure 15) shows the graphic representation of the results obtained for the F3 strain. The concentrations of TPP^+ ion will be analyzed after adding Polymyxin B 9 times. Some membrane damage begins to be recognized when the concentration of the antibiotic is 0.02 mg/mL as it starts to expel TPP^+. To obtain a total expulsion of the ion, a concentration of 0.45 mg/mL is required.

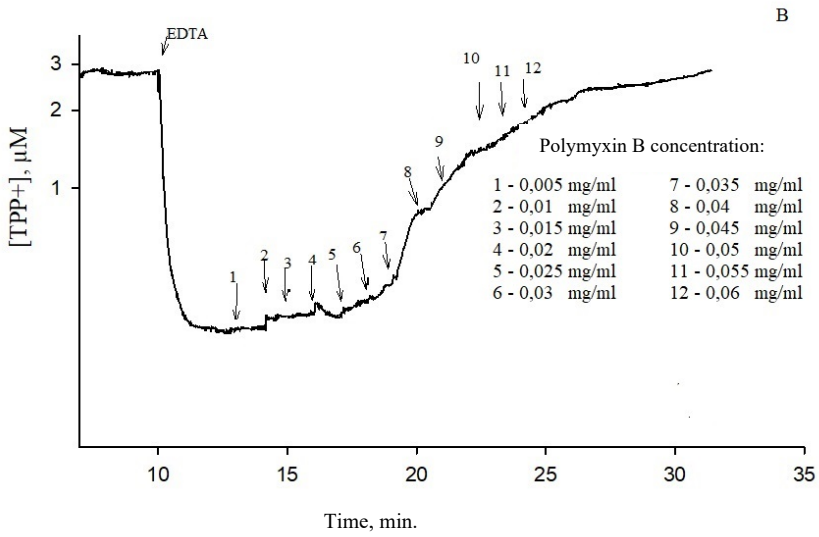

Figure 18: Bacterial membrane potential assay using TPP^+ in F9 strain.

12 inoculations of the antibiotic were necessary for the F9 strain. When the concentration of polymyxin B is 0.035 mg/mL, the bacterium starts to expel TPP^+, getting to expel it completely once the concentration reaches the value of 0.06 mg/mL.

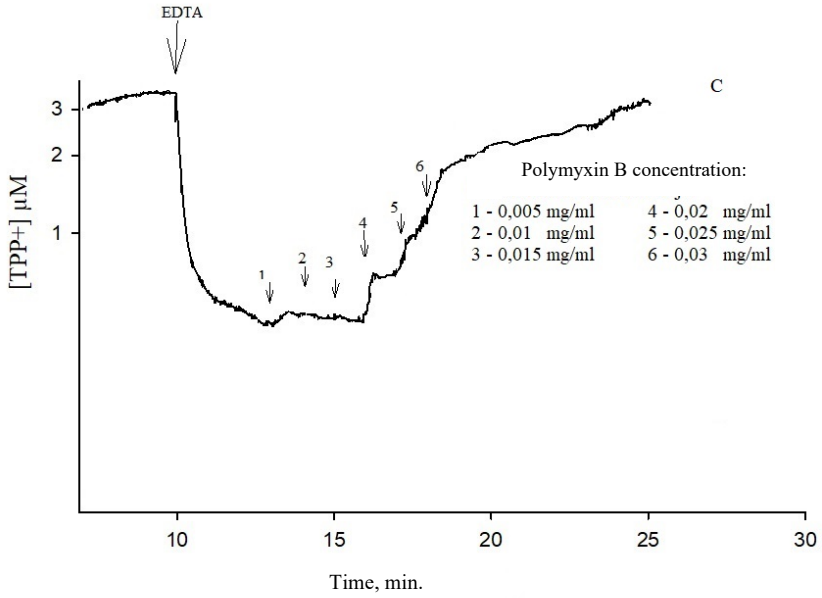

Figure 19: Bacterial membrane potential assay using TPP^+ in F10 strain.

For the F10 strain, 6 inocula of polymyxin B were required, finding the depolarization point when the antibiotic concentration is 0.03 mg/mL. The bacterium begins to expel TPP^+ when the polymyxin is at 0.015 mg/mL concentration.

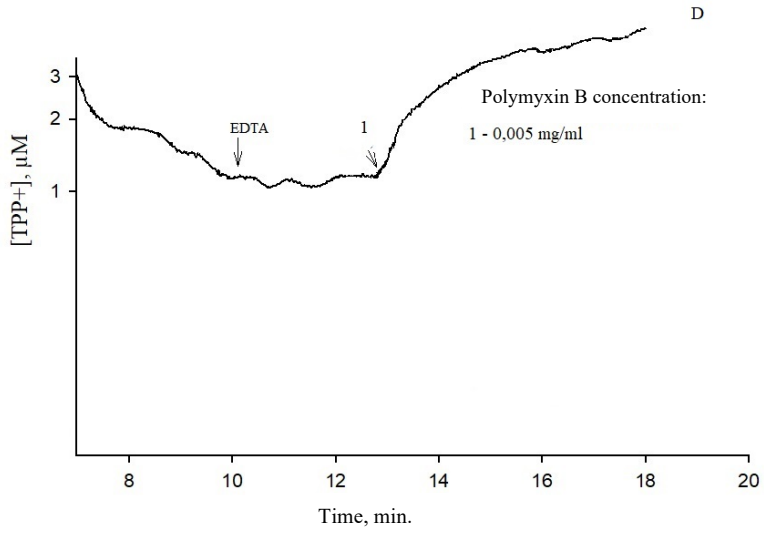

Figure 20: Bacterial membrane potential assay using TPP^+ in F8 strain.

Finally, for the F8 strain only a single addition of polymyxin B was needed. The complete depolarization occurs with a concentration of 0.005 mg/mL, demonstrating that we are facing a bacterium sensitive to the antibiotic used.

As it was possible to verify after obtaining the results for the MIC experiment, the bacteria obtained in the farms show a more noticeable resistance to polymyxin B, having to inoculate said antibiotic a greater number of times.

5. CONCLUSION:

a) After the results obtained in the MIC experiment, it has been possible to verify a clear resistance for the antibiotics chloramphenicol and tetracycline. Ten of the samples studied survived all the antibiotic concentrations used, and could not be found their minimal inhibitory concentration. Most of the resistant strains are those obtained in the farm, being also those that present a stronger resistance.

b) Electrochemical experiments enable us to observe how many inocula of the antibiotic were necessary to completely damage the bacterial membrane. Except for the strain F8 that has been found to be sensitive to the antibiotic used, the rest of the strains required numerous inocula to be affected, again demonstrating resistance this time against polymyxin B.

c) It was confirmed that several of our strains are multiresistant due to the fact that they are not inhibited by any of the three antibiotics used here. Of the twenty strains studied, we have observed a generalized sensitivity for the case of bacteria K1 or F6, however F8 presents sensitivity for polymyxin B but not for tetracycline, and vice versa with F10 and chloramphenicol/polymyxin B. Moreover, the same strains have not been studied the same against the three different antibiotics, hence the results for the three antibiotics for each bacterium cannot be compared.

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