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1542

Final Degree Project

Screening, isolation, and purification of active compounds against *Klebsiella pneumoniae*

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ABSTRACT

The spread of multi-resistant bacteria is a contemporary real threat that constitutes a public health risk. Resistance to antibiotics has increased progressively since their discovery due to their misuse and abuse. Therefore, finding new antimicrobial compounds has become a worldwide task.

By screening a culture collection, this study aimed to find bacteria and fungi able to inhibit the growth of *Klebsiella pneumoniae.* Replica plating, SPE-X columns, disk-diffusion assays, and agar-well diffusion assays are some of the techniques that have been used to isolate and purify the active compounds.

Of all microorganisms tested, *Epicoccum nigrum* (MES 1587) showed the most promising results. It was able to inhibit not only *K. pneumoniae,* but also *S. aureus, P. aeruginosa, B. subtilis, E. faecalis,* and *E. coli* in both disk-diffusion assays and agar-well diffusion assays. It was also shown that its inhibition started upon 8 days of culture and increased over time.

Regarding the rest of the microorganisms that inhibited *K. pneumoniae* in replica plating, it was impossible to reproduce the positive results after SPE-X purification.

1. INTRODUCTION

1.1. Features of Enterobacteriaceae family and *Klebsiella pneumoniae*

Enterobacteriaceae is a heterogeneous family of Gram-negative rod-shaped bacteria. They are ubiquitous and some of them are part of the intestinal flora of humans and animals. Their size, measuring 2 to 4 μm in length and 0.4 to 0.6 μm in width, is among the largest bacteria (Ryan et al., 2004).

Enterobacteriaceae species are further divided into multiple serotypes according to antigenic components of the cell wall and surface. The O antigen is related to the lipopolysaccharide, whose sugar composition determines its antigenic specificity. The K antigen is the polysaccharides that form the capsule. Lastly, the H antigen is associated with the peritrichous flagella that some motile strains possess (Ryan et al., 2004).

Regarding Enterobacteriaceae metabolism, they can ferment glucose, reduce nitrates to nitrites, and are oxidase negative. Some members may be able to ferment lactose, which serves as a differentiation tool for diagnosis by using MacConkey agar in primary isolation. Besides, they grow rapidly under both aerobic and anaerobic conditions. Some may also produce protein exotoxins which damage host cells by inhibiting metabolic pathways and ultimately might cause cell death (Ryan et al., 2004).

Concerning *Klebsiella pneumoniae*, it is a Gram-negative, encapsulated, and non-motile bacterium that belongs to the Enterobacteriaceae family. It ferments lactose and, as a facultative anaerobic microorganism, can perform aerobic respiration if oxygen is present and switch to fermentation if it is absent (Spagnolo et al., 2014).

Humans constitute the first reservoir of *K. pneumoniae* (Ashurst et al., 2022). Apart from living on plants, surface water, and soil, it is known for colonizing human mucosa surfaces of the oropharynx and gastrointestinal tract. Two of the main reservoirs of infection are the hands of the hospital personnel and medical devices. This added to the fact that is one of the most important opportunistic pathogens, it frequently leads to nosocomial outbreaks in sick patients that are being treated for other diseases (Spagnolo et al., 2014). In fact, it is responsible for causing 3 % to 8 % of all nosocomial bacterial infections in the United States (Jondle et al., 2018).

K. pneumoniae can display several virulence factors that lead to infection. The polysaccharide capsule is recognized as its most important virulence factor. So far, 78 different capsular serotypes (K antigen) have been characterized for *K. pneumoniae*. Serotypes K1 and K2 stand out as they are hyper-virulent due to they produce more capsule polysaccharides than the rest of the serotypes. The presence

of a capsule at the cell surface allows it to escape from opsonization and phagocytic cells (i.e., macrophages, neutrophils, or dendritic cells) (Li et al., 2014).

Lipopolysaccharides that cover the outer surface of *K. pneumoniae* are found to be another virulence factor (Ashurst et al., 2022). They release an inflammatory cascade in the host, which inhibits IL-8 production. Fimbriae, another virulence factor, permits *K. pneumoniae* to stick itself onto host cells. Type 1 fimbriae mediate the binding to structures that contain mannose, while type 3 fimbriae bind to collagen. Another virulence factor to be highlighted could be its different iron uptake systems. For *K. pneumoniae* to show full virulence it needs two ABC transporters and three siderophore systems (Li et al., 2014). This allows *K. pneumoniae* to take iron from the host and use it to continue with the infection (Ashurst et al., 2022).

Generally, healthy people don't get infected with *K. pneumoniae*. It has been associated with immunocompromised people, chronic alcoholism, or even diabetes mellitus. It can be acquired by oropharyngeal aspiration. The location of the infection will determine the symptoms and the treatment. Infection in the respiratory tract is characterized by the colonization of the upper lobes of the lungs. However, lower lobes can also be affected. Patients may show cough, fever, chest pain, and shortened breath. Besides, it can also cause urinary tract infections, skin infections, meningitis, and blood infection (Ashurst et al., 2022).

1.2. Antibiotics

A Nobel Prize-winning scientist, Paul Ehrlich defined the 'magic bullet' concept as a compound capable of killing disease-causing microorganisms without harming host cells (Ehrlich, 1960). Therefore, antibiotics can be considered magic bullets.

Generally, antibiotics are low molecular weight compounds (less than 1000 Da) secondary products than can be synthesized by bacteria, fungi, or plants (Fabbretti et al., 2011). They can inhibit bacterial growth or even kill bacteria and other microorganisms. Although antibiotics are natural compounds, some may have been modified by chemical changes to make them more effective.

Antibiotics can be classified according to several criteria. According to the effect they cause on bacteria, they can be defined as bacteriostatic, if they only inhibit growth; or bactericidal, if they not only cause growth inhibition but also kill bacteria (Ryan et al., 2004).

The spectrum of action of an antibiotic describes against which bacteria they can cause inhibition. According to this, some are known as narrow-spectrum agents, if they are only active against a few bacterial species; or as broad-spectrum agents, if they are active against several bacteria species (i.e., against Gram-positive and Gram-negative bacteria) (Ryan et al., 2004).

More important, antibiotics can be classified according to their mechanism of action. We can find four different groups (Ryan et al., 2004) (Jameson et al., 2016):

- **Antibiotics acting on cell wall synthesis**. Two kinds of antibiotics belong to this group: β-lactam and glycopeptide. The peptidoglycan of the bacterial cell wall is formed by a cross-linking structure between short peptide side chains and glycan molecules (N-acetylglucosamine and N-acetylmuramic acid). Both β-lactam and glycopeptide act inhibiting this cross-linking process. In the end, bacteria will die due to osmotic lysis as water will pass from the outside to the interior of the cell.

- **Inhibition of protein synthesis**. Almost every inhibitory-protein-synthesis-antibiotic is characterized by targeting ribosomes. There are two groups: the ones that bind to the 30S subunit (like tetracyclines), and those which bind to the 50S subunit (like macrolides).

- **Inhibition of nucleic acid synthesis**. Again, we have two groups. Those which cause inhibition of DNA synthesis (like quinolones), and those which inhibit RNA synthesis (like Rifampin).

- **Antibiotics act on the outer and cytoplasmic membrane**. Polymyxin B and E belong to this group. They bind to the cell membranes of Gram-negative bacteria modifying their permeability. The outcome is the loss of essential cytoplasmic components of the bacteria and, eventually, cell death. The advantage is that bacteria rarely develop resistance against them. The disadvantage is that that may react against host cell membranes.

- **Inhibition of bacterial metabolism**. They act on the folic acid synthesis pathway. Folic acid is essential for bacteria due to it acts as a cofactor in some nucleic acid and some amino acid synthesis.

1.3. Antimicrobial resistance of *Klebsiella pneumoniae*

Bacterial resistance to antibiotics is defined as the ability of a bacteria to survive antibiotic concentrations that inhibit or kill other bacteria of the same species (Alós, 2015). Among the increasing diffusion of resistant bacteria, what constitutes a crucial risk to public health is the spread of carbapenem-resistance Enterobacteriaceae (Spagnolo et al., 2014). Data from the European Centre for Diseases Prevention and Control (ECDC) shows that 29.5 % of isolated *K. pneumoniae* in Italy is resistant to carbapenems, and 54.3 % to third-generation cephalosporins.

Regarding *K. pneumoniae*, there are progressively more strains resistant to carbapenems (Spagnolo et al., 2014). Carbapenems belong to the β-lactam family, therefore, they act on inhibiting cell wall synthesis. They appear to penetrate easily

through both Gram-negative and Gram-positive bacteria, and they are very resistant to β-lactamases (Ryan et al., 2004).

Resistance to carbapenems is usually exerted by the production of β-lactamases, enzymes that hydrolyses β-lactam rings. In *K. pneumoniae*, the most common β-lactamase is called KPC and is encoded by the *bla*KPC gene. It can be spread to other enterobacteria and even to *Pseudomonas aeruginosa* (Grundmann et al., 2010). Moreover, carbapenem-resistant *K. pneumoniae* strains are associated with a considerable increase in illness and death (Sanchez et al., 2013).

2. OBJECTIVES

The main objective of this project has been to try to find bacteria and fungi that can inhibit the growth of *Klebsiella pneumoniae.* Once the screening of microorganisms has been carried out, active secondary metabolites have been pre-concentrated and tested against *K. pneumoniae*.

3. MATERIALS AND METHODOLOGY

3.1. Preparation of media and bacteria cultures

The screening has been carried out from the UNICAM culture collection which includes ~ 2000 microorganisms isolated from air, soil, and water. The collection was stored at -80 ºC and frozen alongside glycerol, which is a cryoprotectant. The collection is sorted according to an alphanumeric code. 'MES' stands for mesophile microorganisms, therefore, their optimal growth temperature is from 20 to 45 ºC (Prescott et al., 2008).

48 different microorganisms have been tested against *Klebsiella pneumoniae*.

Table 1 shows the media composition used in this work to prepare the bacteria cultures.

Table 1. Culture media used and their composition.

Culture media	Composition per litre
Luria-Bertani (LB)	Tryptone, 10 g Yeast extract, 5 g Sodium chloride, 5 g Agar, 17 g
Potato Dextrose Agar (PDA)	Potato extract, 4g Dextrose, 20 g Agar, 15 g

Culture media	Composition per litre
Reasoner's 2A Agar (R2A)	Casein hydrolysate, 0.5 g Dextrose, 0.5 g Dipotassium phosphate, 0.3 g Magnesium sulfate, 0.024 g Proteose peptone, 0.5 g Sodium pyruvate, 0.3 g Soluble starch, 0.5 g Yeast extract, 0.5 g Agar, 15 g
Tryptone Soya Agar (TSA)	Casein peptone (pancreatic), 15 g Sodium chloride, 5 g Soya peptone (papainic), 5 g Agar, 15 g

The first step was to prepare the Petri dishes where the microorganisms from the collection would be inoculated. A circumference of 3 cm in diameter was drawn in the middle of the dishes. Then, the glycerol was let be melted, and the inoculation of the microorganisms was performed just inside and all over the circle. After that, they were let to grow at their optimal temperature (15 ºC, 30 ºC, or 37 ºC).

Once the inoculated microorganisms had spread inside the whole circle (Fig. 1), a plate containing *K. pneumoniae* was made for the replica process. Liquid LB-medium culture was prepared from a fresh solid culture of *K. pneumoniae* and was let grow overnight at 37 ºC. After an incubation of 12 hours, LB Petri dishes were inoculated with *K. pneumoniae* by using a sterile swab all over the surface. After 6 hours of growth, *K. pneumoniae* was ready for the replica plating.

3.2. Replica plating

For replica plating, a sterile velvet, a block, an elastic band, the plate with the microorganism to test, and the *K. pneumoniae* plate were needed. Firstly, the block and the elastic band had to be sterilized with ethanol. Following this, the sterile velvet is placed on top of the block and fixed with the band. Then, the *K. pneumoniae* plate is placed on the velvet and some light taps are needed to imprint *K. pneumoniae* colonies on it. Lastly, the microorganism to test is placed on the velvet which will contain the imprinted *K. pneumoniae*. The plate is incubated at room temperature for 24 hours. The results can be:

- **No inhibition**. *K. pneumoniae* has grown homogeneously all over the plate and there is no halo of inhibition. This means that the tested microorganism does not produce any metabolites that either inhibit or kill *K. pneumoniae*.

- **Inhibition**. There is a halo of inhibition around the microorganism in the center of the plate. This means that the tested microorganism produces metabolites that either inhibit or kill *K. pneumoniae*.

- **Growth reduction**. There is not a halo of inhibition, but *K. pneumoniae* has not grown uniformly all over the plate (Fig. 2). This means that the tested microorganism produces metabolites that will be required in larger amounts to inhibit *K. pneumoniae* growth.

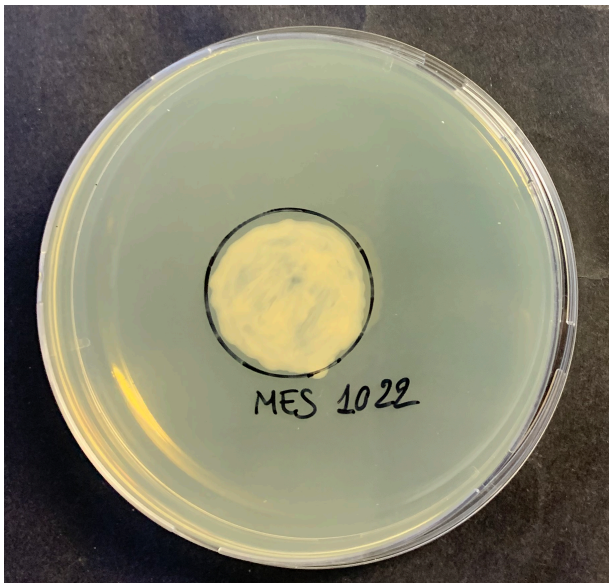


Figure 1. MES 1022 growing in LB medium after defrost from -80 ºC storage.

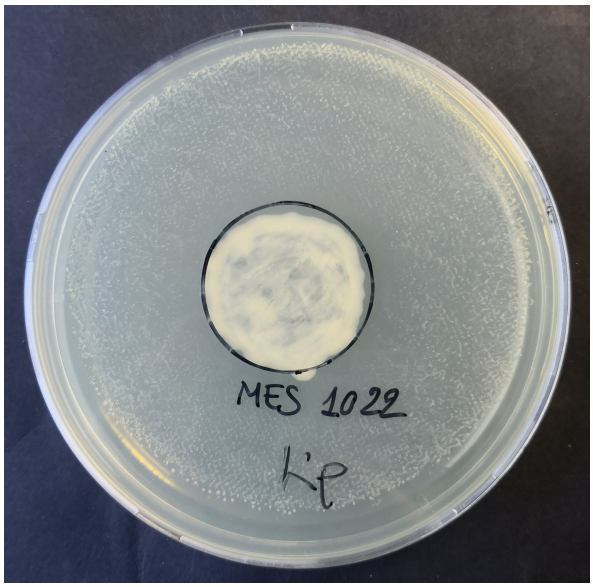


Figure 2. MES 1022 and *K. pneumoniae* after replica plating.

After this screening, the microorganisms that caused inhibition (see Table 5) are selected to perform the pre-concentration of the inhibitory metabolites. Once selected, they were inoculated in 5 ml of liquid medium. When they grew, 1 ml of that inoculum is re-inoculated into 50 ml of medium and is left to grow.

3.3. SPE-X pre-concentration and antibiotic tests

The pre-concentration step (SPE-X column) is performed as soon as they have grown. To remove the cells from the liquid medium, centrifugation at 10,000 rpm for 20 minutes is carried out. The pellet is discarded and the supernatant (50 ml) is loaded onto the SPE-X column. Methanol, water, and 20 %, 80 %, and 100 % of

acetonitrile are used in this process. The protocol for concentration and purification of secondary metabolites by SPE-X can be summarized in 5 different steps:

I. Activation of the column. It is achieved by pouring 2 volumes of the column of methanol.

II. Column wash. 2 volumes of water are used to remove any traces of methanol.

III. The supernatant (50 ml) obtained after centrifugation is loaded. In this step, all the hydrophobic molecules will bind to the column.

IV. Wash again with water to remove all the hydrophilic molecules.

V. Elution step. The hydrophobic metabolites were eluted at increasing concentrations of acetonitrile and 12 fractions of 1.5 ml were collected in 2 ml Eppendorf tubes (Fig. 3). 3 tubes for 20 % acetonitrile, 6 tubes for 80 % acetonitrile, and 3 tubes for 100 % acetonitrile.

100 µl aliquots of each tube were taken and lyophilized. After the lyophilization, fractions were dissolved with 12 µl of dimethyl sulfoxide (DMSO) and tested by diffusion assays (see below).

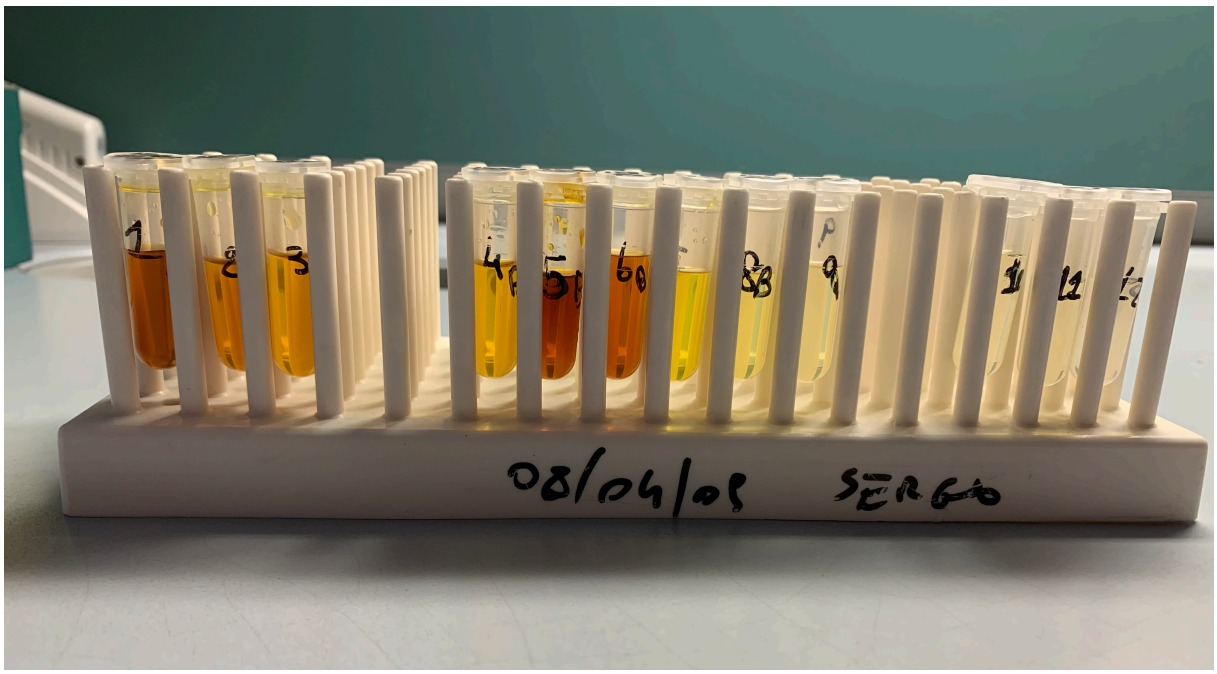


Figure 3. Fractions from the SPE-X collected. The different colours of each fraction can be appreciated due to the variety of molecules present in each one.

To test the antimicrobial properties of the isolated molecules, filter paper disks were placed on an LB Petri dish previously inoculated with *K. pneumoniae*. These disks

were impregnated with 10 µl of the antibiotic-DMSO solution, so all 12 fractions were tested (Fig. 4). The plates were incubated at 37 ºC for 12 hours.

Another test that was carried out to evaluate the antibiotic properties of the isolated molecule was the agar-well diffusion assay (Fig. 5). Like the disk-diffusion assay, a Petri dish was inoculated all over the surface with *K. pneumoniae*. Then, with the help of sterile pipette tips, holes were made in the agar, and 200 µl of the extract solution was poured inside the hole. Results were obtained after 12 hours of incubation at 37 ºC.

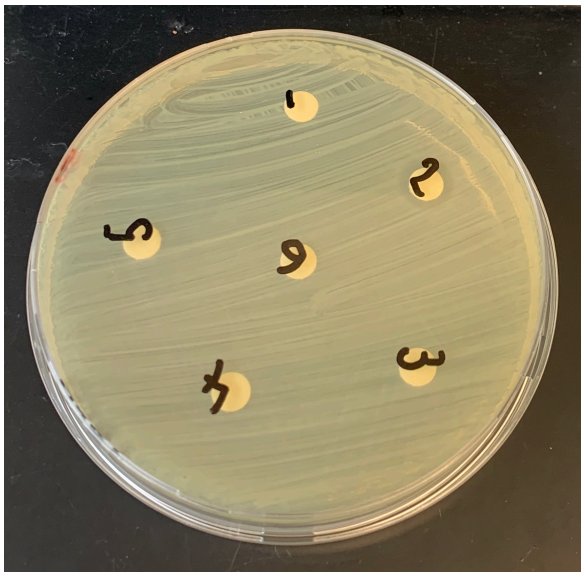


Figure 4. Example of disk-diffusion assay in which there were tested 6 fractions eluted from SPE-X column. A tiny halo of inhibition can be appreciated in fraction number 2.

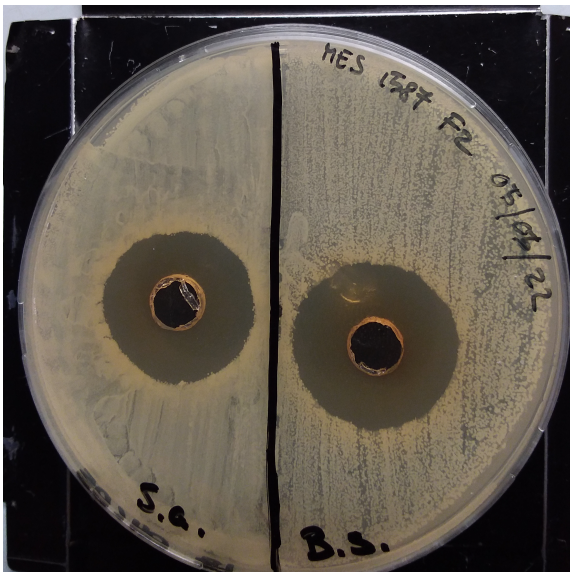


Figure 5. Example of agar-well diffusion assay. Two haloes of inhibition can be appreciated.

3.4. DNA extraction and PCR of MES 1587

By using the screening described above, the microorganism identified with the code MES 1587 was selected for its ability to inhibit *K. pneumoniae* (see the results section). Based on its morphology, MES 1587 was initially classified as fungi. To identify this microorganism at the species level, it was necessary to sequence a particular region of its DNA (Fig. 6).

The chromosomal DNA of MES 1587 was extracted with E.Z.N.A.® Water DNA Kit, which is perfectly adequate for the isolation of fungi DNA. The isolated DNA was used to perform PCR amplification.

First, a mixture containing the PCR buffer, the dNTPs, primer MOL84, primer MOL85, the enzyme Taq Polymerase, and water was prepared. In table 2, the components, their volume, and their concentration are shown.

Table 2. Components, volume and final concentration of the PCR mixture.

Components	Volume	Final concentration
10X PCR buffer	21 µl	1X
2.5 mM dNTPs	8.4 µl	100 µM
10 µM MOL84	8.4 µl	0.4 µlM
10 µM MOL85	8.4 µl	0.4 µlM
5 U/µl Taq polymerase	1.05 µl	0.025 U/µl
H_2O	106.75 µl	-
Final volume	154 µl	

Table 3 shows the volume of MES 1587 DNA, mix, and water that each PCR tube contained.

Table 3. Volume of DNA, mixture and water used in each tube.

Tube	DNA	Mixture	H_2O	Total volume
1	-	22 µl	8 µl	30 µl
2	1 µl	22 µl	7 µl	30 µl
3	2 µl	22 µl	6 µl	30 µl
4	4 µl	22 µl	4 µl	30 µl
5	6 µl	22 µl	2 µl	30 µl
6	8 µl	22 µl	-	30 µl

Lastly, Table 4 shows the temperature and the time for each step of the PCR.

Table 4. Temperature, time and cycles of each step of the PCR.

Step	Temperature	Time	Number of cycles
Initial denaturation	95 ºC	4‘	1
Denaturation	94 ºC	45‘‘	38
Annealing	55 ºC	30‘‘	
Elongation	72 ºC	40’’	
Final elongation	72 ºC	3’	1

Denaturation, annealing, and elongation were repeated 38 times before the final elongation step. The next figure shows the rRNA region which was amplified and the location of the MOL84 and MOL85 primers. The amplified region entails 500 bp approximately.

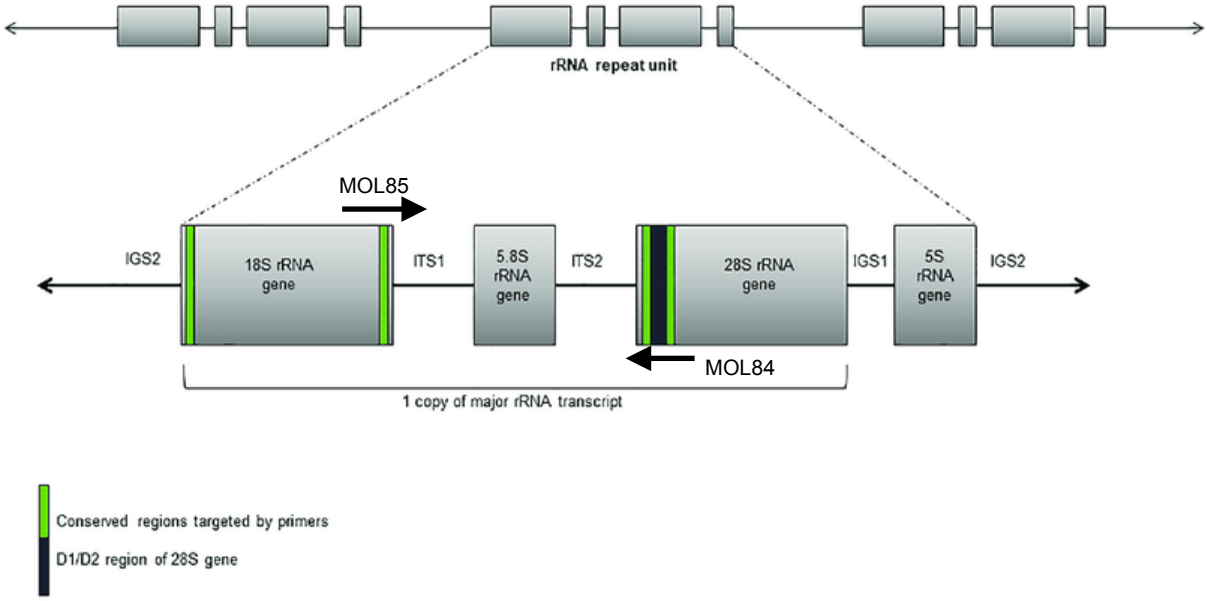


Figure 6. rRNA region which was amplified in PCR, and the location of MOL84 and MOL85 primers. (Kidd et al., 2020).

To see if the samples were correctly amplified, electrophoresis in 1.5 % agarose was carried out. 12 µl of each sample were loaded onto the gel and the results were observed under UV light.

Lastly, 70 ng of amplified DNA was sequenced by Eurofins Genomics.

4. RESULTS AND DISCUSSION

4.1. Replica results.

In this study, a screening of ~ 50 microorganisms belonging to the Unicam culture collection was performed with the aim to find bacteria and fungi that can inhibit the growth of the Gram-negative pathogen *Klebsiella pneumoniae*. Table 5 shows the results obtained by the replica plating technique of all microorganisms tested against *Klebsiella pneumoniae*.

Table 5. Features and inhibition of tested MES against K. pneumoniae.

Code	Environmental sample	Culture medium	Temperature of incubation (º C)	Days of incubation	Inhibition
MES 64	Fresh water	LB	15	7	No
MES 82	NDA	LB	15	2	No
MES 93	Sea water	LB	15	4	Growth reduction
MES 100	Soil	LB	15	10	Yes
MES 101	Soil	LB	15	10	Yes
MES 103	Soil	R2A	15	8	Yes
MES 111	Sea water	LB	15	5	No
MES 151	Waste water	LB	15	4	No
MES 153	Fresh water	LB	15	7	Growth reduction
MES 204	Organic matter	LB	15	No growth	
MES 219	Fresh water	R2A	15	10	Yes
MES 259	Waste water	LB	15	8	No
MES 280	Fresh water	R2A	15	8	No
MES 339	Soil	LB	15	5	Yes
MES 354	Organic matter	R2A	15	7	No
MES 402	Sea water	TSA	15	7	No
MES 488	Fresh water	R2A	15	8	Growth reduction
MES 494	Fresh water	R2A	15	8	Yes
MES 495	Fresh water	R2A	15	8	Yes

MES 638	Fresh water	TSA	15	4	No
MES 643	Fresh water	TSA	15	8	Growth reduction
MES 1037	NDA	R2A	15	6	Growth reduction
MES 1160	NDA	R2A	15	6	No
MES 1161	Fresh water	R2A	30	No growth	
MES 1162	Fresh water	R2A	15	No growth	
MES 1163	NDA	R2A	15	No growth	
MES 1164	Fresh water	R2A	15	6	No
MES 1224	Fresh water	TSA	15	7	No
MES 1295	NDA	PDA	30	No growth	
MES 1296	Soil core	PDA	15	No growth	
MES 1305	Soil core	TSA	15	10	No
MES 1312	NDA	LB	37	6	No
MES 1320	Soil core	PDA	37	4	Growth reduction
MES 1343	Soil core	R2A	15	5	No
MES 1345	Soil core	PDA	15	10	No
MES 1395	Soil core	PDA	15	4	No
MES 1402	Soil core	LB	15	No growth	
MES 1403	Soil core	LB	15	8	Growth reduction
MES 1404	NDA	R2A	15	5	No
MES 1413	Soil core	R2A	15	6	No
MES 1414	Soil core	R2A	30	5	No
MES 1418	Soil core	R2A	30	5	No
MES 1419	Sea water	R2A	30	5	No
MES 1423	NDA	LB	30	5	No
MES 1424	Soil core	LB	30	5	No
MES 1426	Soil core	PDA	30	2	No
MES 1540	NDA	R2A	15	6	Yes
MES 1587	Volcanic soil	PDA	15	21	Yes

The strains that caused inhibition in the initial replica screening were: **MES 100, MES 101, MES 103, MES 219, MES 339, MES 494, MES 495, MES 1022, MES 1540, and MES 1587**.

4.2. Antimicrobial activity test results for inhibitory strains.

Among the positive strains, MES 103, MES 219, MES 494, MES 495, MES 1022, and MES 1540 were selected to perform the SPE-X column and the antimicrobial activity tests.

The agar-disk diffusion assay was carried out in all of them. Just **fraction number 2 of MES 1540** produced a halo of inhibition, specifically, 2 mm wide (Fig. 4). The rest of them brought negative results. Additionally, to exclude that the loss of activity of the molecules is caused by a lack of interaction between these molecules and the SPE-X column, the flow-through of all selected MES was tested with the agar-well diffusion test, but none caused inhibition (Fig. 7).

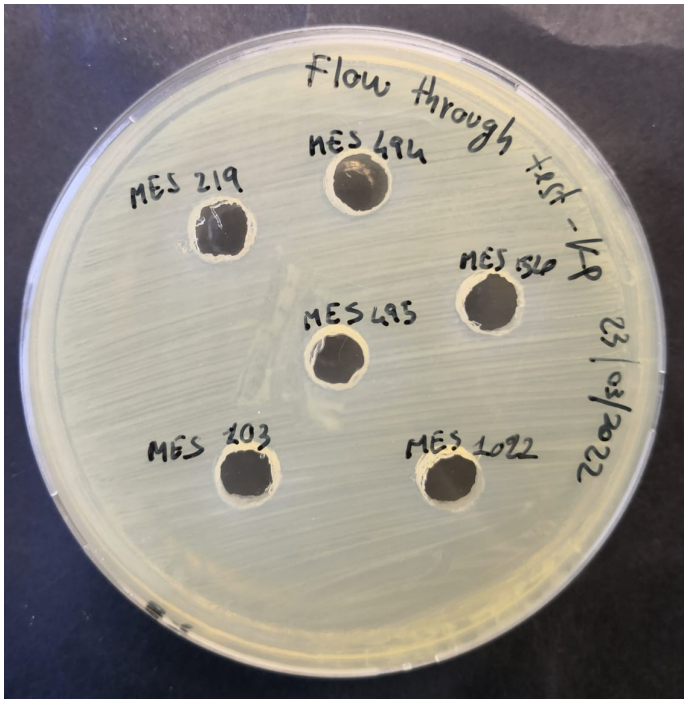


Figure 7. Flow through from all bacteria that were able to inhibit *K. pneumoniae* in replica, tested in agar-well diffusion assay. No one was able to cause inhibition.

There may be many reasons and possibilities that can explain why there was inhibition in replica plating, but there was not either in the agar-disk diffusion assay or the agar-well diffusion assay. Firstly, the conditions in which both experiments were carried out were different. After performing the replica, Petri plates were left at room temperature (23 ºC approximately), while Petri plates in the diffusion assays were

incubated at 37 ºC. Considering the optimal growth temperature of *K. pneumoniae* is 37 ºC (Bengoechea and Sa Pessoa, 2018), *K. pneumoniae* could have grown stronger in the diffusion assays than in replica plating. Therefore, *K. pneumoniae* could be easier to inhibit its growth in replica plating rather than in the diffusion assays.

Another reason could be that the inhibitory molecules are unstable in acetonitrile and they undergo spontaneous degradation during the purification process.

4.3. Results of MES 1587.

MES 1587 is a red mold isolated from volcanic soil. Its optimal growth temperature ranges from 20 to 37 ºC. Figures number 8 and 9 show the appearance of the red mold in liquid and solid culture, respectively.

Figure 8. Appearance of MES 1587 in liquid medium.

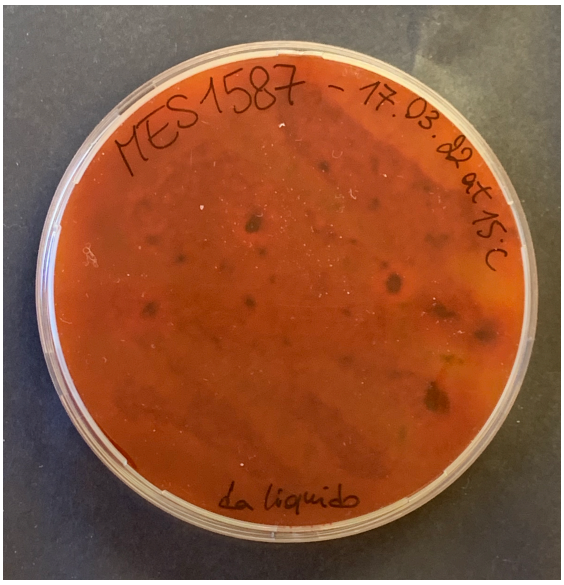


Figure 9. Appearance of MES 1587 on solid culture.

When the inhibitory potential of MES 1587 against *Klebsiella pneumoniae* was discovered, disk-diffusion and agar-diffusion assays were performed. Regarding the first assay, it was performed not only on *Klebsiella pneumoniae* but also on *S. aureus, B. subtilis, P. aeruginosa, E. faecalis,* and *E. coli.* Table 6 and Figure 10 show the size of the halo of inhibition against these bacteria.

Table 6. Halo of inhibition of MES 1587 on bacteria in disk-diffusion assay.

Bacteria	Halo of inhibition (mm)
Staphylococcus aureus	23
Bacillus subtilis	22

Pseudomonas aeruginosa	8
Enterococcus faecalis	8
Escherichia coli	11
Klebsiella pneumoniae	18

Moreover, the inhibitory capacity of MES 1587 on these bacteria was also tested with the agar-well diffusion assay. Table 7 and Figure 11 show the size of the halo of inhibition against these bacteria.

Table 7. Inhibitory halo of MES 1587 on bacteria in agar-well diffusion assay.

Bacteria	Halo of inhibition (mm)
Staphylococcus aureus	24
Bacillus subtilis	26
Pseudomonas aeruginosa	10
Enterococcus faecalis	15
Escherichia coli	11
Klebsiella pneumoniae	21

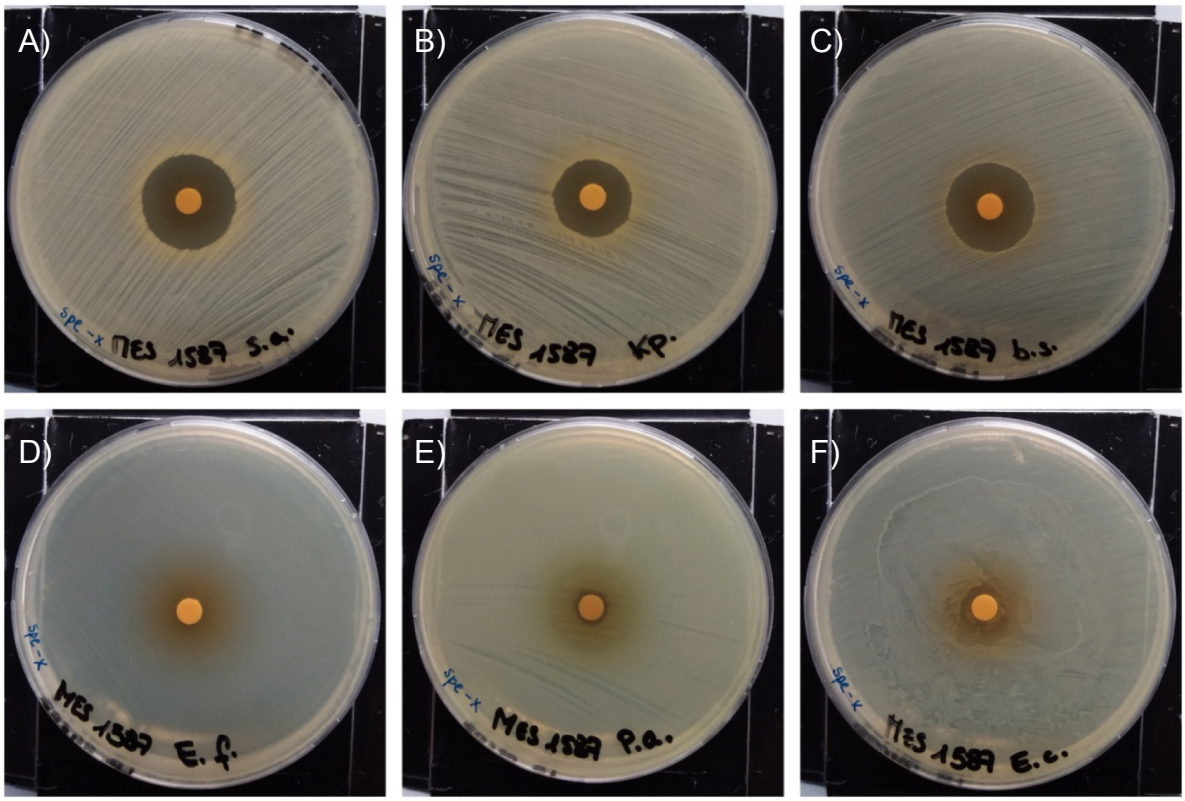


Figure 10. Disk-diffusion assay of MES 1587 against A) *S. aureus, B) K. pneumoniae, C) B. subtilis, D) E. faecalis, E) P. aeruginosa and F) E. coli.*

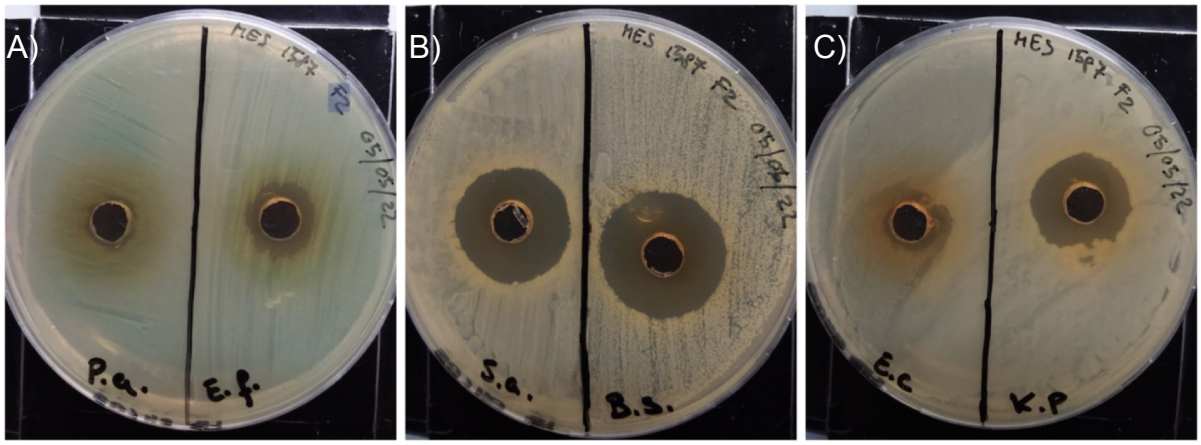


Figure 11. Agar-well diffusion assay of MES 1587 on A) *P. aeruginosa and E. faecalis, B) S. aureus* and *B. subtilis and C) E. coli and K. Pneumoniae.*

From the antimicrobial tests, it can be inferred that the secondary metabolites secreted by MES 1587 are more active against *S. aureus*, *B. subtilis,* and *K. Pneumoniae,* and less active, but still causing inhibition, against *P. aeruginosa*, *E. faecalis*, and *E. coli*. The most impressive result is that it has been able to inhibit both Gram-positive and Gram-negative bacteria.

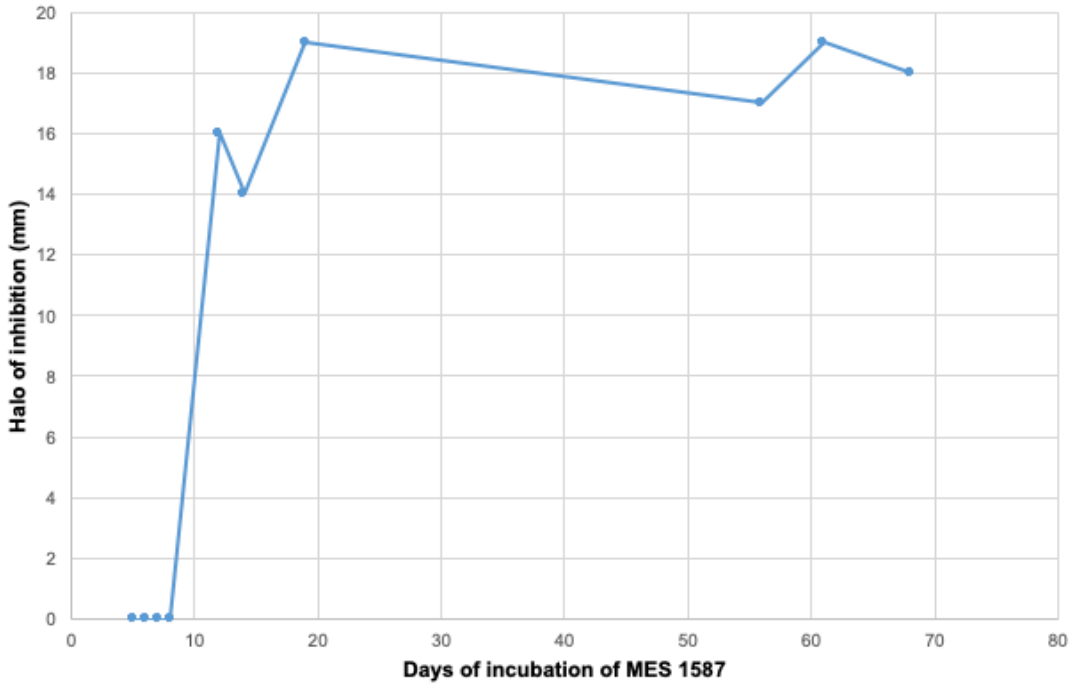
In addition, the inhibitory potential of MES 1587 against *K. pneumoniae* was tested over time. Table 8 exposes how the size of the halo of inhibition changes when increasing the days of culture of MES 1587.

Table 8. Halo of inhibition of MES 1587 over time.

Days of incubation	Halo of inhibition (mm)
5	0
6	0
7	0
8	Growth reduction
12	16
14	14
19	19
56	17
61	19
68	18

Measuring the inhibition haloes of the agar-well diffusion assay allows a graph to be made in which the inhibition can be observed against MES 1587 days of incubation.

Figure 12. Halo of inhibition vs. Days of incubation of MES 1587.



As it can be observed, up until day 7, there was no inhibition at all. On day 8, growth reduction can be appreciated, so it is when the inhibitory molecules are started to be synthesized and accumulated in the medium. On day 12, significant inhibition was already obtained (16 mm). This inhibition increases up to 17-19 mm after three weeks of growth and remains stable over time.

In order to classify MES 1587, DNA extraction and PCR amplification of the ITS region were performed as described in the methodology section. Figure 13 shows the PCR results visualized by agarose gel electrophoresis.

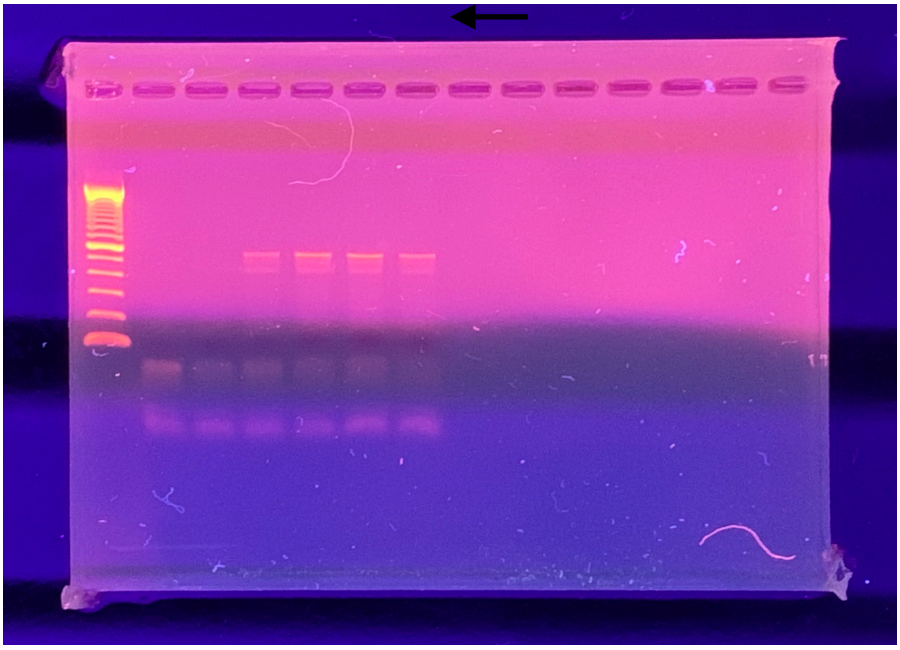


Figure 13. Electrophoresis results of the amplification of MES 1587 DNA made by PCR. Lane 1 corresponds with the marker, and lanes 2 to 7 correspond with tube 1 to 6, respectively. The arrow points at the amplificated region.

As it can be appreciated, tubes 4 and 5 (lanes 5 and 6, respectively) got the greatest amplification and proved that the extracted DNA was enough for Sanger sequencing.

Below, is shown the DNA sequence of the amplified ITS region of the MES 1587.

NNNNCTTTTGGGACGTCGTCGTTATGAGTGCAAAGCGCGAGATGTACTGCGCTC
CGAAATCAATACGCCGGCTGCCAATTGTTTTAAGGCGAGTCTACACGCAGAGGC
GAGACAAACACCCAACACCAAGCAGAGCTTGAAGGTACAAATGACGCTCGAACA
GGCATGCCCCATGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGAT
TCACTGAATTCTGCAATTCACACTACTTATCGCATTTCGCTGCGTTCTTCATCGAT
GCCAGAACCAAGAGATCCGTTGTTGAAAGTTGTAACTATTATGTTTTTTCAGACGC
TGATTGCAACTGCAAAGGGTTTGAATGTTGTCCAATCGGCGGGCGGACCCGCC
GAGGAAACGAAGGTACTCAAAAGACATGGGTAAGAGGTAGCAGACCGAAGTCTA
CAAACTCTAGGTAATGATCCTTCCGCNNNNNNN

Once the results came from Eurofins Genomics, a sequence alignment was carried out with the BLAST tool of the National Center for Biotechnology Information (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment suggests that MES 1587 corresponds to ***Epicoccum nigrum***.

5. CONCLUSIONS

Considering some bacteria were able to inhibit *Klebsiella pneumoniae* in replica plating, but they couldn't either in paper disk-diffusion assay or agar-well diffusion assay, it can be asserted two statements:

1. Replica plating and antibiotic assays need to be performed in similar conditions, especially for those bacteria that are just able to inhibit slightly the growth of *K. pneumoniae.*

2. Both the replica plating, antibiotic tests, and SPE-X should be performed more than once to conclude with certainty that a bacterium does or does not inhibit the growth of Klebsiella pneumoniae or any other microorganism.

Additionally, as it has been shown, *Epicoccum nigrum* has demonstrated a great capacity to inhibit not only *K. pneumoniae,* but also *S. aureus, P. aeruginosa, B. subtilis, E. faecalis,* and *E. coli.* Further investigation of the antimicrobial ability of *E. nigrum* could lead to the discovery of new inhibitor compounds.

6. PERSONAL APPRECIATION

This project has allowed me to improve my general microbiology knowledge. As for the laboratory experience, it has permitted me to practice 'in real-life' those microbiology techniques that I studied in my second year of biotechnology as well as learn new ones that I had never heard about.

I would like to thank my supervisor, Attilio Fabbretti, for guiding me in the course of this project and it has been a pleasure to work with him. The same I can say for Lucia Cimarelli, who is an open microbiology book.

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