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PROSPECTIVE STUDY ON INTESTINAL DECOLONIZATION OF MULTIDRUG-RESISTIANT GRAM-NEGATIVE BACTERIA CARRIERS. (PRECLINICAL STUDY)

THESIS

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TO OBTAIN MEDICAL DOCTORATE

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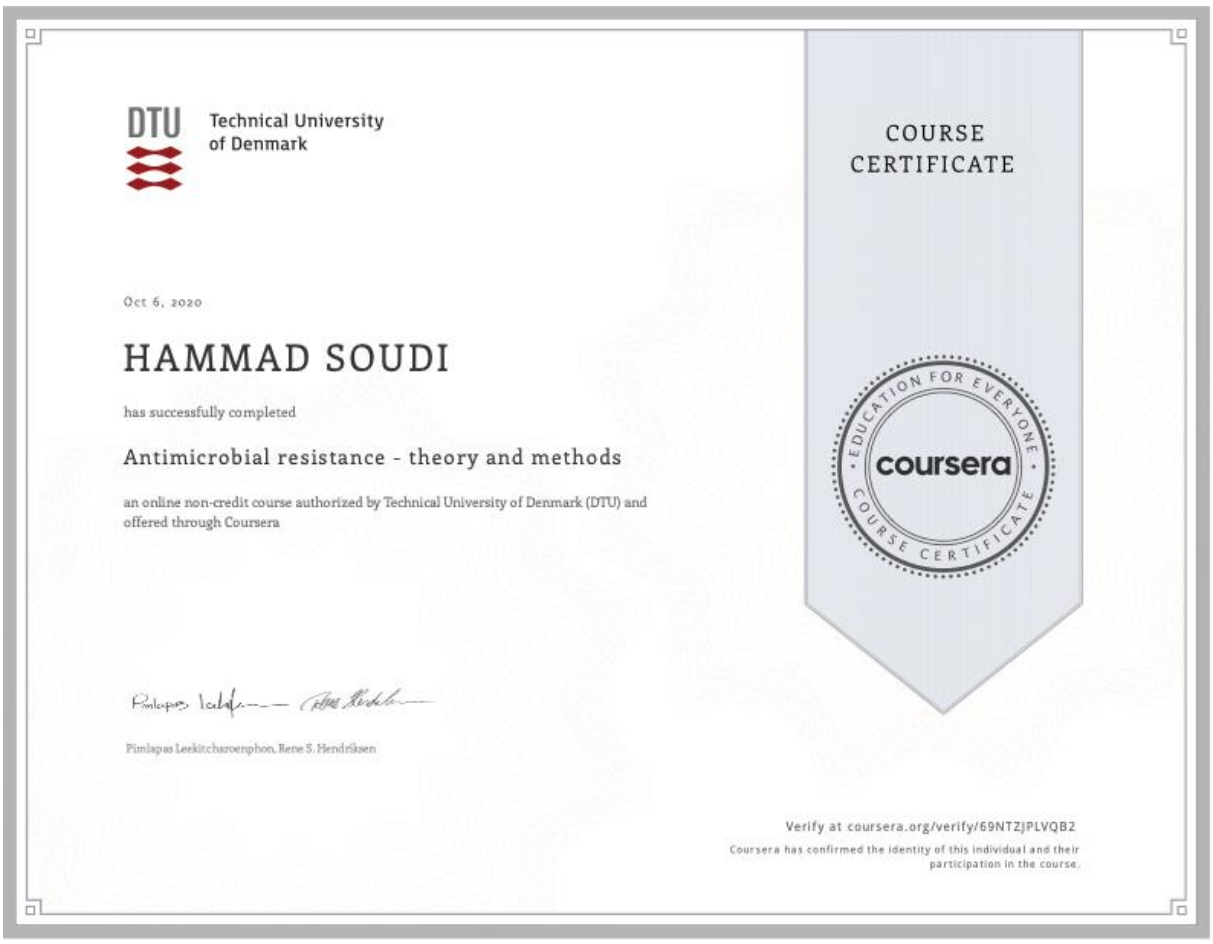

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LIST OF ABBREVIATIONS

AK: Amikacin

AMC: Amoxicillin / clavulanic acid

AMR: Antimicrobial resistance

ASC: Active surveillance cultures

C3G: Cephalosporin 3rd generation

CAZ: Ceftazidime

CDC: Centers for Disease Control and Prevention

CFM: Cefixime

CFR: Cefadroxil

CFU: Colony Forming Unit

CI: Confidence Interval

CIP: Ciprofloxacin

CP: contact precaution

CPE: Carbapenemase- producing Enterobacteriaceae

CTX-M: Cefotaxinase-Munich

E. coli: Escherichia coli

EMB: Eosin methylene blue

EO: Essential oil

EPE: ESBL-producing Enterobacteriaceae

ERT: Ertapenem

ESBL-E: Extended-spectrum beta-lactamase-producing Enterobacteriaceae

FEP: Cefepime

FMT: Fecal microbiota transplantation

ICU: Intensive care unit

IMP: Imipenem

IMVIC: Indole Methy red, Voges Proskawer, citrate

K.p: Klebsiella pneumonia

MDR: Multidrug Resistant

MDRGN: Multidrug resistant GRAM-NEGATIVE

MDRO: Multidrug resistant organisms

MRSA: Methicillin Resistant Staphylococcus Aureus

PLP: Penicillin-binding protein

PRL: Pipracillin

PTZ: Pipracillin-tazobactam

ROC: Receiver operating characteristic

SD: Standard deviation

SXT: Trimethoprim-Sulfamethoxazole

TIC: Ticarcillin

VRE: Vancomycin Resistant Enterococcus

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

General introduction

Bacterial resistance to antibiotics is currently one of the most severe threats to global health[1] [2] [3]. Infectious diseases caused by multidrug-resistant (M.D.R.) bacteria will be the leading cause of death from disease in 2050, according to a report on antibiotic resistance around the world. More than 10 million deaths are expected to occur per year worldwide, compared to 700,000 currently. It is a higher rate than that caused by cancer[4].

Extended-spectrum beta-lactamase-producing Enterobacteriaceae (ESBL-E) and carbapenemase-producing Enterobacteriaceae (C.P.E.) have recently been classified by the World Health Organization (WHO) as the most "critical" priority pathogens for research and development [5]. The classification as a high priority refers to several issues, including the limited antibiotic arsenal available to physicians to treat infections[6]. The overuse of antibiotics, the suboptimal and inadequate implementation of infection control measures, on the other hand, will contribute to a rapidly increasing incidence of infections [7].

Asymptomatic colonization is the first step for developing an ESBL infection. As the ESBL-carrier rate in the population increases and nosocomial transmission becomes more common, the risk of being infected increases. Organisms that produce ESBLs or carbapenemases are a common cause of empiric antibiotic therapy failure, with severe consequences for patients [8] [9]. Furthermore, infections associated with ESBL reduce treatment effectiveness, more extended hospitalization, higher costs, and higher morbidity and mortality. Moreover, ESBL-producing Enterobacteriaceae (EPE) often displays multidrug-resistant phenotypes, further limiting the therapeutic options.

ESBLs also constitute a burden on health care systems conferring prolonged hospital stay. De Kraker and co-workers describe BSIs in Europe caused by E. coli isolates

resistant to extended-spectrum cephalosporins. They estimate the cost to >2,700 excess deaths and 18.1 million EUR, represented by >120,000 excess days of hospital stay[10].

Patients with severe infections are highly dependent on receiving adequate treatment early in the course of infection for a benign outcome. Many times the diagnostic procedures available are too slow, and empirical treatment is necessary. Knowledge of the local epidemiology is therefore very fundamental. For a patient infected by resistant bacteria, such as an ESBL-producer, administration of an ineffective antibiotic can be lethal. Changing to an effective regimen after treatment failure might be too late[11].

The spread of multiresistant bacteria is also related to its propagation from the hospital to the community. Therefore it is critical to eradicating these patients for several reasons. We notice an individual and global benefit when limiting the spread of the multiresistant bacteria, infection, and all their consequences.

Several studies have performed the decolonized MDRGN bacteria carriers patients, which eradicated MDR with varying percentages using many protocols, primarily antibiotics. However, the risk of recontamination is higher. As a necessity, a practical protocol is critical to reducing eradication and limiting the consequences of multiresistant bacteria carriage.

The antimicrobial activity of essential oils has been widely described. Their use is increasing in treating infections, specially multiresistant bacteria, as described in several studies, thus the interest in guiding this work.

This study's primary goal is to decolonize the digestif tract using rabbits carrying M.D.R. strain.

This goal had conducted in several steps;

- Firstly the selection of MDR by antibiotic use, as well as spontaneous resistance;
- Secondly, the resistance induction via direct inoculation of bacteria obtained from the bank strain of the laboratory;
- Studying decolonization and all the parameters surrounding ;
- Is it possible to obtain decolonization if the administration of the inoculum is maintained?
- Studying correlation between decolonization (bacterial load) and other factors like fever and weight gain.

This work was performed on an animal model using a new product called AMC booster by cineol, studying the kinetics digestive decolonization of multiresistant bacteria and all the issues surrounding it, as reported in this preclinical study.

Bibliographical context

Chapter I: Antibiotic resistance and infection caused by MDR bacteria

I. Antibiotic resistance

Antibiotics are substances that work by inhibiting or limiting the activity of microorganisms. The usage of these molecules has resulted in an increase in life expectancy, a decrease in newborn mortality, and a significant role in surgical procedures such as prophylaxis. However, the rapid rise of antibiotic resistance has created a global public health crisis. Bacteria are becoming increasingly resistant, and in some cases multi-resistant, to antibiotic treatments, reducing the available pool of effective drugs and even resulting in therapeutic dead ends.

1. How to explain resistance to antibiotics

Antibiotic resistance is as old as antibiotics. Thus, scientists discovered penicillinase only a few years after the first antibiotic (penicillin) (in the 1920s). Multiple approaches can explain it.

a. Individual-level

The exposition of an antibiotic disrupts the coexistence of resistant and non-resistant strains. Indeed, the antibiotic promotes the growth of resistant strains at the expense of non-resistant variants. The growth in new resistant bacteria then disrupts the commensal flora of the host. It is especially true in immunocompromised persons receiving antibiotics, as their weaker immune systems have a tough time fighting off the infection [12].

b. Community-level

Numerous studies at the community level demonstrate a link between antibiotic consumption and antibiotic resistance [13], implying that antibiotic overuse is to blame for the rapid rise of clinical antibiotic resistance.

Numerous domains associated with antibiotic exposure may play a role in the emergence and dissemination of bacterial multidrug resistance:

- Overprescribing and misuse in human medicine

In Europe, the UK has a low outpatient antibiotic use[14]. However, approximately 20% of antibiotic prescriptions are unnecessary[15]. Multiple factors explain this issue; physicians provide an inaccurate diagnosis, prescribe antibiotics as a precaution, or use broad-spectrum antibiotics.

Public Health England's in a recent report[16] revealed that most antibiotic prescriptions were for the urinary tract or respiratory infections. However, almost 30% had no clinical reasoning. Although non-compliance patients contribute to the misuse of antibiotics[17], this includes discontinuing treatment. One of the main reasons for misuse is the fear of extended drug use causing side effects. Incomplete treatment primes bacteria with sub-lethal concentrations leading to acquired resistance[18].

Many countries lack regulatory and legislative control, which would typically govern antimicrobial distribution[19]. In developing regions where healthcare is not provided consistently, there is less control of antibiotic use, with varying regulatory guidelines between countries[20]. There is no control over the antibiotic supply. Self-medication is common[21] [22] and may overuse antibiotics, allowing to say that many antibiotic

prescriptions are unneeded[23]. Antibiotic misuse creates a severe worldwide problem for public health and is considered one of the biggest challenges to many health care systems.

- <u>environmental health and veterinary medicine</u>

Antibiotics can be used as "growth promoters" in farm animals, which has led to massive exposure worldwide for decades and continues in many countries [24]. there is an increase in antibiotic use in animals in countries such as China, India, Pakistan, and Egypt[25]. The consumption selects resistance genes in the animal flora and then transmitted to humans, either by direct contact or indirectly via food or even contaminated soil or water following the dissemination of these genes in the environment.

The primary transmission route for MDR organisms is the food chain[20], as sublethal antibiotic doses are in constant use in agriculture, farming, and fisheries, for treating infections, preventing diseases, and growth promotion.

Furthermore, antibiotics are used as a pesticide in the plant-producing process, biocide additives to home and other cleaning products [26,27], and veterinary medicine for preventative or curative purposes [12,28].

Additionally, antibiotic manufacture contributes to environmental release and promotes resistance selection. For instance, the research found that Indian rivers surrounding the city of Hyderabad, which is home to multiple pharmaceutical companies, are contaminated with ciprofloxacin[29].

The acquisition of novel resistance to antibiotics by bacteria is an equilibrium process dependent on the antibiotic's action method and the bacteria's defence mechanisms. Each mode of action corresponds to a microorganism's resistance mechanism.

II. Resistance Mechanisms of bacteria

1. Resistance acquisition at the bacterium scale

Two processes contribute to resistance acquisition: mutation and horizontal transmission. Bacteria can develop resistance through spontaneous chromosomal gene mutation. These chromosomal genes encode antibiotic-targeting ribosomal proteins, proteins involved in the formation of the cell wall or membrane, or proteins involved in metabolic processes. Second, gene transfer via mobile elements such as plasmids or transposons is a mechanism by which bacteria of the same species acquire resistance against bacteria of different species (cross-transmission). Antibiotic exposure exerts selection pressure on the host flora and results in exchanging resistance genes between bacteria[12]. Transferring resistance genes across bacteria of different species in a hospital setting is a significant public health concern. The hospital environment promotes the concentration of many organisms in more vulnerable hosts.

2. Mechanism action against antibiotics

Many mechanisms, natural or acquired, can conduct bacterial resistance.

a. Antibiotic prevention reaching the target

- **Reduce permeability**

Porins are proteins found on the surface of bacterial membranes that allow various molecules, such as antibiotics, to enter. Thus, these non-specific porins may be a route of entry, particularly in Enterobacteriaceae. Gram-negative bacteria employ strategies to decrease membrane porin production or substitute more selective channels (whose wall is poor in peptidoglycan).

- **Increase the rate of the flow**

Bacterial efflux pumps are proteins that are responsible for removing harmful chemicals from the cell. By increasing their production, it is possible to develop resistance to antibiotics by expulsion. Overexpression of this type of "specific substrate" efflux pump system is a tactic used by many hospital Enterobacteriaceae, including Pseudomonas aeruginosa and Staphylococcus aureus.

b. Mutation and alteration of the antibiotic's target

Consider methicillin-resistant staphylococci (MRSA), which acquire a mec chromosomal cassette to acquire methicillin resistance (SCCmec). The mecA gene encodes a penicillin-binding protein (PLP2a), which inhibits the activity of penicillins. Indeed, PLP is the target protein for B-lactam antibiotics, and PLP2a has a low affinity for them. This gene is located in a movable element (the chromosomal cassette)

integrated into the bacterial chromosome. It confers resistance to the microorganisms to all B-lactam antibiotics, including methicillin and oxacillin[30].

c. Protect the target

Consider quinolone resistance. The gene is located on several pathogens' plasmids. The qnt gene encodes a pentapeptide repeating protein (PRP) that binds to and protects topoisomerase IV and DNA gyrase from quinolone toxicity.

d. Direct modification of the antibiotics

- Inactivation of antibiotics by hydrolysis

There are several hundred enzymes that break down and modify antibiotics. It is the case with β-lactamases which degrade β-lactams. Extended-spectrum β-lactamases (ESBLs) are active on several subclasses. They can hydrolyze penicillins and 1st, 2nd, third, and 4th generation cephalosporins. According to the classification based on the peptide sequence of the Amber enzyme site, there are four classes of β-lactamases: A, B, C, and D[31]. The different types of β-lactamases mainly found around the world are:

- TEM-type ESBL (Temoneira);
- ESBL type SHV (Sulfhydryl Variable) (Sulfhydryl Variable);
- ESBL type CTX-M (Cefotaximase-Munich);
- ESBL type OXA (Oxacilinase) (Oxacilinase);
- β-lactamases AmpC;
- Carbapenemas.

- Chemical group transfer inactivation

By adding chemical groups to the antibiotic, it prevents it from adhering to its target. Aminoglycoside antibiotics are particularly affected by this type of resistance by acetylation of the antibiotic, reducing the target's avidity.

e. Coresistance

Antibiotic resistance can be mediated by a variety of different resistance mechanisms concurrently. The acquisition of resistance to one class of antibiotics can sometimes be accompanied by resistance to other classes, especially when the acquisition is through a plasmid. For example, the CTX-M plasmids often carry other resistance genes (aminoglycosides, tetracycline, sulfonylurea, and trimethoprim). Transmission via plasmids facilitates the spread of multiple resistance genes, contributing to the current problem of multidrug resistance, which is particularly prevalent in hospitals.

III. Multidrug-resistant bacteria associated with care setting

1. the global prevalence of multidrug-resistant bacteria and costs

In March 2018, the WHO published a list of resistant bacteria for which new antibiotic research and development are critical[32]. This list is based on a variety of criteria, including the bacterium's mortality rate, its impact on healthcare facilities and the community, its transmissibility, the prevalence of resistance, the tendency to acquire resistance genes over ten years, the impact of prevention on healthcare facilities and the community, the availability of treatment, and the recent and ongoing development of new antimicrobial agents. As illustrated in Table 1, carbapenem-

resistant bacteria Priority bacteria include Acinetobacter baumannii, carbapenem-resistant Pseudomonas aeruginosa, and C3G and carbapenem-resistant Enterobacteriaceae. E. coli and K. pneumonia are the two major Enterobacteriaceae found worldwide[33].

Antibiotic resistance costs Europe 1.5 billion euros per year [34] and the United States between 21 and 34 billion dollars per year [33].

Table 1: The WHO published a list of resistant bacteria classified by priority for research and developing new antibiotics in 2018.

Priority	Species
Critical	- Acinetobacter baumannii, carbapenem-resistant. - Pseudomonas aeruginosa, carbapenem-resistant. - Enterobacteriaceae*, carbapenem-resistant, 3 rd generation cephalosporin-resistant.
High	- Enterococcus faecium, vancomycin-resistant. - Staphylococcus aureus, methicillin-resistant, vancomycin-intermediate, and resistant. - Helicobacter pylori, clarithromycin-resistant. - Campylobacter, fluoroquinolone-resistant. - Salmonella spp., fluoroquinolone-resistant. - Neisseria gonorrhoeae, 3 rd generation cephalosporin-resistant, fluoroquinolone-resistant.
Medium	- Streptococcus pneumoniae, penicillin-non-susceptible. - Haemophilus influenzae, ampicillin-resistant. - Shigella spp., fluoroquinolone-resistant.

2. Infections caused by MRD in care settings

MDR constitutes a significant cause of healthcare-associated infections and community-acquired infections [35]. Bacterial resistance has increased in recent years, particularly resistance to third-generation cephalosporins (C3G), fluoroquinolones, and carbapenems [33,36], increasing morbidity and mortality associated with nosocomial infections and complicating treatment of infected patients. The recent emergence of resistance to colistin among Gram-negative bacteria is a significant issue [37], as colistin is a last-resort antibiotic used to treat multidrug resistance.

a. MDR transmission and reservoirs

Nosocomial MDR bacteria can be acquired in several ways by a patient:

- The patient's flora, via bacteria, is transferred to a location other than their natural habitat. Thus, commensal bacteria can be transferred from the intestinal flora to the urinary flora, resulting in a urinary tract infection. Bacteria naturally found in the gastrointestinal tract are frequently responsible for surgical site infections as well;

- Another patient's or staff member's flora. Bacterial transmission between individuals can occur in a variety of ways:

o Through direct contact (hands, saliva, or bodily fluids).

o Through indirect contact, frequently airborne (droplet, contaminated dust). Healthcare workers may act as transient carriers of the bacteria, passing it from patient to patient [38].

- The natural environment. Certain bacteria, such as Enterobacteriaceae, can survive in the environment for several days to several months [39,40]. The hospital environment

is a breeding ground for numerous bacteria, including water and humid environments (Pseudomonas aeruginosa), linen, medical equipment and rooms, food, and fine dust.

b. Infection in ICU and risk factors

Compared with patients in the general hospital population, ICU patients are susceptible to increased selective and colonization pressure[41,42].

- ICU infection

Although most studies of ICU-associated infections come from industrialized countries, the rates of infection may even be higher in developing countries, as illustrated by a multicenter prospective cohort surveillance study of 46 hospitals in Central and South America, India, Morocco, and Turkey[43].

An overall rate of 14.7% (or 22.5 infections per 1000 ICU days) observed. The following rates were found for specific devices:

- Ventilator-associated pneumonia (VAP); 24.1 cases per 1000 ventilator days (range 10.0 to 52.7 cases);

- Catheter-related bloodstream infection (CRBSI); 12.5 cases per 1000 catheter days (range 7.8 to 18.5 cases);

-Catheter-associated urinary tract infections (CAUTI); 8.9 cases per 1000 catheter days (1.7 to 12.8 cases);

- Risk factors

Specific characteristics increase the risk of infections with multidrug-resistant pathogens in ICUs by contributing to increased selective pressure (leading to the emergence of multidrug-resistant organisms) and increased colonization pressure

(leading to ineffective containment of these organisms) [42,44,45]. Specifically, risk factors for resistant infections reported from ICUs include the following [46–48].

- Presence of underlying comorbid conditions (diabetes, renal failure, malignancies, immunosuppression) and higher severity of acute illness indices;

- Long duration of hospitalization before the ICU admission, including interinstitutional transferring (particularly from nursing homes);

- Frequent encounters with health care environments (hemodialysis units, ambulatory daycare clinics);

- Frequent contact with health care personnel concurrently caring for multiple patients, whose hands can serve as vehicles for transferring pathogens between patients. Shared equipment and contaminated environments can also serve as reservoirs vectors that contribute to the acquisition of infections in the ICU;

- Presence of indwelling devices such as central venous catheters, urinary catheters, and endotracheal tubes, which bypass natural host defence mechanisms and serve as portals of entry for pathogens;

- Recent surgery or other invasive procedures;

- Receipt of antimicrobial therapy before the ICU admission, which creates selective pressure, promotes the emergence of multidrug-resistant bacteria. Several studies and various methodologies have demonstrated the association between prior receipt of antibiotics and infection with drug-resistant organisms. In case-control studies, antibiotic exposure has consistently been associated with the emergence of resistance to that same or a different class of antimicrobial agent[49]. For example, the receipt of fluoroquinolones allows the emergence of piperacillin-resistant P. aeruginosa[50].

Antibiotic exposure was the strongest single predictor for infection with extensively drug-resistant gram-negative pathogens.

- <u>Factors linked to antibiotics choose</u>

Patients with infections due to multidrug-resistant organisms usually are chronically or acutely ill and at risk of dying from underlying severe and complex medical illnesses. However, many factors related to the difficulties of choosing antibiotics for multidrug-resistant bacteria independently predispose to poor outcomes. These include the following:

- Multidrug-resistant pathogens are more frequently resistant to empiric antimicrobial regimens than are susceptible organisms. Wherefore, there are often delays in initiating appropriate, effective antimicrobial therapy in treating multidrug-resistant organisms[51]. These delays are independent predictors of mortality in severe sepsis and thus contribute to the increased mortality rates associated with resistant infections[52–56]. As an example, in a study of patients with septic shock, each hour of delayed appropriate therapy in the first six hours of infection was associated with an average decrease in the survival rate of 7.6% [57];

- Antimicrobial resistance often precludes the use of optimal "first-line" antimicrobial agents. It necessitates using "second-line" agents with inferior bactericidal activity and unfavourable pharmacokinetic and pharmacodynamic properties[58]. When "second line" agents are required to treat a resistant organism, adverse patient outcomes sometimes result in [59,60]. For example, vancomycin is commonly used to treat MRSA since anti-staphylococcal penicillins (nafcillin) and first-generation cephalosporins (cefazolin) are not active against the organism. However, vancomycin does not possess the intense bactericidal activity and is associated with an increased risk for

renal insufficiency compared with beta-lactams. In several clinical studies, vancomycin was inferior to beta-lactam agents in treating methicillin-susceptible S. aureus infections[61].

Another factor that may contribute to poor outcomes among patients with infections due to specific multidrug-resistant pathogens is the virulence properties of the organism. However, this issue is subject to continuous debate [62].

Chapter II: Microbiota intestinal and colonization with MDR bacteria

I. Intestinal Microbiota Role and properties exerted by intestinal microbiota in health

The gut microbiota contains a variety of bacteria, ranging from 10 to 10^{12} per gram of faeces. Furthermore, a healthy person has more bacteria, about 10^{13} to 10^{14}, than human cells 10^{12} [63]. The number of bacteria and species rises from the duodenum to the rectum [64].

Due to its significant genetic content and metabolic complement, the gut microbiota exerts various beneficial impacts on the host. It can be considered a real organ, playing several vital tasks, including maintaining the mucous barrier and metabolic and immune functions. Furthermore, a practical immune function requires interaction between the commensal microbiota and the mucosal immune system[65].

1. Barrier effects and immune function

a. Barrier effects

The intestinal mucosa surface comprises enterocytes, which are cells with villi connected by tight junctions. Bacteria from the intestinal flora attach to the mucosa's surface, preventing harmful bacteria from colonizing the mucosa through a phenomenon known as competition at adhesion sites. Pathogens are less adapted to gut ecology than commensal bacteria. They generate a protective layer on the intestinal epithelium's surface. When pathogenic bacteria are detected, bacteria in the intestinal flora can stimulate the production of antimicrobial peptides such as

bacteriocins by intestinal epithelial cells. These peptides have an antibiotic activity that is either bactericidal or bacteriostatic.

b. Immune function

The microbiota can also stimulate the immune system's secretory IgA production, co-localize with gut bacteria in the outer mucus layer, and help limit bacterial exposure to the epithelial cell surface[66].

The immune system does not mature correctly in the absence of microbiota, lymphoid tissue is underdeveloped, and lymphocyte populations in the intestine reduce and cannot proliferate[67].

2. Metabolic function

In the intestine, microorganisms can perform various functions such as degradation, transformation, and synthesis.

a. Carbohydrate metabolism

Depending on the individual and their diet, the amount of fermentable carbohydrates (found in grains, fruits, and vegetables) reaching the colon ranges from 10 to 60 grams per day. The anaerobic degradation of these substrates implicates various bacterial groups of the human colonic microbiota. Glycolytic bacteria allow carbohydrate transformation. Most species use glycolysis to convert carbs to pyruvate, converted into fermentation products such as short-chain fatty acids (acetate, propionate, butyrate). Propionate is rapidly absorbed by the colonic epithelium and metabolized both locally and distantly. They provide energy and stimulate colonic absorption of sodium. Butyrate is the primary nutrient for colonocytes.

Moreover, it inhibits the growth of cancerous colonic cells[68]. It also has immunomodulatory properties at the local level. Short-chain fatty acids help to stimulate regulatory T lymphocytes in the intestinal mucosa[69].

b. Vitamin synthesis

The gastrointestinal microbiota is essential for the synthesis of vitamins that the host cannot produce [70]. Lactic bacteria play a crucial role in the production of vitamin B12[71]. Bifidobacteria are the primary folate producers[72]. Vitamin K, riboflavin, biotin, nicotinic acid, panthotenic acid, pyridoxine, and thiamine are additional vitamins synthesized by the human gut bacteria[73].

II. Intestinal microbiota as a reservoir of antibiotic resistance genes

1. Resistome

The gut resistome is a collection of resistance genes found in the gut microbiota. It consists of the endogenous or "resident" resistome and the exogenous or variable resistome.

The resident resistome is typically composed of chromosomal resistance genes that are not associated with mobile structures. On the other hand, the variable resistome is frequently associated with mobile structures (such as plasmids or transposons) that can be exchanged with the host's resident bacteria.

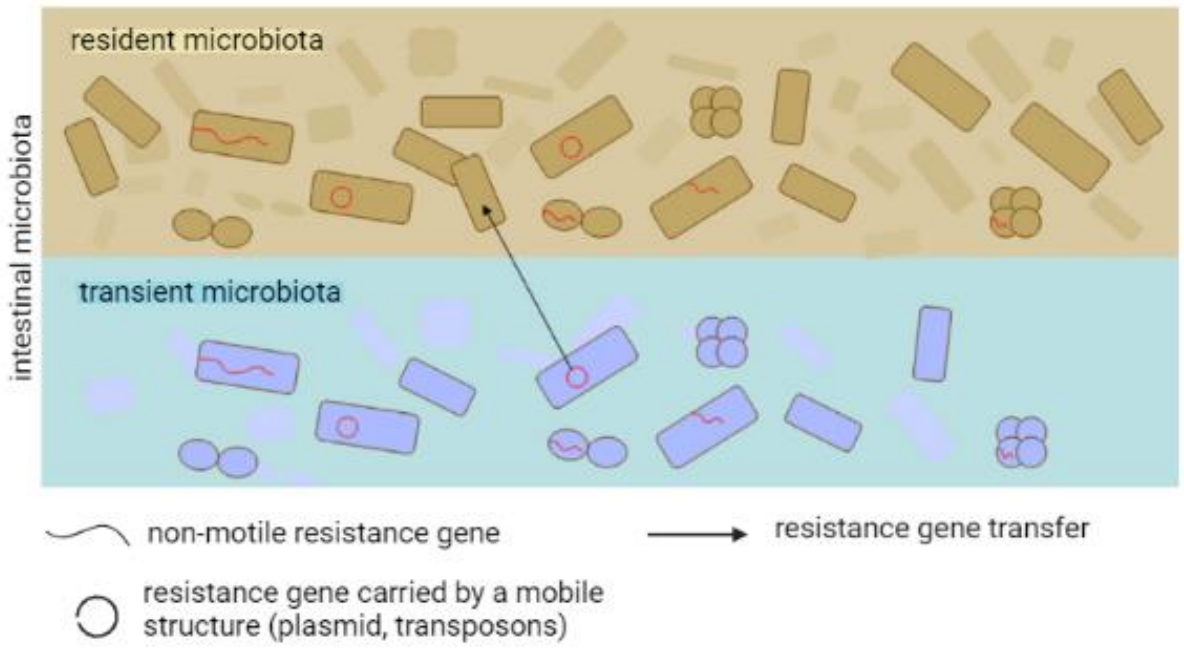


Figure 1: Schematic representation of the intestinal resistome consists of two parts: the resident resistome and the variable resistome.

2. Methods for studying intestinal resistance

There are numerous approaches to studying gut microbiota (Table 2). The most common method is to culture a stool sample or a rectal swab on antibiotic-containing agar media. This method has the advantage of being very sensitive because it theoretically allows for the detection of bacteria in low concentrations (10^2 CFU / g stool). However, this method only identifies cultivable bacteria and does not assess the diversity of resistance genes in the intestinal microbiota. Another option is to perform PCR on D.N.A. extracted from the stool. However, even if this technique is sensitive, it relies on primers based on genes already known in databases, making it impossible to discover new resistance genes. Finally, two metagenomics approaches can be used: direct sequencing metagenomics and functional metagenomics.

The sequences of these D.N.A. fragments are compared to databases, and genes encoding resistance can be identified or suspected based on similarity with previously known genes. Furthermore, it lacks sensitivity because it can only detect bacteria at concentrations greater than 10^6 and ignores minority populations. Potentially dangerous bacteria such as S. typhi, Yersinia enterocolitica, and Tropheryma whipplei, found in human feces at concentrations below 10^5 CFU per ml, are among these overlooked populations[74] [75]. However, this method has the advantage of detecting genes in non-cultivable bacteria; it does not detect genes that are already known or are close to genes that are already known. It also does not confer the resistance phenotype conferred by gene expression. By combining metagenomics and functional selection, the diversity of the resistome could be better appreciated.

Table 2: Methods of studying intestinal resistance.

Methods	Descriptions	Advantages	Disadvantages
Culture in selective media	Culture of a stool sample in the media containing antibiotics.	- Sensitive (10^2 CFU / g saddle). - Provides strain and select genes that are expressed. - Fast and inexpensive.	- Only cultivable bacteria. - No direct identification of the gene: further testing is required (PCR, cloning).
Stool DNA PCR	Specific PCR detecting DNA on the total stool.	- Sensitive Direct gene identification - Allows targeting of non-cultivable bacteria. - Fast, inexpensive.	- Do not donate the host bacteria. - It only finds genes that primers have specifically targeted. - Non-functional: does not provide information on the gene's expression.
Metagenomics	Total stool D.N.A. was sequenced using a high-throughput sequencing method, and genes were identified by analogy with known genes.	- Massive amount of data - Allow for the targeting of non-cultivable bacteria. - Direct identification of the gene (already sequenced) - appreciate the diversity of genes.	- Not particularly sensitive (> 10^5 CFU / g saddle). - Incomplete sequences can occur at times. - Do not provide information on the gene's expression or activity spectrum.

		- Discover new genes. - Can provide details about the host bacteria.	- Only look for genes that are similar to those that are already known (sequence analogy) - Expertise in bioinformatics is required.
Functional Metagenomics	It enables massive cloning of entire stool DNA into a vector (Escherichia coli) and selection on an antibiotic-containing medium.	- Detect new resistance genes. - Obtaining resistance phenotypes (in Escherichia coli) - Allows targeting of non-cultivable bacteria - Allows to appreciate the diversity of genes	- Challenging technique. - The genus must confer resistance on itself. - For the time being, only Escherichia coli is being cloned. - Sensitivity?

III. The dynamics of multiresistant bacteria colonization in the gastrointestinal tract

1. Pre-colonization phase: determinants of resistant enterobacteria acquisition

Resistant Enterobacteriaceae, particularly EBLSEs, can be acquired by faecal peril when travelling in endemic areas[76 77], by manual transmission (for example, in a hospital setting)[78 79], contaminated water[80], food [81 82], or even from a previous patient in the hospital room[83]. To enter the intestinal microbiota, resistant Enterobacteriaceae must first overcome the stomach's acidic pH; the use of anti-acids, such as anti-H2 antihistamines, has also been recognized as a risk factor for carrying ESBLE[84 85]. The barrier effect in the gut microbiome prevents long-term implantation[86 87]. Certain antibiotics could have a crucial role in boosting multidrug-resistant bacteria's installation and proliferation if the barrier effect is removed [88 89]. New research reveals that taking ciprofloxacin did not reveal quinolone-resistance mutations in the gut microbiota's enterobacteria but instead aided the secondary implantation of resistant exogenous strains[90]. These several exposure variables (travel to endemic places, substantial morbidity, chronic renal disease, hepatic failure, and chronic infections) and selection variables (antiH2 antihistamines, history of antibiotic use) link to EBLSE portage[76 77 85 91 92 93].

Figure 2: Schematic representations of the intestinal microbiota's central role in the spread of multiresistant Enterobacteriaceae (red): pre-colonization (green), colonization (blue), and post-colonization (orange).

2. Colonization phases

The colon's native bacteria play an essential role in the host's defence by preventing the colonization of the potentially harmful pathogens. This defence mechanism, known as "colonization resistance," can prevent potential indigenous pathogens from overgrowing and prevent exogenously introduced organisms from colonizing[94]. The dominant anaerobic microbiota typically maintains low population densities of Escherichia coli, a component of the indigenous colonic microflora[95].

The effect of antibiotics on the gut microbiota is determined by faecal concentrations (and active metabolites) and antibiotic activity spectrum. For this purpose, antibiotics affect the intestinal microbiota[96 97].

a. Effect of antibiotics on intestinal microbiota

One of the consequences of antibiotics is a loss of species diversity: sensitive species are killed (bactericidal effect) or no longer multiply (bacteriostatic effect), and only resistant bacteria, particularly exogenous bacteria, survive. The loss of the barrier effect is especially noticeable when antibiotics active against anaerobic bacteria are used. One study discovered that taking antibiotics active against anaerobic bacteria increased the intestinal densities of multidrug-resistant Enterobacteriaceae[89]. The barrier effect could be considered at the species level, such as E. coli. The E. coli population in healthy subjects comprises one or more clones with varying relative abundances[98]. If one of them is resistant to a particular antibiotic, using that antibiotic could favour that clone over the others, modifying the relative abundances of E. coli clones without affecting the density of all the E. coli bacteria present in the intestinal flora[99].

b. Selective antibiotic pressure

Antibiotic exposure is not believed to be a direct factor in the development of resistance mechanisms. Antibiotic therapy exerts selection pressure by promoting the proliferation of antibiotic-resistant gram-negative bacteria while simultaneously reducing the number of competing but non-resistant species. For example, ceftazidime therapy can eradicate sensitive gram-negative bacteria while promoting the establishment of novel ESBL mutants such as Klebsiella pneumoniae or a preexisting Enterobacter species subpopulation[100 101 102].

3. Post-colonization phases; bacteremia and infection process

The gut microbiota may play a role in the relationship between antibiotic exposure and infection with resistant Enterobacteriaceae. Because EBLSEs are typically resistant to other antibiotic families, including quinolones and cotrimoxazole[103], taking many antibiotics can favour these bacteria and enhance the chances of being present in an infectious process.

The movement of bacteria from the digestive tract to the mesenteric lymph nodes is known as digestive translocation[104]. This passage allows bacteria to colonize other areas, such as the bloodstream. Bacterial translocation occurs physiologically in healthy subjects, but only to a limited extent and with no adverse repercussions. In contrast, in neutropenic, malnourished, or hemorrhagic shock patients, sustained bacterial translocations are observed, leading to severe infections [105 106]. Bacterial density is essential in digestive translocation because the dominant bacterium is more likely to translocate [97 106].

Furthermore, bacterial translocation has been recorded in immunocompromised mice treated with various antibiotics (penicillin, clindamycin, and metronidazole) due to an increase in intestinal bacterial density (from 3 to 5 log UFC/g selles)[107]. Antibiotics that cause an increase in bacterial density in the digestive tube may put some patients at risk of multiresistant bacterial translocations.

Chapter III: MDRO management

The management of the spread of MDRO has been summarized in a perfect set of guidelines titled "Management of Multidrug-Resistant Organisms in Healthcare Settings" by the Healthcare Infection Control Practices Advisory Committee (HICPAC)[108].

Various types of interventions implicate in controlling or eradicating MDRO. Administrative support, judicious antimicrobial use, surveillance (routine and enhanced), screening strategies (Standard and Contact Precautions), environmental measures, education, and decolonization are among them.

I. Administrative support

Several MDRO control interventions necessitate administrative commitments of fiscal and human resources. One example is the use of A.S.C. Other interventions include:

- Implement system changes to ensure early and efficient communication, such as computer alerts to identify patients previously known to be colonized/infected with MDROs[109].
- Provide a sufficient number and appropriate placement of handwashing sinks and alcohol-containing hand rub dispensers in the facility.
- Maintain staffing levels that are proportionate to the level of care required.
- Enforcing adherence to recommended infection control practices for MDRO control (e.g., hand hygiene, Standard and Contact Precautions).

- A "How-to Guide" for implementing change in I.C.U.s, which includes an analysis of structure, process, and outcomes when designing interventions, can aid in identifying administrative interventions that are required[110].

An effective strategy that requires administrative support is vital for reducing emerging or rising MDRO problems[111].

II. Education

Numerous effective research incorporates facility-wide, unit-specific, and informal educational interventions. The interventions were designed to increase understanding of the MDRO problem the facility was attempting to control to promote behaviour change. Whether the desired change involved hand hygiene, antimicrobial prescribing habits, or other outcomes, enhancing awareness and developing a culture that encouraged and promoted the desired behaviour was considered essential to the intervention's success. Educational programs to improve hand hygiene adherence and other prevention measures in different healthcare settings have reduced MDRO transmission[112].

III. Judicious use of antimicrobial agents

Treatment options for M.D.R. gram-negative infections are limited. Antimicrobial stewardship programs aim to improve outcomes for patients with infections caused by M.D.R. gram-negative organisms, slow the progression of antimicrobial resistance, and reduce hospital costs by optimizing the appropriate use of currently available antimicrobial agents[113].

The C.D.C. Campaign to Prevent Antimicrobial Resistance, which began in 2002, offers evidence-based guidelines for judicious use of antimicrobials and tools for implementation. This effort is directed at all healthcare settings. Focuses on the efficient treatment of infections with antimicrobials by using narrow-spectrum agents, avoiding prolonged therapy, and avoiding using broad-spectrum or more potent antimicrobials to treat serious infections when the pathogen is unknown or when other effective agents are unavailable.

A systematic review of controlled studies identified several promising practices. These include social marketing (i.e., consumer education), practice guidelines, authorization systems, formulary restrictions, mandatory consultation, and peer review and feedback. It further suggested that online platforms that provide clinical information, standardized order entry, and decision support are promising strategies[114]. Organizational and multidisciplinary antimicrobial control programs are the most effective way to achieve these improvements [115].

IV. MDRO surveillance

Surveillance is a critical component of any MDRO control program, allowing for detecting newly emerging pathogens, monitoring epidemiologic trends, and evaluating intervention effectiveness. We have many MDRO surveillance strategies, ranging from monitoring clinical microbiology laboratory results obtained as part of routine clinical care to using A.S.C. to detect asymptomatic colonization.

1. Active surveillance culture

A recent review of the Active surveillance culture program in asymptomatic patients discusses using an A.S.C. program as an infection prevention strategy to contain M.D.R.-GNB[116].

In asymptomatic MDR-GNB colonization, the intended population and timing of an A.S.C. are unidentified. Depending on the at-risk area, the targeted patients with a high risk of MDR-GNB colonization may be identified in many settings (e.g., I.C.U., NICU, or burn unit with high MDR-GNB rates or colonization pressure).

Furthermore, the best time for an A.S.C. is unspecified. Admission to the hospital or intervention unit and transfers to and from approved units (e.g., I.C.U.) can result in culture acquisition. An A.S.C. can be obtained in periodic cultures to discover silent transmission or follow-up cultures in the targeted population (e.g., weekly or twice-weekly).

Some MDRO control reports describe healthcare personnel surveillance cultures during outbreaks. This strategy should be reserved for settings where specific healthcare personnel implicate in MDRO transmission.

2. Resources required

For the effective implementation of an A.S.C. program for asymptomatic colonization as part of an infection prevention and control (I.P.C.) program, additional resources are required, including (1) personnel to acquire suitable cultures, (2) microbiology laboratory personnel to process the cultures, and (3) a mechanism to communicate results to caregivers. (4) further measures to prevent infection (e.g., contact precautions), and (5) procedures to ensure that additional infection prevention

measures are followed. A.S.C. as part of infection prevention measures has been influential in the United States and Europe.

Restricted or inadequate I.P.C. in resource-constrained settings is due to many factors, including low sensitivity with a single site culture, higher cost, the need for selective plates, administrative difficulties, delayed turnaround times, and weak compliance with the A.S.C. policy.

3. Tools to obtain A.S.C

Methods for obtaining A.S.C. must be carefully considered; MDR-GNBs have been detected using various approaches, including peri-rectal or rectal swabs alone or in conjunction with oro-pharyngeal, endotracheal, inguinal, or wound cultures. The lack of standardized screening media for various gram-negative bacteria makes isolating a specific MDR-GNB a time-consuming operation. Commercially available chromogenic enzyme substrate-containing media (chromID ESBL) have been demonstrated to have high sensitivity and specificity for ESBLE identification. They allow easy discrimination of different colonies based on their color, particularly useful in specimens containing resident-associated flora. Furthermore, it significantly reduces the need for unnecessary validation tests. ChromID ESBL allows considerable time and cost savings for ESBL screening[117].

V. Screening strategies

The screening strategies have been summarized in a perfect literature review entitled: "Multidrug-resistant organisms: when and who do we need to screened ?" by Gabriel Birganda, Jean-Christophe Lucetand, and her team[118].

The first step in the decolonization process is to develop a screening approach for suspected carriers.

1. Prioritize situations for screening

Three criteria are taken into account in the graduation of the risk the epidemiological circumstance. Screening strategies must take into account the frequency of occurrence of the event at the local level.

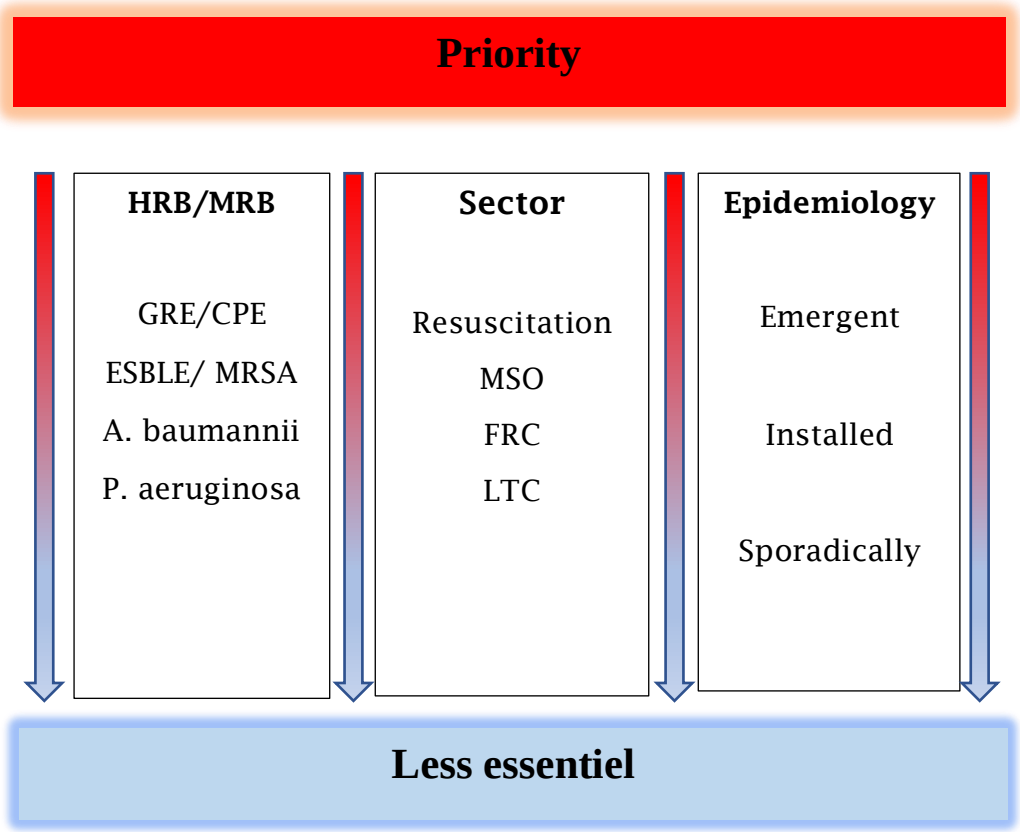


Figure 3: Interest of screening based on the type of bacteria, the hospital area and the epidemiological. situation

Abbreviation: H.R.B.: High resistant bacteria; M.R.B.: Multiresistant bacteria; GRE: glycopeptide resistant enterococci; C.P.E.: Carbapenemase-producing Enterobacteriaceae; ESBLE: Extended-spectrum beta-lactamase-producing Enterobacteriaceae; MRSA; Methicillin-resistant Staphylococcus aureus. MSO: Medicine/Surgery/Obstetrics. F.R.C.: follow-up and rehabilitation care; L.T.C.: long term care

a. Epidemiology situation

In sporadic cases, we screen patients who exhibit factors that increase their risk of exposure.

When we identify the patient, the strategy is to place him in strict touch precautions as quickly as the admission. In recent non-controlled epidemics, this notion can be employed to slow down transmission dynamics. In an established epidemic, a more traditional strategy is recommended, including touch precautions and post-exposure prophylaxis.

A more conventional approach, which involves touch precautions and varying degrees of rigorous screening, is recommended in the event of an existing outbreak[119].

b. The commensal or saprophytic character of bacterial species

Understanding the dynamics of transmission and control strategies requires an understanding of bacteria's commensal or saprophytic nature.

Commensal bacterial species in the digestive tract, such as E. coli, serve as a significant reservoir of often resistant strains that persist in the microbiota for long periods. The median duration of carriage for EBLSEs has been evaluated at 6.6 months[120]. Due to the commensal nature and prolonged carriage of EBLSEs, transmission to the family circle is possible, about 17% of household members in a Spanish study[121]. Another study concludes that the community could be a reservoir of these ESBL-producing bacteria and enzymes[122].

Saprophytic pathogens such as A. baumannii and P. aeruginosa mainly affect intensive care units, which combine invasive procedures, and significant antibiotic selection

pressure. As a result, the vast majority of outbreaks take place in intensive care units[123].

M.R.B. and H.R.B. have the potential to spread resistance genes between Enterobacteriaceae. CTX-M ESBLs were initially found only in E. coli but are now commonly found in K. pneumoniae and Enterobacter spp [124].

2. Objective of screening

Screening can have both individual and collective benefits in preventing infection by detecting and preventing the spread of bacteria.

a. Individual impact

The purpose of individual screening is to prescribe preventive or curative treatment to avoid serious adverse events.

The precautions include:

- Knowing the status of careers in high-risk areas and preventing infection through decontamination or decolonization,
- Adjusting antibiotic-prophylaxis during surgical interventions,
- Choose the appropriate antibiotic therapy in the event of an infectious disease.

Table 3: Preventive measures and expected benefits based on microorganisms.

	MRSA	ESBL-E	CPE
What is the point?			
Contact precautions	+	+	++
Decolonization	++	+	+
Specific measurements (antibiotic-prophylaxis)	++	?	?
What advantages are there?			
Individual	++	+	?
Collectively	++	++	+++

b. Collective impact

The goal of collective interventions is to control the spread of resistance. From the most basic to the most stringent, the measures are as follows:

- Avoid cross-contamination,
- screening to determine the status of patients "contacts,"
- Patients and contacts are divided into two districts, each with dedicated staff in the event of an epidemic.

3. The levels of strategy prevention measures for cross-transmission

The intensity of the measures to restrict cross-patient transmission is increasing[125].

a. First level: Standard precautions

Colonization patient with MDROs is frequently undetected. Therefore, Standard precautions must be used, based primarily on maintaining hand hygiene through Alcohol-based hand rub (ABHR) before interaction with each patient or immediate environment [108 126 127 128].

b. Second level: Standard precautions+contact precautions

Contact precautions are applied to standard precautions to restrict transmission from patients carrying specific pathogens, such as MRSA, and EBLSE[108 129]. In a systematic review published by Cohen and all, the utility of contact precautions alone against transmission of any MDRO among adult acute care patients were evaluated. They looked at six studies that evaluated the effectiveness of contact precautions in preventing MDRO infections. In one study, the number of cases decreased in phases where isolation precautions were employed compared to no intervention for A. baumannii colonization or infection[130]. In addition, an observational study conducted by Jean-Ralph Zahar found that additional C.P. interventions had little effect on the occurrence of ESBL-E in hospital settings[131]. These can be used simply for MDR bacteria carriers patients in clinical samples or in conjunction with a screening policy to identify the entire reservoir of these carriers.

c. Third level: search and isolate

The third level of precautions is implementing maximum "search and isolate" measures, the objective of ensuring the absence of circulation of H.R.B., C.P.E., and ERG. A comprehensive screening policy around index cases, clustering of cases in dedicated areas with management by specially qualified staff, monitoring contact patients for carriage, and careful elimination of suspected carriage after exposure to ERG patients has ended with at least three consecutive negative screenings are among these strategies [132].

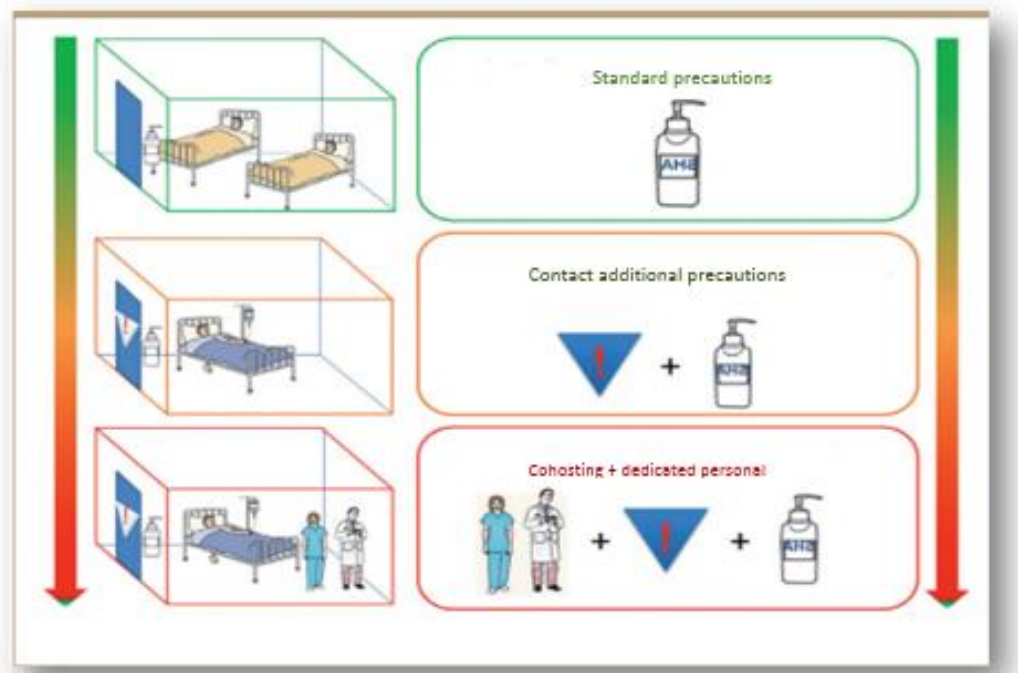


Figure 4: Strategies for controlling hand-carried cross-transmission of multi- and highly antibiotic-resistant bacteria.

4. Screening tools

Screening for ESBL-E and C.P.E. carriages in the intestines presents many technical and practical hurdles. Phenotypic or genotypic approaches can be utilized to describe resistance in Enterobacteriaceae. Both have drawbacks, and they can give drastically different results[133].

The benefit of phenotypic or culture-based screening approaches is that they use tools commonly used in clinical microbiology laboratories. However, the turnaround time (often several days) is lengthy, and the sensitivity of specific cultural approaches is suboptimal. Direct culture has been shown to miss up to 26% of ESBL-E rectal carriers due to improper rectal swabbing procedures or ESBL-E colonization densities below detection thresholds. Conversely, a genotypic approach based on nucleic acid amplification techniques (PCR) can only detect known ESBL or carbapenemase-encoding genes[134]. As a result, it is critical to understand the prevalence and types of resistance enzymes in a given situation. There is still some controversy regarding the best methods. However, a combination of culture- and PCR-based tests is currently recommended by experts to detect all phenotypic resistance and to confirm the underlying genetic mechanisms, at least for CPE[133].

VI. Environmental measures

Several studies have looked into the function of environmental reservoirs, including surfaces and medical equipment in the transmission of VRE and other MDROs. Although environmental cultures are not systematically recommended, they have been used in several studies to document contamination. They have contributed to measures such as the use of dedicated non-critical medical devices, reassignment

of dedicated cleaning personnel to the affected patient care unit, and increased cleaning and disinfection of frequently touched surfaces (e.g., bed rails, bedside commodes, and door handle). As a result, improved environmental cleaning and disinfection could reduce MDRO accumulation in the environment and suppress MDRO colonization in I.C.U.s, lowering nosocomial infections and improving adverse patient outcomes[135].

VII. Decolonization

Enterobacteriaceae, which colonize the human gut is the main reservoir of infections by these organisms, and patients usually carry resistant strains as part of their gut microbiota before infection develops[136]

Decolonization may have many advantages, including lowering the risk of infection from the resistant strain in the person carrier and preventing the bacteria from spreading to other patients (cross-transmission). Moreover, Gram-negative M.D.R. bacteria such as ESBL-E and C.P.E. can serve as a reservoir of mobile genetic elements for other species of the Enterobacteriaceae family so that decolonization could restrict the possibility of a horizontal gene in the intestinal tract [137].

There is no clear definition of decolonization. The number of negative samples used to define decolonization impacts decolonization rates; decolonization rates are higher when only one negative sample is used, so more than one negative sample is needed[138]. Suggesting a patient has been decolonized with just one sample may lead to a false impression of success because ESBL-E and C.P.E. may only have been suppressed below detection levels. Standard methods, including genotypic methods, are incapable of distinguishing between carriage persistence and reacquisition. A

patient can be recolonized by a new strain with a similar resistance profile that can only be distinguished by high-resolution analysis, such as whole-genome sequencing, which is difficult to perform regularly. The body site to sample for screening is also a bone of disagreement. The gastrointestinal tract is the primary human reservoir for Enterobacteriaceae.

Nonetheless, the urinary and inguinal ESBL-E carriage has been detected as a reference. As a result, different reservoirs are possible, and decolonization regimens must be active against extraintestinal sites of colonization. More high-quality prospective data and a consensus concept for decolonization are urgently needed.

1. Decolonization of ESBL-producing Enterobacteriaceae

a. Spontaneous decolonization

In a recent prospective cohort study, 101 Norwegian patients with ESBL-E-related community-acquired urinary tract infections were included[139]. Stool swabs are taken every three months for a year to monitor the progression of ESBL-E carriages in the gut. If two consecutive negative samples were obtained, faecal ESBL-E clearance was reported. To recap, the natural history of ESBL-E colonization is uncertain, but there is evidence that colonization persists over time in a large proportion of patients.

b. Decolonization strategy

Several strategies investigate for ESBL-E decolonization:

- **Probiotics**

Probiotics are defined by the World Health Organization (WHO) as "live microorganisms that, when administered in adequate amounts, confer a health benefit

on the host" [140]. Over the last decade, there has been increasing public and scientific interest in using live micro-organisms to prevent or treat disease.

In Brazil, a single-centre, double-blind, placebo-controlled trial was performed to see whether a probiotic product could decolonize patients with any multidrug-resistant Gram-negative bacilli (Enterobacteriaceae (n=53), Acinetobacter baumannii, Pseudomonas aeruginosa, Burkholderia cepacia, Serratia marscescens[141]. Adult patients colonized (as determined by a rectal swab) or infected with M.D.R. Gram-negative bacteria were randomly assigned to either a probiotic or a placebo for seven days (2x/day). The probiotic supplement (10^{10} Lactobacillus bulgaricus and 10^{10} Lactobacillus rhamnosus units) was given orally or through a Nasoenteric tube. Decolonization was defined, a negative rectal swab within 24 hours of the end of treatment (or at discharge if discharged before). There was no difference in the rate of decolonization between the two groups (16.7% (8/48) in the treated group and 20.7% in the control group (11/53), p=0.60). Another placebo-controlled trial was conducted among older people living in long-term care facilities in New-Zeeland[142]. Sixty-nine patients were infected with M.D.R. E. coli received either a probiotic strain of E. coli ($5x10^{9}$ -$5x10^{10}$ Escherichia coli «Nissle 1917» (Mutaflor®)) (n=36) or placebo (n=33). There was no discernible difference between the two Accepted Manuscript groups (58% of the placebo group versus 77% of the treatment group remained positive). So far, no evidence exists for the impact of probiotics on decolonization, and caution should be exercised before using probiotics for this purpose in routine clinical practice because manipulation of the microbiome could result in unintended consequences. Probiotics may also be the source of infections, particularly in immunocompromised individuals and the critically ill[143].

- <u>Faecal microbiota transplantation</u>

Faecal microbiota transplantation (F.M.T.) is a crude form of bacteriotherapy that utilizes a healthy donor's diverse microbial gut community. During F.M.T., faecal material enriched with commensal microorganisms is transferred to the patient's gastrointestinal tract from a healthy donor. This therapeutic approach is now recognized as a safe and successful treatment for patients with recurrent Clostridium complicated infection, and it is recommended by international guidelines[144] [145]. The scientific literature on ESBL-E/CPE decolonization in humans is still limited to case reports and small, unregulated studies.

Huttner and all performed a multicenter, internationally randomized controlled trial to evaluate a 5-day course of oral antibiotics (colistin and neomycin) followed by frozen FMT for no intervention[146]. Thirty-nine patients with ESBL-E (n=36) and CPE (n=11) colonization were included in the study. Non-absorbable antibiotics followed by FMT reduced ESBL-E/CPE carriage slightly (9/22, 41%) compared to controls (5/17, 29%). However, the difference was not statistically significant, possibly due to the trial's early termination and failure to reach the intended sample size. Singh and all investigated FMT for ESBL-E carriage decolonization in 15 patients in an uncontrolled cohort study[147]. It was discovered that three out of fifteen (20%) patients were ESBL-negative at 1, 2, and 4 weeks after the first transplant, while six out of fifteen (40%) were negative after the second transplant. Bilinski and all carried out an uncontrolled study among 20 patients with haematological disease colonized by CPE, ESBL-E, or another MDRO. They reported a successful eradication of intestinal carriage in 15 out of 20 patients (75%) at one month after FMT and 13 out of 14 (93%) patients at six months[148].

- **Antibiotics**

The principal reservoir for ESBL-E is the intestine. For intestinal decolonization, antibiotics are not absorbed orally and have no activity against anaerobic microflora, such as aminoglycosides, polymyxins, or the rifamycin-based antibiotic rifaximin.

Three randomized controlled trials (R.C.T.s) were carried out to evaluate the efficacy of an oral antibiotics regimen for ESBL-E decolonization. Huttner et al. randomly assigned 58 patients for ten days to either oral colistin sulfate [1.26 million units four times daily] or neomycin sulfate [250 mg (salt) four times daily] (plus nitrofurantoin for five days in the event of urine detection) or placebo[149]. The Rectal carriage was slightly lower in the treatment group than in the placebo group at the end of treatment, but the outcome disappeared after seven days post-treatment and at 28 days post-treatment. However, there was no significant difference in the ESBL-E detection 28+/-7 days after treatment. These findings suggest that antibiotics can temporarily suppress ESBL-E carriage below the detection level, highlighting the rapid reemergence of detectable colonization. Stoma et al. conducted a controlled randomized trial with 62 hematologic patients in a tertiary haematology centre (Minsk, Republic of Belarus) randomly allocated to either a colistin decolonization regimen (2 M.I.U., four times per day P.O. for 14 days) or a placebo. They were followed up with rectal swabs performed on day 14 and day 21[150]. They found similar results in the first study, with a temporary suppression of decolonization on day 14 of the treatment, which was then interrupted on day 21.

Several uncontrolled cohort studies have also been carried out. In a Swiss prospective cohort study[151], 35 patients were treated with antibiotics (oral paromycin, chlorhexidine mouth rinse, and oral antibiotics for urinary tract colonization) and

followed for 12 months. Patients with persistent ESBL-E carriages were treated with one to four courses of antibiotics until they were decolonized. Decolonization is defined as at least one set of negative screening samples, including rectal, throat, and any previously ESBL-positive site, without further positive screenings. This research revealed that repeated decolonization treatment for chronic carriage significantly improved decolonization performance. Decolonization was accomplished by 48.6% (18/35) of people after one (median; range: 1-3) course and by 62.9% (22/35) of people after a median of 15 months. It is difficult to separate the effect of repeating the decolonization regimen from the passage of time [spontaneous loss of carriage]. The overall results should be interpreted with caution because most patients were infected with an unknown colonization rate. In addition, half of the patients did not adhere to the decolonization regimen, and the loss of carriage cannot be distinguished from spontaneous loss of carriage in the absence of a control group.

In the two randomized trials comparing decolonization therapy with placebo (one for ESBL-E[149] and one carbapenem-resistant Enterobacteriaceae (C.R.E.)[152], high-quality evidence), the R.R. for persistent colonization at the end of decolonization therapy was 0.42. However, this effect was non-significant after one month (RR=0.72).

Many of the studies listed above have weak study designs, particularly the nonrandomized design, making it challenging to provide high recommendations. Furthermore, most studies assessed the effectiveness of decolonization strategies at the end of the therapy or for a brief period (one month). It appears critical that studies assess the long-term impact of decolonization to interpret the results correctly.

Routine decolonization of ESBL- Enterobacteriaceae is not recommended in the latest ESCMID-EUCIC recommendations on decolonization of multidrug-resistant Gram-

negative bacteria carriers[153]. The guidelines suggest undertaking a trial for high-risk patients for whom a temporary suppression would be clinically relevant, based on limited evidence of the possible efficacy of short-term decolonization.

Chapter IV: Essential oil as an alternative to combatting MDR

I. Combatting antimicrobial resistance

Researchers can prevent the antimicrobial resistance (AMR) crisis by improving education and knowledge of AMR, boosting investments, providing support for research on novel antimicrobials, and implementing strategies to combat misuse and reduce the use of antibiotics worldwide[154].

The challenge of AMR's threat is worldwide. The USA pledged to invest 1.2 billion dollars in preventing AMR, almost doubling its funding in 2015[155]. The UK's anti-AMR strategies began in 2000[156] and employ economics expert O'Neill (2014). O'Neill (2014) suggested that ten interventions are needed immediately to mitigate AMR. The suggestions focus on improving awareness of AMR worldwide by using programs and campaigns, focusing on improving sanitation and hygiene, increasing surveillance of antimicrobial consumption and resistance, encouraging and supporting infectious disease researchers, investments in new drugs, advancing existing drugs, and rising non-commercial research funding[157]. These efforts need to be undertaken concurrently with other alternatives to antibiotics and the generation of a global alliance.

The UK's latest strategy to combat AMR reported only a 7% reduction in human consumption of antibiotics but a 40% reduction in agricultural antibiotics use during 2013-2018[158]. During this period, there was a 35% increase in bloodstream infections due to resistant bacteria[159].

II. Essential oil treating infections

1. EO definition

According to the European Pharmacopoeia 2011[160], essential oil is a fragrant product with a complex composition obtained from a raw fragrant vegetal material, water vaporization, dry distillation, or a proprietary mechanic process that does not require drying. Today, we have over 3000 EO, of which 300 are commercially available for use in pharmaceutics, perfumery, and cosmetics. In addition, the usage of Essential oil can be in various applications, including phytosanitary products, flavouring products, and, finally, human and animal food[161]. Furthermore, it has many benefits, demonstrated by experimental studies in various domains, including antimicrobial, antioxidant, and anti-inflammatory properties[162].

2. Antimicrobial activity

So far, the antimicrobial activity of EO has been recognized[163,164]. It links to their lipophilic property [165]. Terpenoids and phenylpropanoids can penetrate the bacterial membrane and reach the internal part of the bacterial membrane and cell due to their lipophilicity, which causes a destabilization of the structure and increases membrane permeability. These changes lead to the leakage of ions and intracellular compounds [166]. The loss of cytoplasmic material results in cell bursting. Nevertheless, it has also been proposed that properties such as functional groups and aromaticity are responsible for the antibacterial activity[165].

The essential oil has dual antibacterial activity against Gram-positive and Gram-negative bacteria in sessile and motile conditions[167,168]. Of 53 EOs screened, all exhibited activity against pathogenic bacteria and yeasts such as Bacillus subtilis, Escherichia Coli, P. aeruginosa, S. aureus, and Candida albicans[169]. As low as 0.02% EO, an effective antimicrobial property against E. coli is recognized by thyme, clove, lemon myrtle, bay laurel, lemongrass, cinnamon, and tea tree oregano, and rosewood[170].

Due to EO's volatility, the vapour phase has potential antimicrobial properties. Early studies of 133 EOs found that the vapour of cassia, cinnamon, cherry laurel, origanum, and thyme inhibited a wide range of bacteria[171]. More recently, EO vapours have been used to eradicate bacteria that cause pneumonia[172], inhibit moulds in food products [173], and combat biofilm-forming bacteria [174].

Another revolutionary revolution, the synergism of EOs combined with antibiotics, can prevent AMR transmission[175], with antibiofilm activities [176 177].

III. EO as an alternative against MDR

The spectrum of action of HE is vast. They work against a wide range of bacteria, including multiresistant bacteria. This activity is also variable from one essential oil to another and from one bacterial strain to another[178].

Several teams are working on the resistance problem, attempting to develop alternative treatments for the ESBL 3Gcephalosporinase and other multiresistant bacteria. Essential oils are one of these alternatives because of their potential antimicrobial properties.

1. Research in a worldwide using EO against MDR

Experimental work carried out by Buckova et al[179], the essential oils against multidrug-resistant gram-negative bacteria. The primary goal of this study was to look into the antimicrobial activity of five plant essential oils against multidrug-resistant Gram-negative bacteria.

The antibacterial activity of seven essential oils, oregano, thyme, arborvitae, cassia, lemongrass, and tea tree, was investigated by the agar diffusion method followed by MIC and MAC concentration against five multidrug-resistant isolates, namely Pseudomonas aeruginosa, E.Coli, Enterobacter cloaceae, Morganella morganii, Proteus mirabilis. Ty and OR Eos exhibited excellent responses against all MDR bacterial species, reaching the best MIC values. This study demonstrates the potential of investigated essential oils as natural alternatives for further application in hospital therapies to retard or inhibit bacterial growth.

In addition, a work realized by Mulyaningsih et al[180]. On the title Antibacterial activity of essential oils from *Eucalyptus* and of selected components against multidrug-resistant bacterial pathogens, all the oils tested and the components were hardly active against MDR Gram-negative bacteria. Aromadendrene was found to be the most active, followed by citronellol, citronellal, and 1,8-cineole.

Furthermore, 1,8-cineole is shown to potentiate the activity of other antibacterial compounds when combined. It increases the permeability of the wall, which causes an imbalance in the membrane of the bacteria and thus facilitates the penetration of other more active compounds into the latter.

2. Essential oil as colonization-fighting

As we see before, the colonization by MDR bacteria is a critical issue; an urgent solution is needed to limit the spread of the multiresistant bacteria. Therefore multiple studies investigated the EO as a solution to the colonization problem.

In vitro study realized by Gadisa and all[181]; on the title “Evaluation of Antibacterial Activity of Essential Oils and Their Combination against Multidrug-Resistant Bacteria Isolated from Skin Ulcer.” This study found that the combined effect of EOs has significant antibacterial activity on wound colonizing bacteria and reduces delaying wound healing as that of modern drugs tested in parallel. Hence, further structural elucidation of active compounds helps us properly design or synthesize topical antibiotics for wound care.

There is little work on using EO to fit the colonization issues of multidrugresistant bacteria as a treatment, so more studies are needed to establish a novel protocol and recommendation to combat the colonization/infections of the digestive tract by MDR Gram-negative bacteria.

3. Local research using different EOs against MDR

Numerous studies test the activity of EO against multiresistant bacteria, mainly to establish a practical protocol, with the purpose is to use EO as an alternative treatment on multiresistant bacteria.

Remmal and his team have worked on EO's activity against multiresistant bacteria in multiple studies published or not. Like the work done by El abed and all[182], the title"Carvacrol and thymol components inhibiting Pseudomonas aeruginosa adherence and biofilm formation." This study aimed to investigate whether carvacrol

and thymol can interfere with adherence phenomena act on biofilm formation. The tests on P. aeruginosa strains showed that carvacrol and thymol interfere with the starting phases of adherence with P. aeruginosa biofilms.

More, on another work done by Hriouech and all [183], entitled “the title The Antistaphylococcal Activity of Amoxicillin/Clavulanic Acid, Gentamicin, and 1,8-Cineole Alone or in Combination and Their Efficacy through a Rabbit Model of Methicillin-Resistant Staphylococcus aureus Osteomyelitis”. The results demonstrated that 1,8-cineole showed a synergistic effect combined with AMC and gentamicin, which offer possibilities for reducing antibiotic usage. Also, the AMC associated with 1,8-cineole could be used to treat MRSA osteomyelitis.

An important fact is included in a study conducted by the same author and all[184], entitled "In Vitro and In Vivo Comparison of Changes in Antibiotics Susceptibility of E. coli and Chicken's Intestinal Flora after Exposure to Amoxicillin or Thymol. " It demonstrated that exposure to amoxicillin induced a selection of antimicrobial resistance in TAMF and intestinal *E. coli*, whereas exposure to thymol did not.

This work is a continuation of the work cited before or not, a preclinical animal model study investigating the decolonization/infections of carriers of multiresistant bacteria.

Materiel and methods

I. Study design and quality criteria

1. Study design

The decolonization process of multidrug-resistant gram-negative bacteria carriers in the digestive tract was the main focus of this preclinical research.

The realization of this study lasted over one year, which the first months dedicated to protocol development, followed by the pre-realization period, installation of all necessary materials, and finally, the protocol's implementation, which took several months.

This preclinical study was arranged at the faculty of medicine, pharmacy, and dentistry medicine fez's animal house and laboratory of microbiology and molecular biology.

The progress of this study was in stages. The first step was to promote colonization, which accomplishes by using antibiotics to select the BMR based on the selection pressure process and directly introducing the germs orally, resulting in MDR gram-negative bacteria colonization of the digestive tract. The final step is to eradicate/discharge the digestive tract using the oral treatment AMC+cineol. To conclude, the purpose was to decolonize/treat the MDR gram-negative bacteria carriers.

2. Quality criteria-animal house

To ensure the highest possible quality of the study, we elucidated the quality criteria used in this study for the animal house based on the recommendations of the Commission of the European Communities for guidelines for the housing and care of animals used for experimental and other scientific purposes[185].

Before the experiment, the animal house and all the breeding equipment were disinfected with bleach and detergent. Then, for water and food, we build a safe, easy-to-use, and disinfectable system.

Regarding the frequency of cleaning, consideration gives to the type of compartment, the population density, and the capacity of the ventilation systems to maintain adequate air quality for all the criteria mentioned above. We obtained for one on four/six days.

Rabbits housed in groups have been staying in the same litter and together since weaning.

During handling, we emphasized the importance of minimizing disturbances to our animals or their environment in the compartment by limiting excessive contact and developing a simple and effective system for the oral administration of drugs or inoculum.

II. Materiels

1. Antimicrobial Agents

The antimicrobial agent used is oxytetracycline for the selection of MDR-GRAM negative bacteria[186]. AMC, and AMC-1,8 cineol for decolonization and infection treatment.

2. Inoculum preparation

We chose E. coli MDR-Gram negative from the microbiology and molecular biology laboratory bank strain at Fez's Faculty of Medicine. We confirmed the species with an adequate biochemical identification test and a profile of sensibility. We then proceeded to successive dilutions until we produced a dilution at a rate of 10^5 (figure 5). We will subsequently feed this inoculum to the rabbits through the alimentation feed to each cage to avoid any complications.

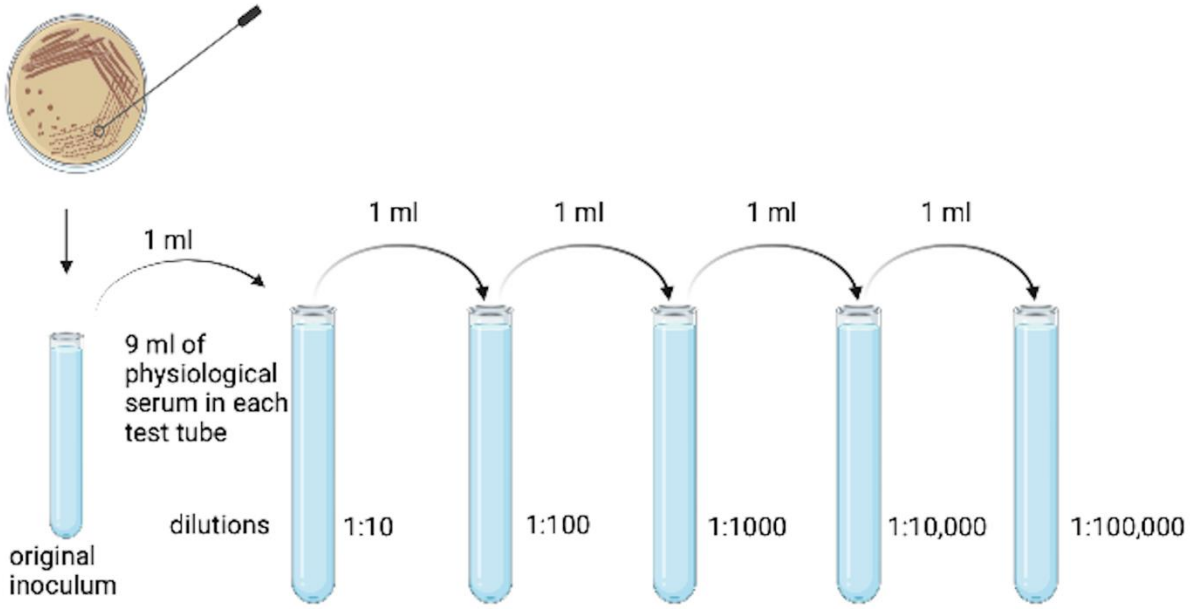


Figure 5: A series of dilutions from a mother inoculum to a diluted inoculum at a rate of 10^5.

3. Culture media

Mueller-Hinton agar (MHA, Biokar®), tryptic soy agar (TSA, Oxoid®), and Eosin methylene blue agar (EMB, Oxoid®) were prepared and sterilized according to the manufacturers' instructions.

4. Animals

We got the rabbits from a vendor who specializes in rabbit breeding and distribution. The rabbits were in the faculty of medicine's animal lab. We admitted 19 rabbits weighing between 1 and 1.2 kg and divided them into four groups.

The rabbits were given feed and water ad libitum and treated following the National Health and Research Council Ethics Committee guidelines[187].

Adequate ventilation was provided, and the environmental temperature was constantly maintained at 21°C ± 3°C. The photoperiod was adjusted daily to 12 h of light and 12 h of darkness. For acclimatization, the animals of the experiment were kept for a week.

5. Groups of Animals

The animals were randomly divided into four experimental groups:

Groups 1: animals colonized with ESBLE E. coli and decolonized with AMC-1,8 cineol at an 80 mg/kg dose.

- Cage 1: 2 rabbits
- Cage 2: 3 rabbits

Groups 2: Control group: colonize with ESBLE E. coli, spontaneous decolonization.

- Cage 3: 3 rabbits
- Cage 4: 1 rabbit

Groups 3: Animals groups still received ESBLE E. coli and were decolonized with AMC at a dose of 80 mg/kg.

- Cage 1: 2 rabbits
- Cage 2: 3 rabbits

Groups 4: Animals groups still received ESBLE E. coli and were decolonized with AMC-1,8 cineol at a dose of 80 mg/kg.

- Cage 1: 2 rabbits
- Cage 2: 3rabbits

As previously stated, all of the animals had been given oral oxytetracycline for 12 days.

The way of administration[188], in the all administration we choose the oral administration.

The doses administered were calculated according to the weight by imitating the recommended human dose for each drug: 80 mg/kg/twice daily AMC, AMC-1,8 cineol, and for tetracycline at a dose of 25mg/kg.

6. Sample collection

Fresh rabbit faeces specimens were collected in the middle of the day with a mosquito net placed under each cage (figure 6).

Figure 6: Under each cage, a mosquito net is collecting faecal excrement.

We collected 5 g of faeces from each cage and placed it in a Centrifuge Tube Plastic of 50ml with 45 ml of physiologic serum. After that, we were transported to the Fez Faculty of Medicine and Pharmacy's Laboratory of Microbiology and Molecular Biology (Morocco).

III. Microbiologic analysis

The samples were immediately processed in the faculty of medicine's microbiological laboratory. They were instantaneously homogenized using a vortex mixer for at least 5 minutes and inoculating with approximately 10 µl in the EMB media (figure 7).

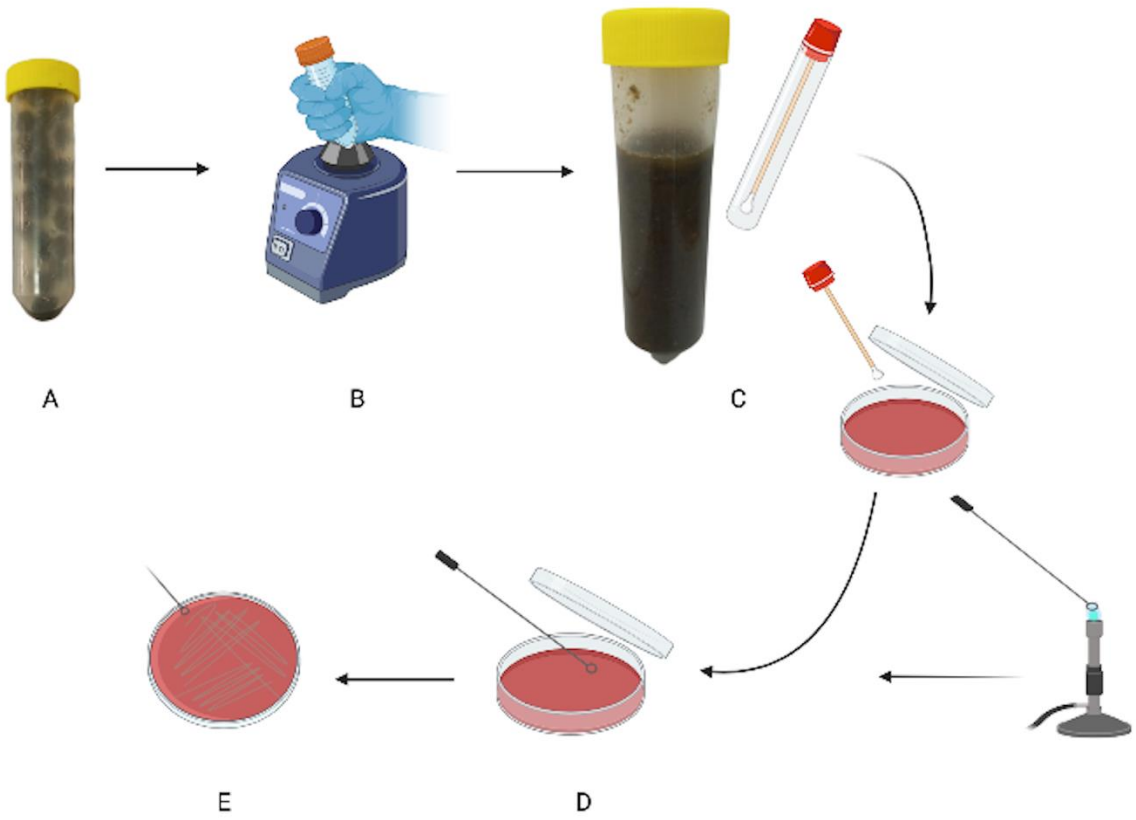


Figure 7: The processes go from a faecal sample to an inoculated EMB petri dish.

A: 5 g of faeces, add 45 ml physiologic serum in a Centrifuge Tube Plastic of 50ml. B: vortex mixer to homogenize the tube. C: take about 10µl from the tube by using

a swab. D: make a separate strip for better bacteria isolation. E: an inoculated Petri disk.

The sample was inoculated on selective culture media: Eosin methylene blue (EMB). For financial reasons, chromogenic media were not routinely used, but in the beginning, we matched our protocol by using it (figure 8). The EMB medium is used as a selective GRAM-negative bacillus, particularly enterobacteria isolation. The sample was inoculated on two culture media: Eosin methylene blue (EMB) and EMB medium with ceftazidime. Incubation was carried out at 37 ° C for 24 h. the following comparison validated the culture medium; The EMB agar supplemented with 2 mg/L of ceftazidime was assessed compared to chromID ® ESBL medium as a reference. Ceftazidime powder used in the experiment was provided by manufacturer [189].

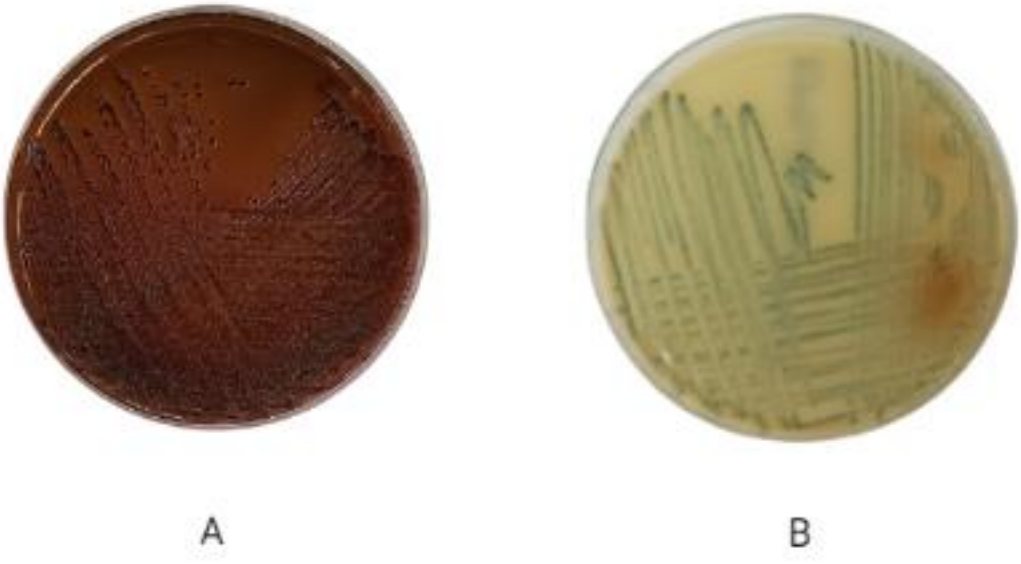


Figure 8: Validation of the protocol by comparing A and B.

A: EMB agar supplemented with 2 mg/L of ceftazidime. B: ChromID ® ESBL medium.

1. Enterobacteria isolation and purification

Macroscopic Aspect, GRAM staining, biochemical identification tests IMVICs (indole, Methy Red, Voges Proskawer, citrate), and API20E confirmation of identification were performed on the colonies grown on EMB media (figure 9).

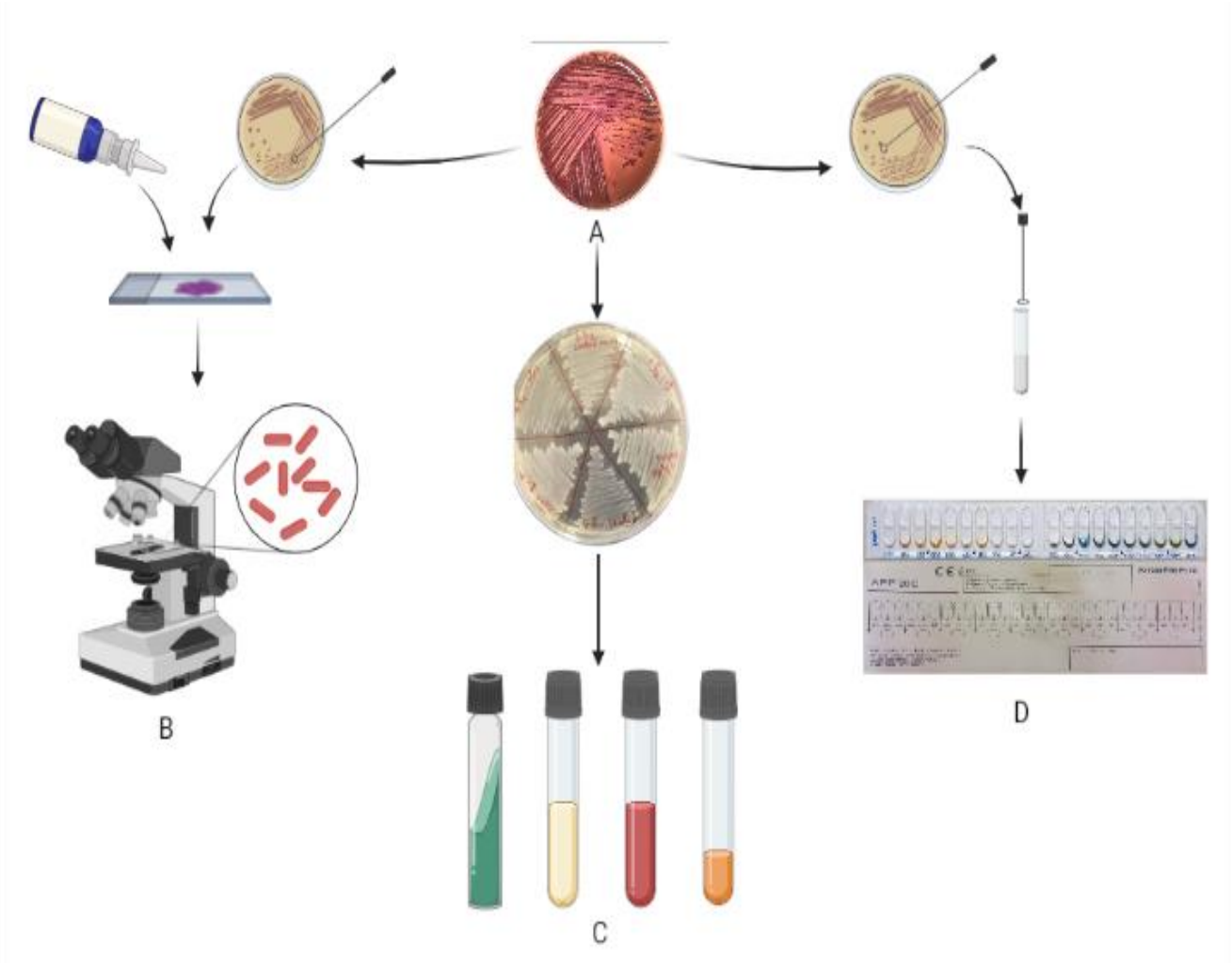


Figure 9: All the steps carried out leading to bacterial identification.

A: Macroscopic Aspect. B: gram strain. C: IMVIC test. D: gallery API20E

2. Macroscopic aspect and GRAM strain

a. Macroscopic aspect

The macroscopic appearance of enterobacteria allows Escherichia coli to be distinguished from other gram-negative bacteria[190].

In general, E. coli colonies are 2-3 mm flat, dark purple with a metallic sheen. In contrast, Klebsiella colonies are large convex roses with fusing mucous membranes, and Serratia grows in small colonies, 1 to 2 mm greyish in diameter.

b. GRAM strain

It is a colouring that allows the proprieties of the bacterial wall to be highlighted to distinguish and classify them. The procedure is as follows: The slide is spread as thinly as possible with a handle, dried, and then completely covered with gentian violet for one minute. The slide is then soaked in Lugol for one minute, washed with distilled water, decorated for 20 seconds with acetone alcohol, rinsed with distilled water, covered with fuscine solution for one minute, rinsed again with water, and dried in room air or on a hotplate[191].

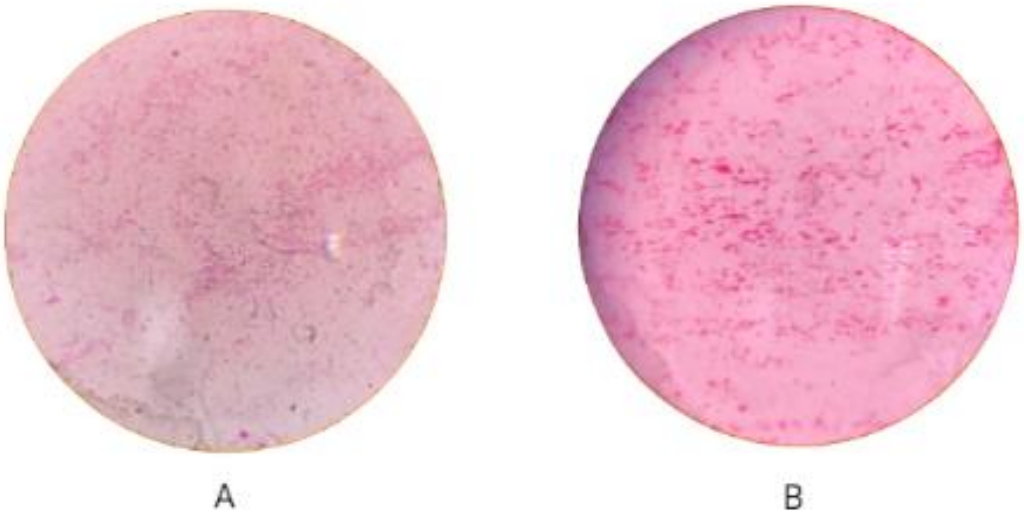


Figure 10: Example of gram-negative in the gram strain.

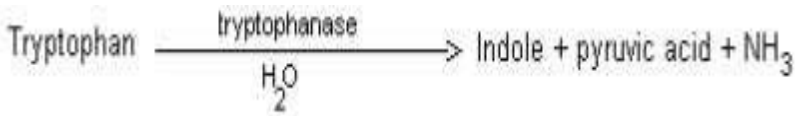
A: E. coli. B: K.P

3. Biochemical identification IMViC

It is a set of tests used to distinguish various gram-negative intestinal bacilli belonging to the Enterobacteriaceae family. IMVIC tests are composed of four different tests. Each letter in the word "IMViC" represents one of these tests[192 193].

a. Indole production

Depending on the section, microorganisms break down tryptophan to indole in the presence of a tryptophenase.



Kovac's reagent is used to detect indole production. Indole reacts with the reagent's aldehyde to produce a red colour. The red colour is concentrated as a ring at the top by an alcoholic layer.

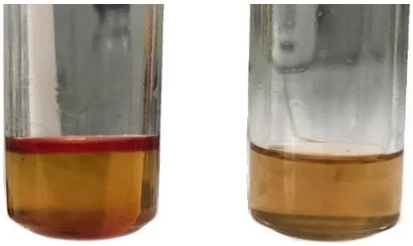


Figure 11: Indole test after adding Kovac's reagent.

A: positive test: red color is concentrated as a ring at the top. B: negative test

b. **Methyl red test**

It allows assessing an organism's ability to produce and maintain stable acid end products from glucose fermentation. Some bacteria produce so many acids from glucose fermentation that they overwhelm the system's buffering action. Methyl Red is a pH indicator that remains red at pH levels of 4.4 or lower.

Glucose → Pyruvic acid → Mixed acid fermentation (pH 4.4)
↓
Red color with methyl indicator

The bacterium to be tested is inoculated into glucose phosphate broth which contains glucose and a phosphate buffer, and incubated at 37°C for 24 hours

A B

Figure 12: Methyl red test after adding methyl indicator.

A: positive test: red colour. B: negative test: no change in colour.

c. VOGES PROSKAUER (VP) TEST

Acetyl-methyl carbinol (acetoin) is an intermediate in the production of butylene glycol. In this test, two reagents, 40% KOH and alpha-naphthol, are added to test broth after incubation and exposed to atmospheric oxygen. If acetoin is present, it is oxidized in the presence of air and KOH to diacetyl. Diacetyl then reacts with guanidine components of peptone via alpha- naphthol to produce a red colour. The role of alpha-naphthol is that of a catalyst and a colour intensifier.

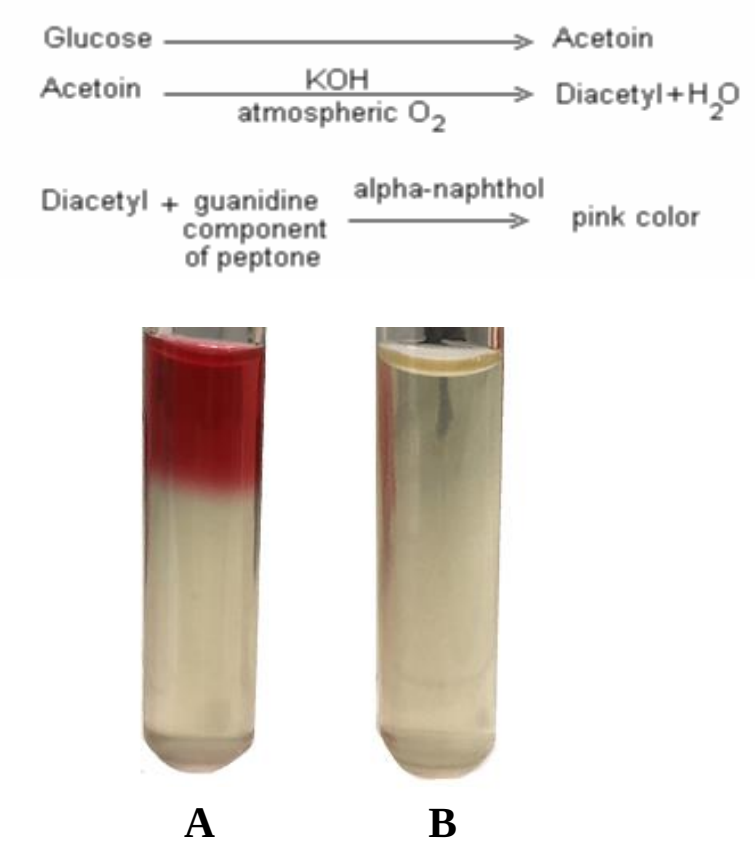
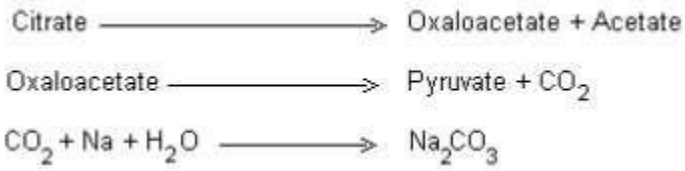


Figure 13: VP test after adding KOH and alpha-naphthol.

A: Positive test: showing a change in colour to red colour. B: Negative test: no change in colour.

d. <u>Citrate test</u>

This test detects an organism's ability to use citrate as its sole source of carbon and energy. Bacteria are grown in a medium that contains sodium citrate and the pH indicator bromothymol blue. The medium also contains inorganic ammonium salts, which are used as the sole nitrogen source.



Citrate is used by the enzyme citritase, which converts citrate to oxaloacetate and acetate. Oxaloacetate is further degraded to produce pyruvate and CO2, the production of Na2CO3 and NH3 from sodium citrate and ammonium salt results in an alkaline pH. As a result, the colour of the medium changes from green to blue.

A **B**

Figure 14: Citrate test.

A: positive showing blue colour. B: negative: there is no change in colour.

e. Examples IMVIC tests

The identification results are correlated with reference results such as the pocket atlas book of microbiology[192].

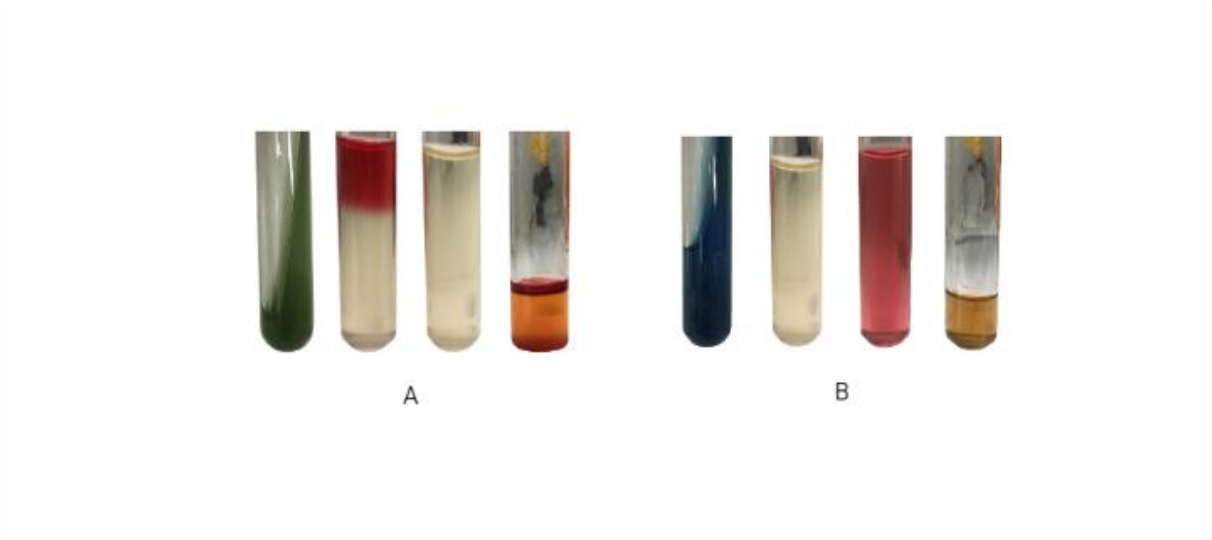


Figure 15: Example of IMVIC test.

A: E.coli. B: KP

4. API20E

The API 20 E constitutes a standard system for identifying Enterobacteriaceae, such as bacilli, a gram-negative fermentants[194]. His principle is based on the fermentation and oxidation of ten carbon sources and the application of ten enzyme tests. Each gallery contains 20 dehydrated substrate-filled microtubes. A bacterial suspension is inoculated into the microtubes. The reactions during the incubation period cause spontaneous colour changes or are revealed by adding the reagents. The reading table is then used to interpret the reactions, and the analytical catalogue (biomérious) or recognition software is used to identify them[195 196].

a. Gallery preparation

The gallery's preparation begins with entering the strain's reference on the box's side tab, followed by placing the gallery in the incubation box. After that, distribute 5ml of distilled water around the gallery's alveolus to create a humid atmosphere. A colony from a young culture of 18 to 24 hours is inoculated in a sterile tube containing 5 ml of sterile physiological saline, creating a homogenous bacterial solution. The gallery is inoculated with the prepared bacterial suspension by filling the tubes and cups of the CTI, VP, GEL tests, and only the tubes of the other tests, and finally by filling the wells of the ADH, LCD, ODC, URE, and H2S tests with paraffin oil to create anaerobiosis. Finally, close the incubation box and place it in the incubator for 18-24 hours at 36 °C +/- 2°C.

b. Gallery reading and interpretation

The digital profile is created using a results sheet. The tests are classified into triplets; each is assigned a 1, 2, or 4. The triplet's three outcomes are summed together. The sums of each triplet read from left to right produce a code of at least seven digits corresponding to the microorganism's metabolic profile. Identifying this bacterium is often attainable by comparing its code to those found in the bioMérious database. If the digital code received is not in this database, it could be a profile of an unreferenced microbe, a technological fault, or a mutation during bacterial development.

c. Gallery example

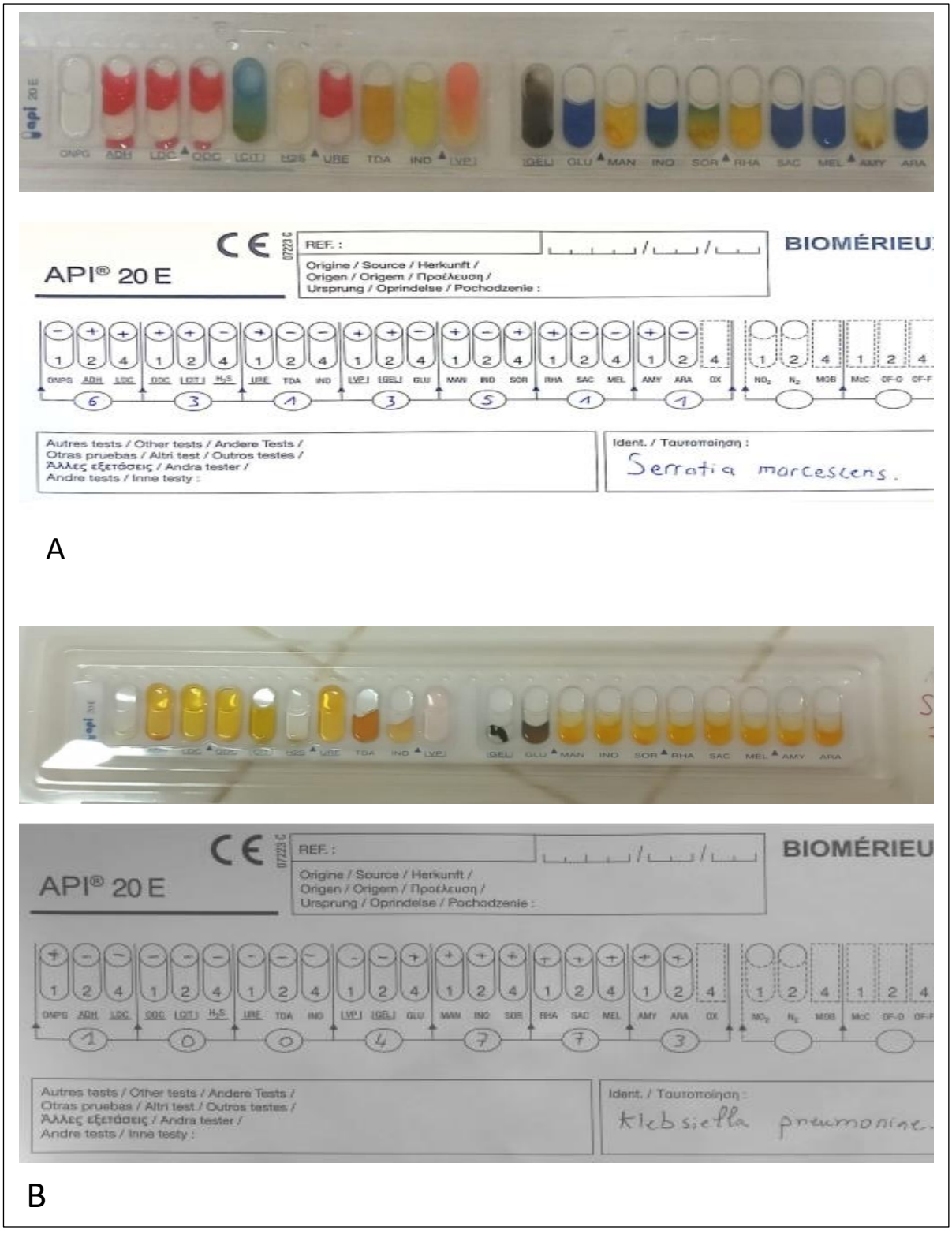


Figure 16: API20E gallery of some strain tested. A: Serratia marcescens and B: Klebsiella pneumoniae.

IV. Antibiotic Sensitivity Test

The antibiotic sensitivity test is a test that determines a bacteria's sensitivity to several antibiotics. It is carried out using the diffusion method on agar medium, as suggested by the antibiogram committee of the French society of microbiology [197].

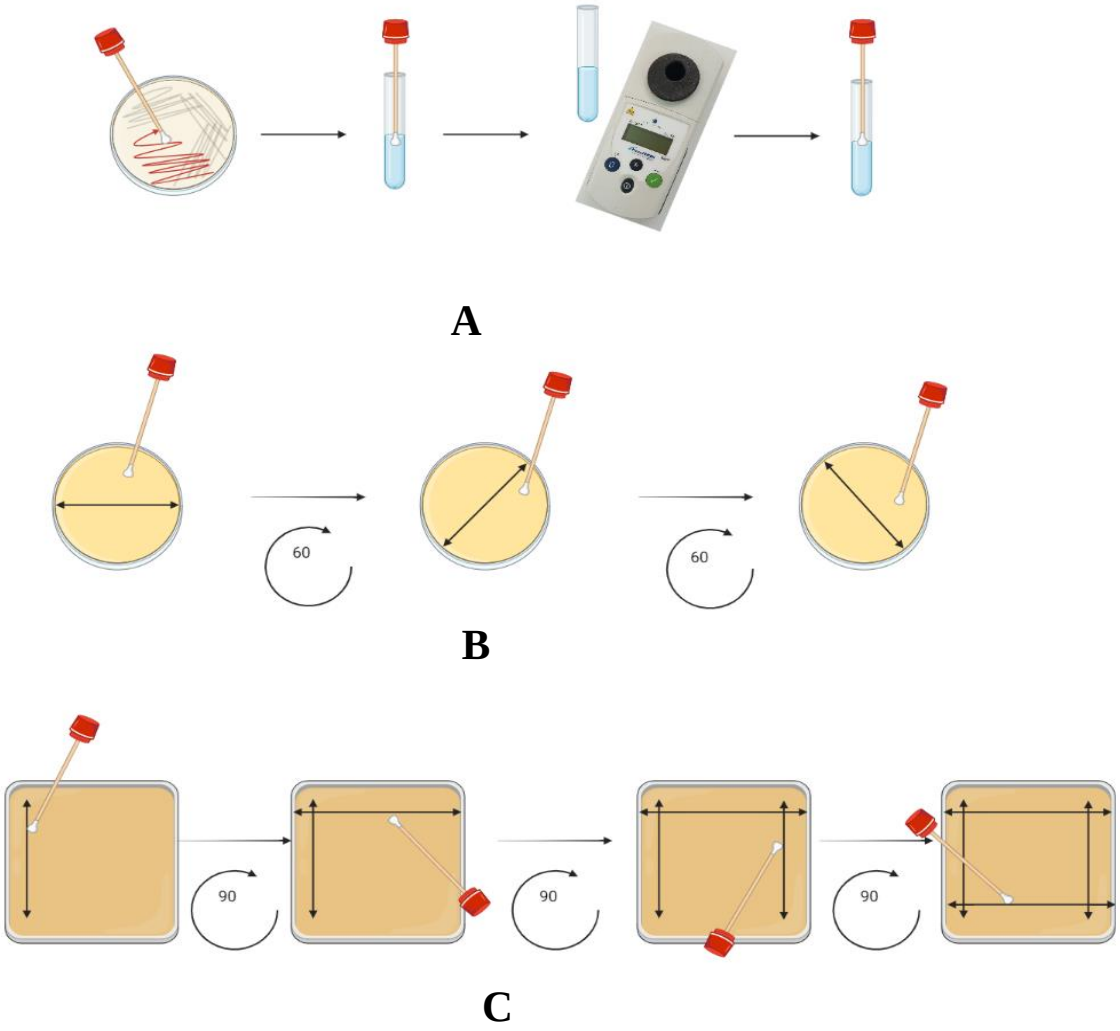


Figure 17: Steps of carrying out an antibiogram.

A: inoculum preparation. B: inoculation of Petri dish of 90mm. C: inoculation of Petri dish of 160mm.

1. Culture Media

Muller Hinton (MH) media was evenly distributed in the Petri dishes at a depth of 4 mm.

2. Preparation of bacterial inoculum

The bacterial suspension is prepared by homogenizing 4-5 colonies of a new 18-24 hour bacterial culture on a non-selective agar medium (TSA) in 0.9 percent sodium chloride, resulting in an Mc standard bacterial inoculum. The Mc Farland device determined Farland 0.5 (10^8 CFU/ml) (figure).

3. Swab inoculation

After obtaining the bacterial solution, a swab was used to inoculate the entire surface of the MH medium. Three passages with an orientation offset of 60 degrees were accomplished for the Petri dish measuring 90 mm. Four passages with an orientation offset of 90 degrees were conducted for the Petri dish measuring 160 mm, followed by a 24 hours incubation at 37°C (figure).

4. Disk application

The antibiotic-soaked discs were placed using flamed forceps, gently pressing them to ensure that they stuck nicely to the agar surface, taking into consideration depositing them at a minimum distance of 15 mm from the edge of the box. The distance between the two discs had to be at least 30 mm so that the zones of inhibition did not overlap.

5. Incubation

The boxes were then placed in a hot air oven set to 37°C for 18-24 hours, and the results were recorded by measuring the diameters of the inhibition zones around the antibiotic disks. The results are expressed as susceptible, intermediate, or resistant according to the criteria recommended by the EUCAST[197].

6. Choice of antibiotics

The antibiotic susceptibility profile of the Gram-negative isolates was determined using the standard Kir-by-Bauer disk diffusion method.

These antibiotics with their respective disk concentrations are as follows: B_lactam group, the penicillins; including Amoxicillin-acid clavulanic (20-10 µg), Piperacillin(30 µg), Piperacillin-tazobactam (30-6µg), Ticarcillin (75 µg). Cephalosporin; including Cefadroxil (30 µg) ceftazidime (10 µg), and cefepime (30 µg), Ceftazidime (10 µg), Cefixime (10 µg). Aminoglycosides group, including amikacin(30 µg). Carbapenems group, including imipenem (10 µg); Ertapenem (10 µg). Quinolones group, including Ciprofloxacin (5 µg). Also, Trimethoprim-sulfamethoxazole (1,25- 23,75 µg) is recommended by the EUCAST[197].

7. Example of the antibiotic sensibility profile

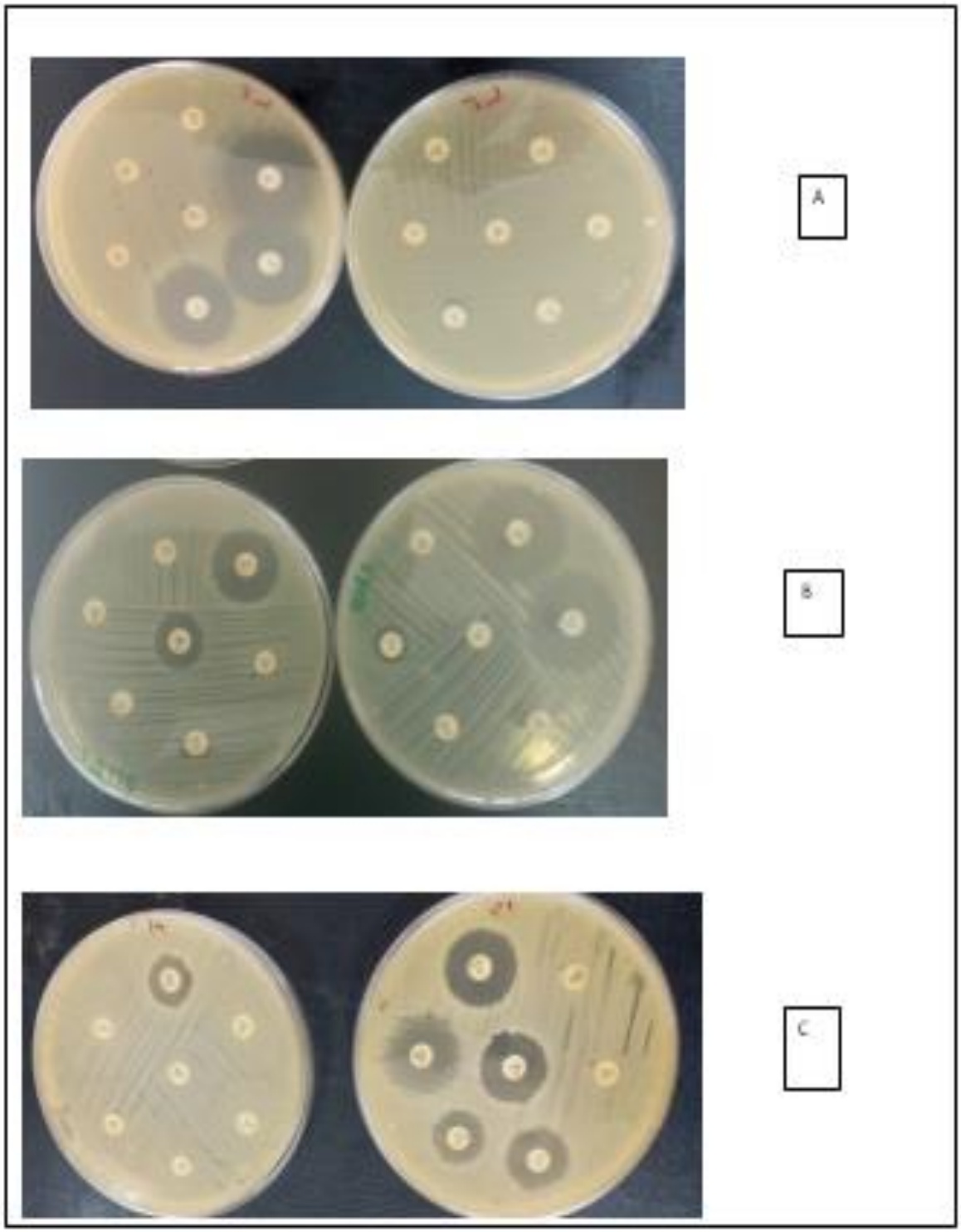


Figure 18: Example of an antibiotic resistance profile.

A: Serratia marcescens, B: E.coli, and C: Klebsiella pneumoniae.

V. Experimental Design

The protocol study was established according to the criteria cited in "a Practical Guide for Health Researchers"[198].

Within a week of their arrival, the rabbits were settled in and familiar with our animal lab. We collected a faecal sample to determine the resistance status of our rabbits' intestinal flora, even though we had earlier checked with our salesperson that they had not received any antibiotics.

In this experiment, we chose the fact that taking an antibiotic allows selecting multiresistant bacteria. This step is justified for several reasons, recognized in the bibliography section. To summarize, antibiotics are widely used, particularly for self-medication, which is on the rise. Also, the COVID-19 pandemic has contributed to increased antibiotic prescriptions and, as a consequence, antibiotic resistance.

We chose oxytetracycline based on a study conducted in Ivory Coast with the title Effect of oxytetracycline and colistin administration on the antibiotic resistance of Escherichia coli in piglets allowed to select for ESBL E.coli using tetracycline[186].

The duration of treatment was established in fewer than two weeks. We took a sample before, in the middle, and at the end of the antibiotic therapy.

The following step was to take an ESBL E.coli from the bank strain of our laboratory, which was conserved under optimal conditions. A profile of resistance was performed after culture to ensure that it is a high-class ESBL. Following a series of dilutions to achieve the desired concentration of 10^5, this concentration was chosen because it is not pathogenic; however, a progressive introduction was made. The administration

mode was fixed on the food to prevent accidental contamination, such as an introduction into the lungs.

The bacteria were given to all of the rabbits in our study. The duration of bacteria administration was maintained at 2 to 3 weeks. A series of samples were collected until a representative bacterial load was found.

The decolonization of the digestive tract was the final and most important step; in this regard, several protocols were tried without any clear recommendations, as described in the bibliography. We had provided a well-defined protocol along with a brand-new antibiotic that was not yet available on the market. The protocol was as follows: we divided our rabbits into four groups, two of which had their ESBL E. coli administration interrupted, one had AMC-1.8 cineol administered, and the other had no administrations. The other two groups gave the ESBL E. coli, but one got AMC-1.8 cineol and the other just AMC. Several samples, up to five, were collected to determine the decolonization process, achieving an optimal eradication that committed with recommendation criteria.

Noting that in all these steps, two parameters were follow-up punctually: weight and fever.

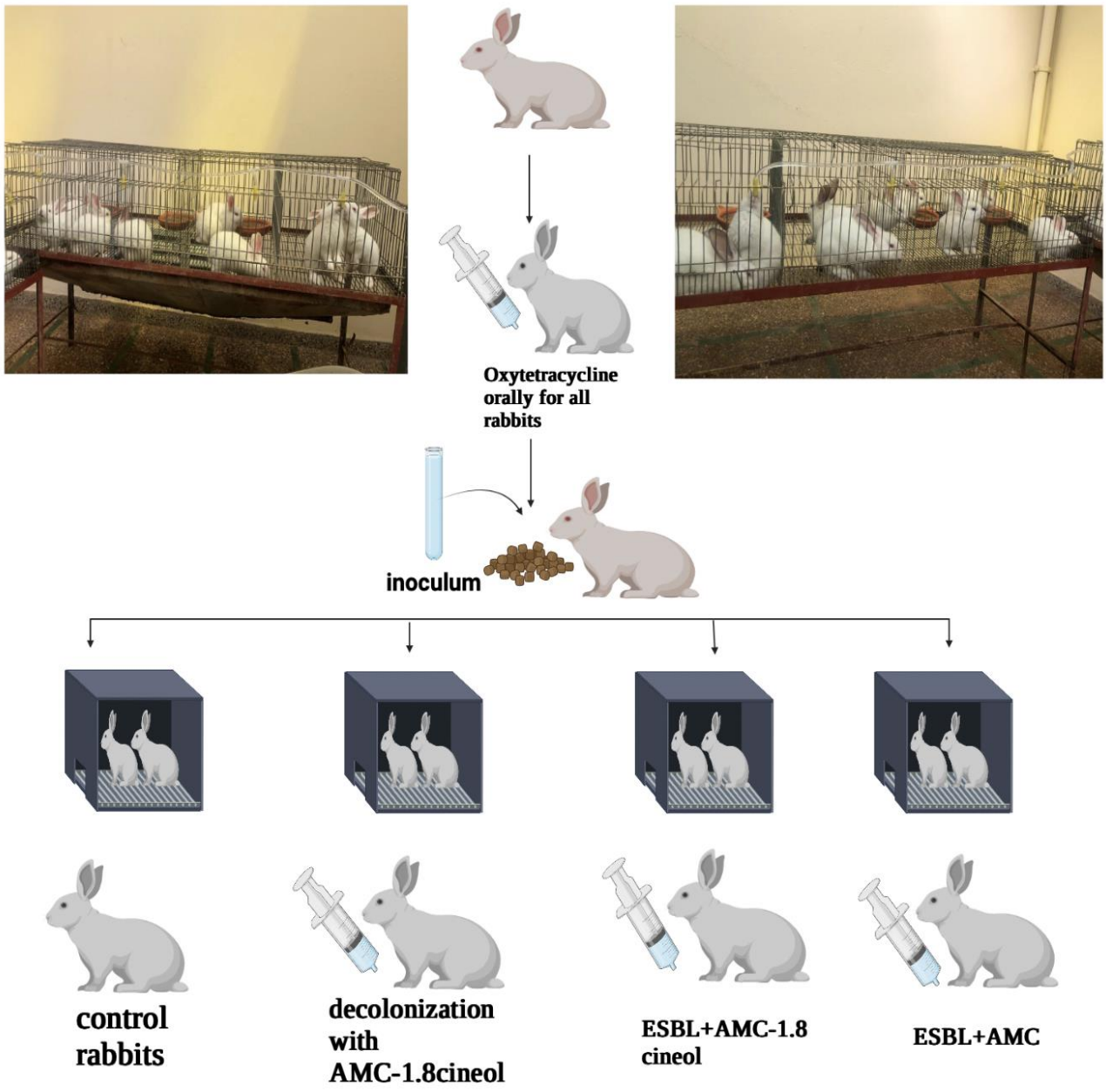


Figure 19: Protocol progresses from rabbits acquisition to the final step, decolonization.

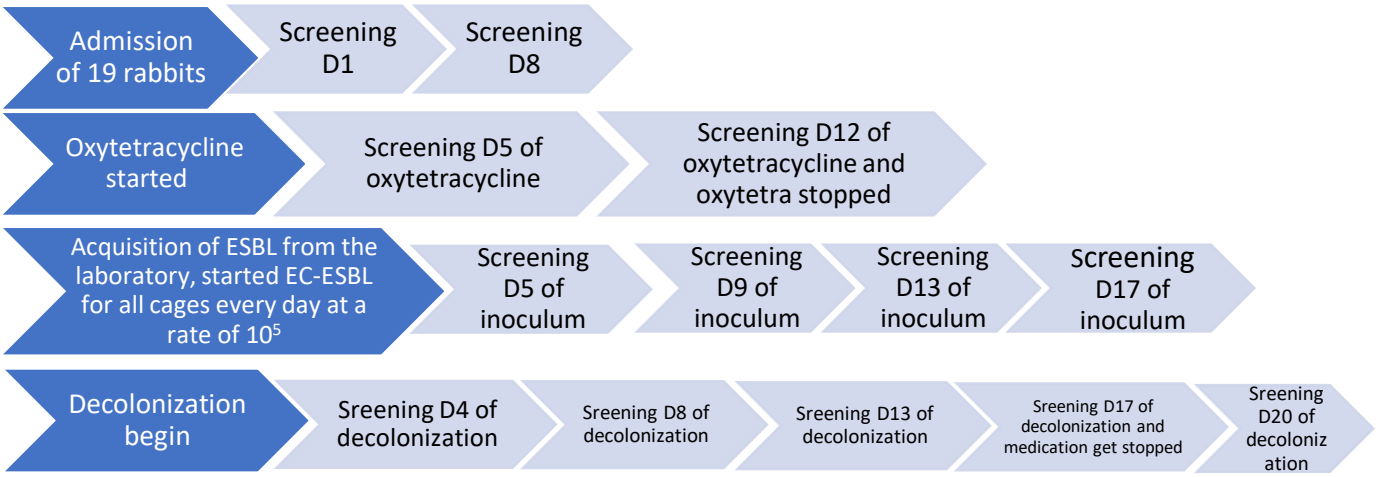


Figure 20: Chronological successive of the protocol.

VI. Clinical parameter

1. Weight

Individual weighings were performed at the start of the experiment and again during this study's phases.

The following formula is used to calculate the average live weight:

Average live weight (g) = total weight of rabbits in a cage/ the number of rabbits in this cage

The following formula is used to calculate live weight gain:

The increase in live weight (g) equals the weight of the following sample minus the weight of the previous sample.

2. Temperature

Individual rectal temperatures were measured in all rabbits during the decolonization period using a flexible digital thermometer placed at the rectal level in

each rabbit. The temperature mentioned for each cage is the sum of the temperature of the entire cage divided by the number of rabbits.

VII. Statistical Analysis

All data were collected and organized in MS Excel® (2019) for windows spreadsheets before being uploaded to and analyzed using GraphPad Prism 8.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com, and the results were expressed as a standard deviation average. Analysis of variance (one-way ANOVA) with Tukey's Multiple Comparison Test was used to evaluate the treatment's effects on the bacterial load (graph pad prism). Other parameters were evaluated by a descriptive analysis of colonization, weight, gain weight, temperature, and antibiogram.

Also, a ROC curve was realized to match our protocol and evaluated the process of screening

We realize a correlation between 3 parameters: temperature, weight or gain weight, and bacterial load.

Cumulative eradication curves were prepared using the Kaplan-Meier method, and univariant eradication distributions were compared using the log-rank test.

All for the statistical analysis realized, we considered p-value <0.05 to be statistically significant.

Results and discussion

Results

In the results section, we present the result following the divisions performed in the experimental design. Begin with the initial screening to know the resistance profile and the outcome of the oxytetracycline administration, following the colonization induction by the E. coli strains chosen from the bank of the laboratory. And finally, the most important is the decolonization party.

Reminding that we have the same distribution of our groups above in all parties of this study:

- group 1: AMC+cineol
- group 2: control
- group 3: bacteria +amc
- group 4: bacteria+amc+cineol

We quote the evolution of the parameters (eight and fever) following each section of the results.

We will present the results of the correlations, bacterial load between and follow-up parameters such as fever and weight, and the eradication with all the details associated with it.

I. Colonization screening and resistance selection

1. Initial screening

The results of the intestinal initial screening, which was done twice, once at the time of our rabbits' admission and again at the start of the second week, show that none of the 16 isolates tested did grow in the selected medium, allowing us to conclude that none of our rabbits had multiresistant bacteria in their intestinal gut.

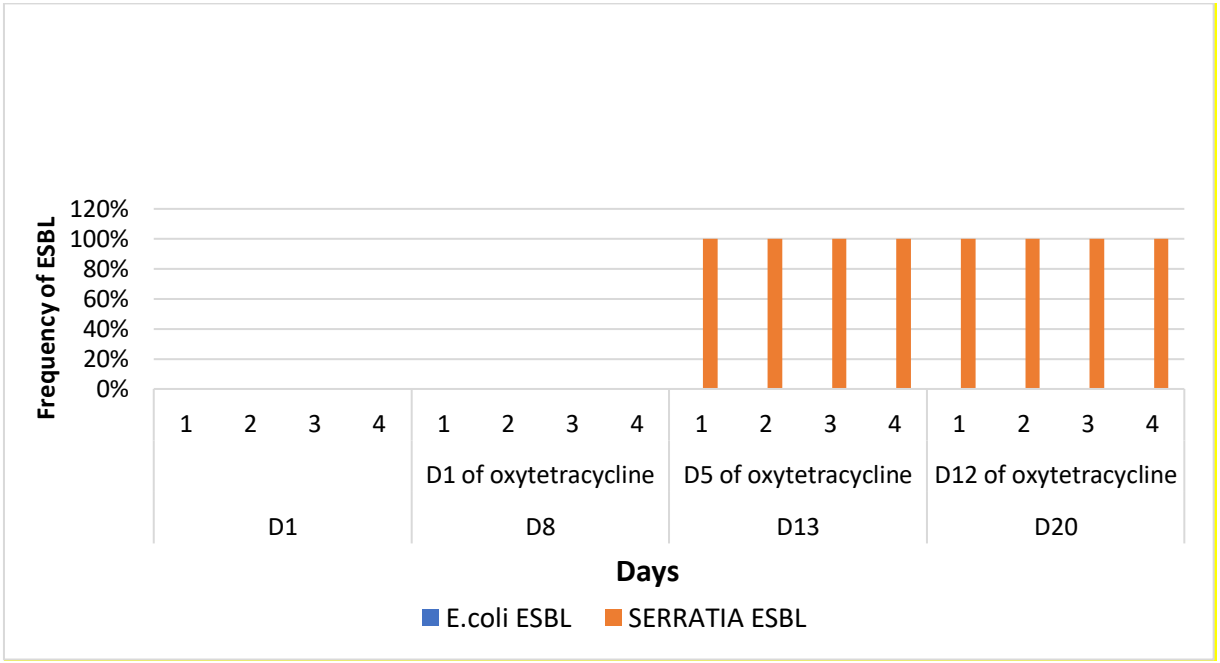


Figure 21: Results of ESBL screening and oxytetracycline administration during the first step of the protocol.

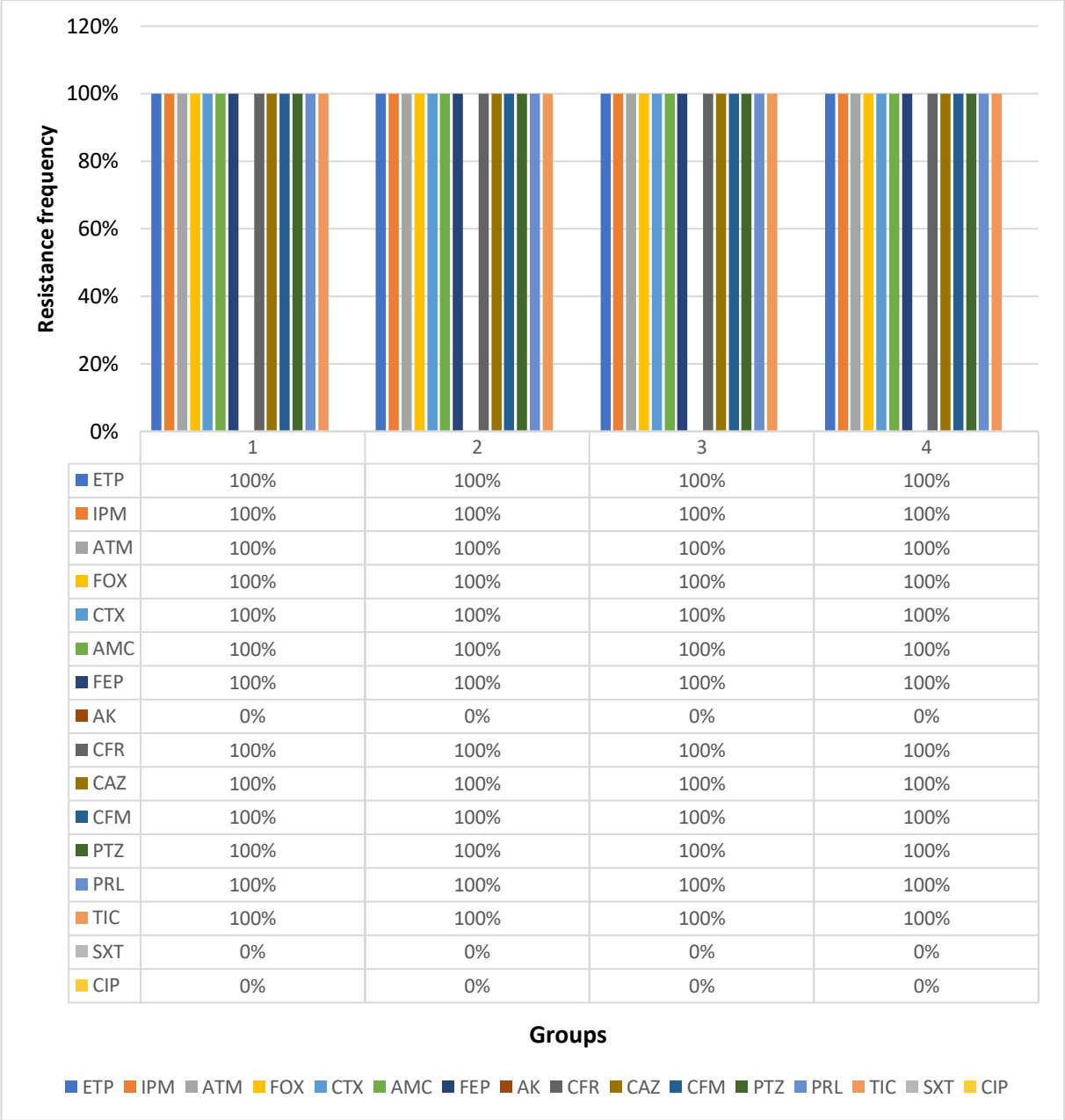
The resistance distribution detected during the first part of the protocol shows that no ESBL bacteria are detected on days 1 and 8. However, Serratia ESBL has been detected since day 5 of treatment with tetracycline.

2. Selection of resistance

Following the initial screening, the results of the next stage were committed to the introduction of oxytetracycline to select multiresistant bacteria.

Remembering that all groups received oxytetracycline, the results of this screening for MDR colonization were as follows: identification of a new strain, *Serratia marcescens*, which was identified on the supplemented medium from day 5 of its administration, and the susceptibility profile realized on 16 isolates between days 5 and 12 was as follows: resistant to all b-lactam antibiotics except amikacin, sulfamethoxazole/trimethoprim, and ciprofloxacin as shown in figure 20.

We may conclude that this bacteria is classed as ESBL 4Gcephalosporinase based on this result.



	1	2	3	4
ETP	100%	100%	100%	100%
IPM	100%	100%	100%	100%
ATM	100%	100%	100%	100%
FOX	100%	100%	100%	100%
CTX	100%	100%	100%	100%
AMC	100%	100%	100%	100%
FEP	100%	100%	100%	100%
AK	0%	0%	0%	0%
CFR	100%	100%	100%	100%
CAZ	100%	100%	100%	100%
CFM	100%	100%	100%	100%
PTZ	100%	100%	100%	100%
PRL	100%	100%	100%	100%
TIC	100%	100%	100%	100%
SXT	0%	0%	0%	0%
CIP	0%	0%	0%	0%

Figure 22: Resistance profile of Serratia.

This figure shows the distribution of antibiotics and their percentage of resistance compared to each group. We have resistance to most antibiotics, except for AK, SXT, and CIP, which applies to all of our groups.

3. Resistance profile of E.coli

from the first day of screening until the last day of oxytetracycline, we isolated 32 isolates of E.coli from a non-selected medium. We conducted a sensitivity profile of each one.

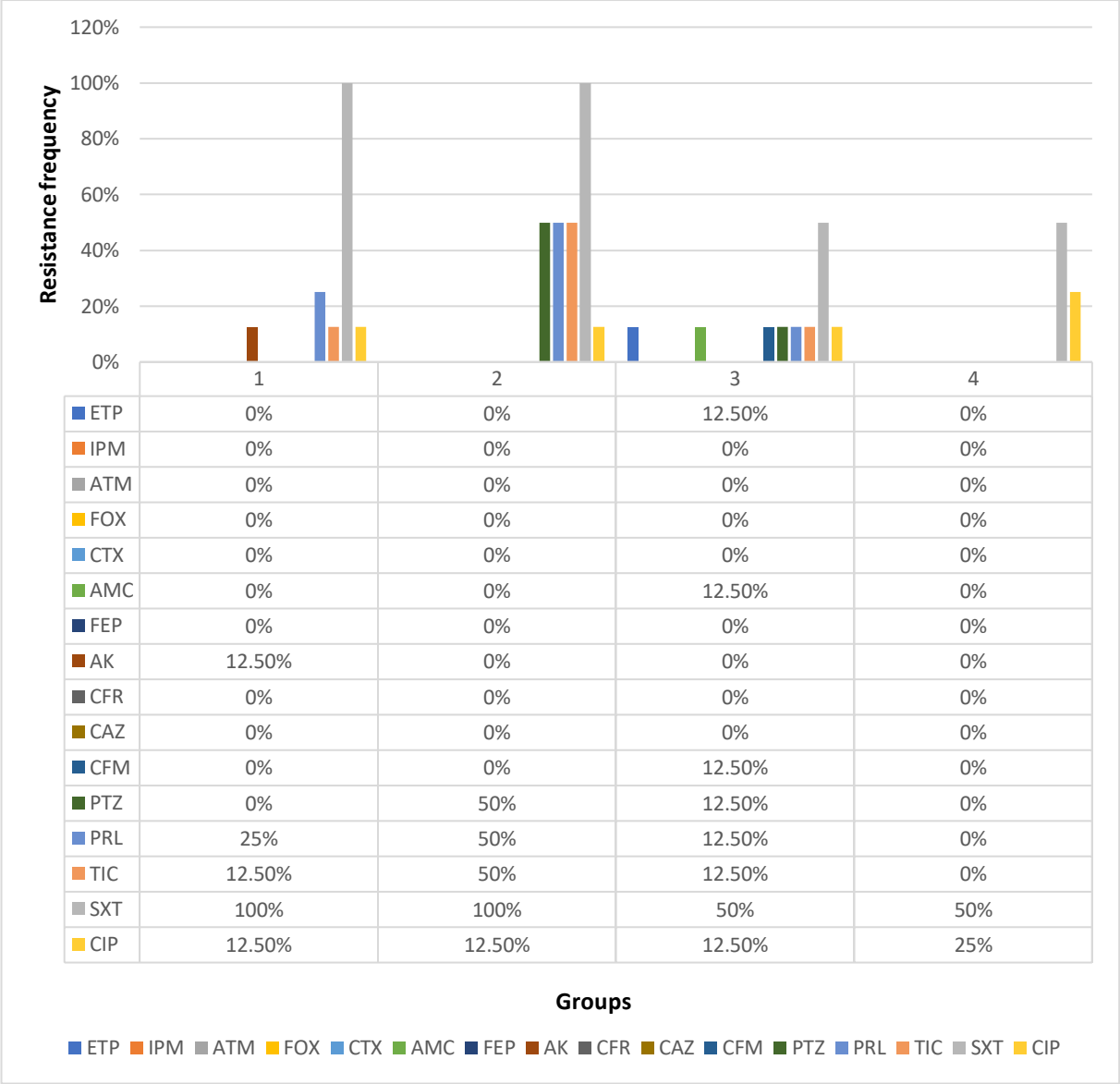
The profile of sensibility reveals an isolated resistance for all the groups.

The resistance distribution in group 1 reveals 100% resistance for the SXT, 25% resistance for the PRL, and 13% resistance for the AK, tic, and CIP.

We found 100 percent resistance for the SXT in the group 2 distribution. There is also 50% resistance to PTZ, PRL, and TIC and 13% resistance in the CIP.

The resistance distribution in group 3 shows 50% of resistance to the SXT, and 13 % of resistance to ETP, AMC, CFM, PTZ, PRL, TIC, and CIP.

Moreover, in group 4, the resistance distribution, we noticed 50 % resistance to SXT and 13% resistance to CIP.



	1	2	3	4
ETP	0%	0%	12.50%	0%
IPM	0%	0%	0%	0%
ATM	0%	0%	0%	0%
FOX	0%	0%	0%	0%
CTX	0%	0%	0%	0%
AMC	0%	0%	12.50%	0%
FEP	0%	0%	0%	0%
AK	12.50%	0%	0%	0%
CFR	0%	0%	0%	0%
CAZ	0%	0%	0%	0%
CFM	0%	0%	12.50%	0%
PTZ	0%	50%	12.50%	0%
PRL	25%	50%	12.50%	0%
TIC	12.50%	50%	12.50%	0%
SXT	100%	100%	50%	50%
CIP	12.50%	12.50%	12.50%	25%

Figure 23: Resistance profile of E.coli.

The figure depicts the distribution of antibiotics and their percentage of resistance concerning each group during the first part of the protocol. Furthermore, as shown, we have a majority resistance to SXT. For the other antibiotics, we have sporadic resistance, such as TIC, PRL, and TPZ, which have a resistance of 50% in group 2, and AMC, which has a resistance of 13% in group 3.

4. Clinical monitoring parameters

Every day, we examined each of our groups' eating habits and reactions using the appropriate tools.

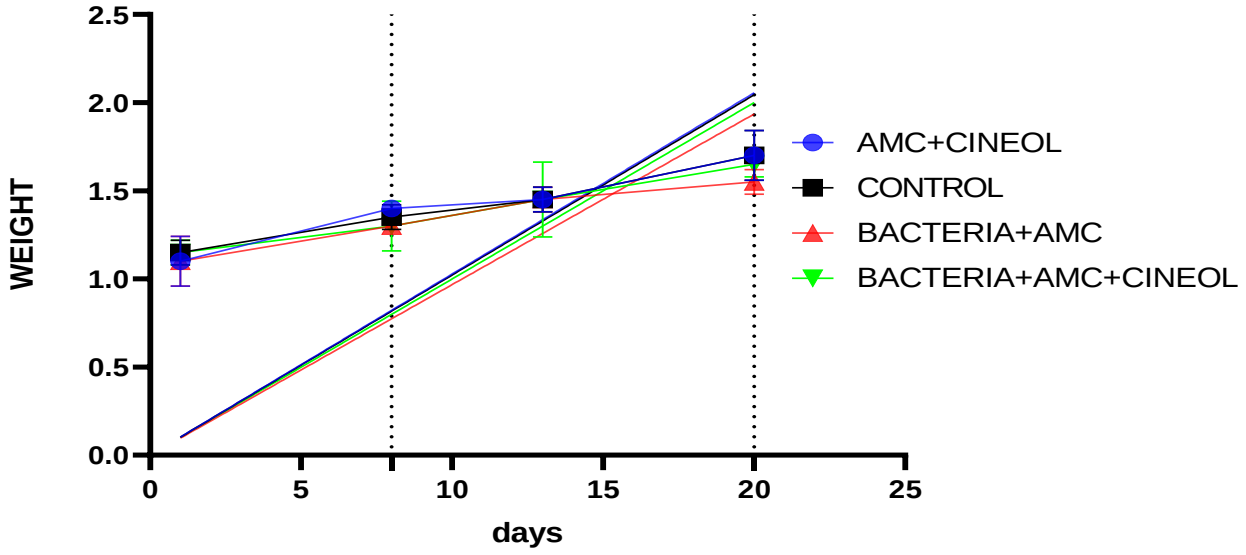
We noted that our rabbits' septic parameters were stable throughout the 20-day course of oxytetracycline.

The weight surveillance:

Take note that each time a sample was taken, the weight was determined. As illustrated in figure 4, our groups almost have an average and standard deviation (SD) of approximately 1.4 +/- 0.2kg (the details are in figure 24).

The continued food administration allows the rabbits to gain weight as long as this evolution is justified in time and the amount of food administered.

A



B

	AMC+CINEOL	CONTROL	BACTERIA+AMC	BACTERIA+AMC+CINEOL
Number of values	4	4	4	4
Minimum	1,100	1,150	1,100	1,150
Maximum	1,700	1,700	1,550	1,650
Range	0,6000	0,5500	0,4500	0,5000
Mean	1,413	1,413	1,350	1,388
Std. Deviation	0,2462	0,2287	0,1958	0,2136
Std. Error of Mean	0,1231	0,1143	0,09789	0,1068
Lower 95% CI of mean	1,021	1,049	1,038	1,048
Upper 95% CI of mean	1,804	1,776	1,662	1,727

Figure 24: Weight evolution during the first phase of the protocol is depicted in graph part A and table part B.

A: Curve of weight evolution The X-axis represents the number of days, and the Y-axis represents the weighted average. The curve has a linear progression. B: table displaying the minimum and maximum ranges, mean, standard deviation, and confidence intervals for each group. Our groups almost have an average standard deviation (SD) range between 1.3 +/- 0.2kg and 1.4 +/- 0.25kg.

II. Induction of colonization

Taking count that E. coli is the most common pathogen in the community and nosocomial infections, whether urinary or pulmonary infections. As a result, the following findings will concentrate primarily on E. coli.

The isolate of E.coli was obtained from our laboratory's strain bank. This strain can be classified as ESBL 4G cephalosporinase. The sensitivity profile performed demonstrates resistance to the 1, 3, and 4 generations cephalosporins tested as FEP, CAZ, and CFM, as detailed in Table 4.

Table 4: E.coli resistance profile used for colonization induction.

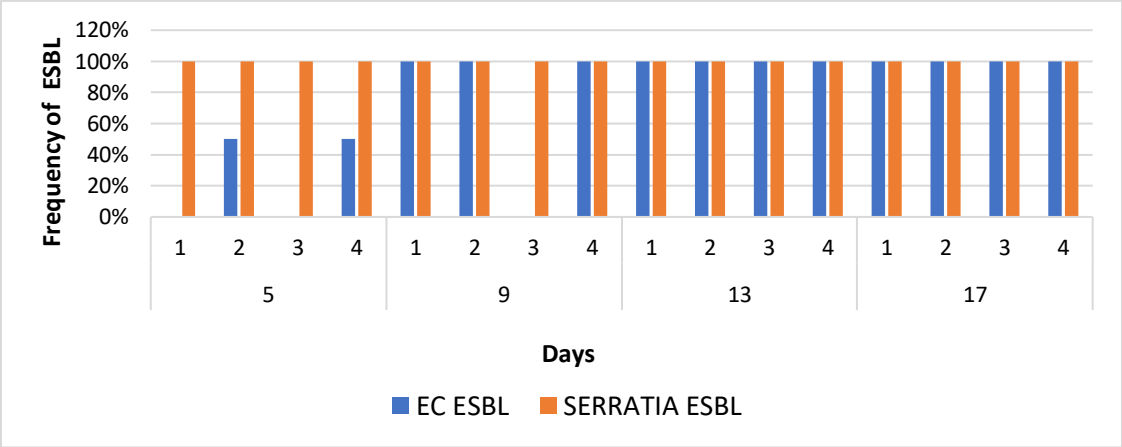
	ETP	IPM	FOX	CTX	AMC	FEP	AK	CFR	CAZ	CFM	PTZ	PRL	TIC	SXT	CIP
ESBL-E.coli	R	S	S	R	R	R	R	R	R	R	R	R	R	R	S

The daily administration of this inoculum for an average of two weeks and the collection of samples in parallel on days 5, 9, 13, and 17 revealed that ESBL-E.coli gradually colonized our group. On day 5, we have 50% colonization in two groups, groups 2 and 4, and the administration continues to increase the frequency of colonization, resulting in 100% in all groups on days 13 and 17. The Serratia found previously is still present in our entire group (figure 25).

On the same days of the sample taken, we determinate the bacterial load. The results suggest that on day 5, the bacterial load did not surpass two colonies, especially in groups 2 and 4. On day 9, the bacterial load in groups 1, 2, and 4 was determined to be greater than 20 colonies. Finally, on day 17, all groups had a bacterial load of

between 200 and 300 colonies. The average charge between these groups ranges between 90 and 105, and all of this information is in detail in Figure 26.

A



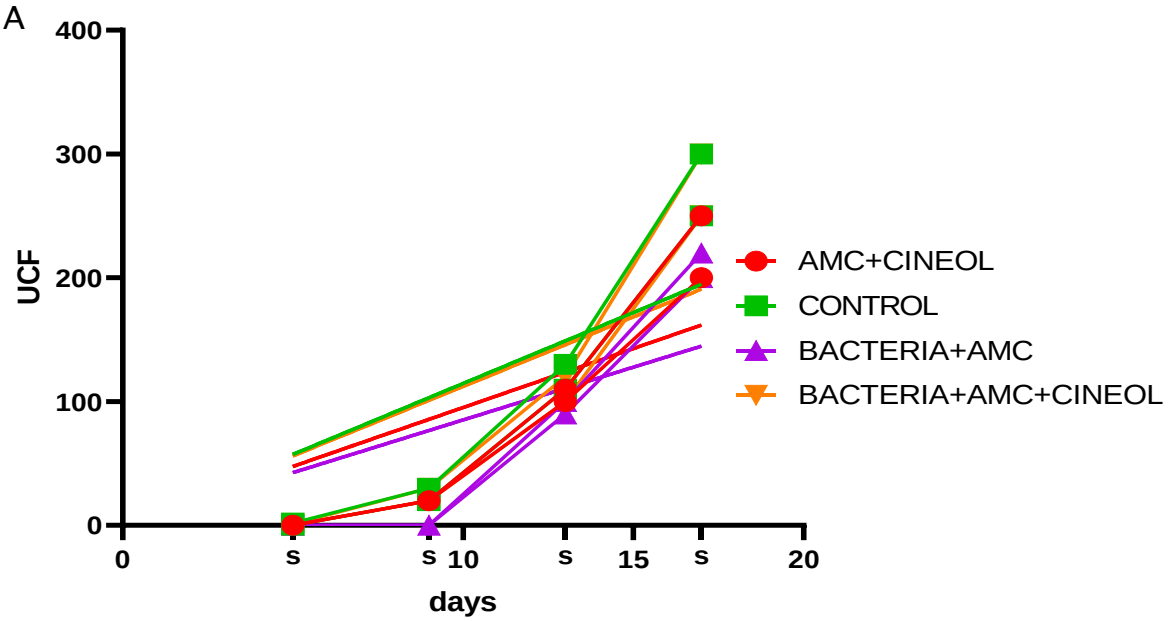
B

Days	Screening D5				Screening D9				Screening D13				Screening D17			
GROUPS	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
E.coli ESBL	0 %	50%	0%	50%	100 %	100 %	0%	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %
SERRATIA ESBL	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %	100 %

Figure 25: Screening of ESBL during the second part of the protocol.

The distribution of resistance detected during the second part of our protocol, which is colonization by ESBL-E, is shown in graph A and table B. We began to detect ESBL-E on day 5 in two groups, 2 and 4, and they became detectable in all groups within 13 days. However, Serratia ESBL continued to be detected throughout this second part of our protocol.

A



B

	AMC+CINEOL	CONTROL	BACTERIA+AMC	BACTERIA+AMC+CINEOL
Number of values	4	4	4	4
Minimum	0,000	1,000	0,000	0,5000
Maximum	225,0	275,0	210,0	275,0
Range	225,0	274,0	210,0	274,5
Mean	87,50	105,3	76,25	102,6
Std. Deviation	102,3	124,3	99,78	124,1
Std. Error of Mean	51,17	62,14	49,89	62,06
Lower 95% CI of mean	-75,36	-92,52	-82,52	-94,89
Upper 95% CI of mean	250,4	303,0	235,0	300,1

Figure 26: Bacterial load progression during ESBL-E. Coli colonization. The illustration is divided into two sections: A and B.

A: The curve depicts a linear form of bacterial load growth from 0 to 250 in the AMC + cineol group, 2 to 300 in the control group, 0 to 275 in the bacteria + AMC group, and 1 to 275 in the bacteria + AMC group + cineol group.

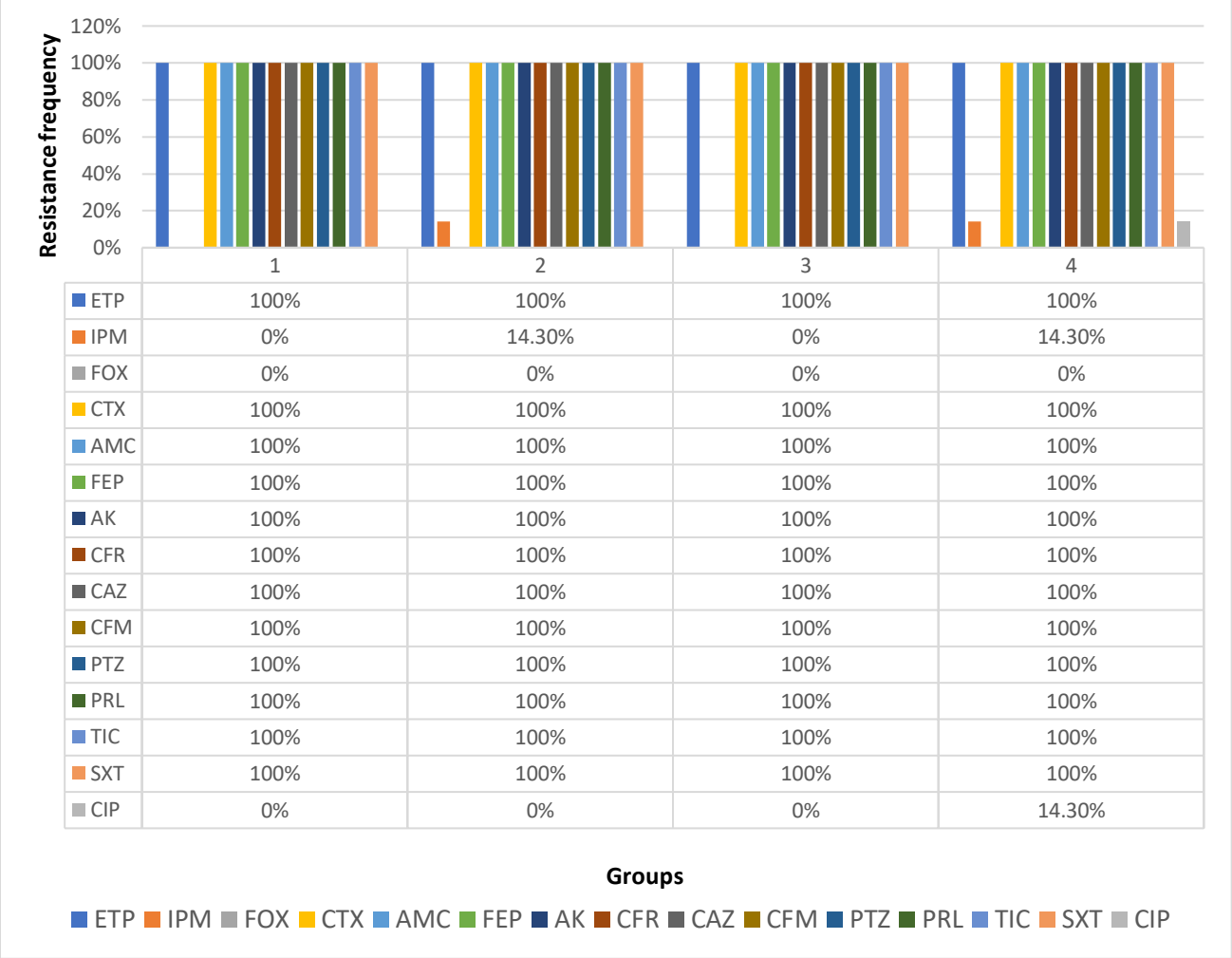
B: The table depicts a descriptive analysis of the progression of this bacterial load, including the min, max, mean, SD, and e CI, indicating that the bacterial load ranges from 1 to 275, with a mean of around 100 for all groups.

1. E.coli resistance profile

We carried out the sensitivity profile concurrently with the bacterial load measurement. The goal was to test the sensitivity profile throughout the colonization induction, which lasted more than two weeks. A total of 23 isolates were purified and tested.

The sensitivity profile, as shown in Figure 25, follows the same resistance profile as the inoculum. Except for the 2nd generation, we have complete resistance to the first, second, and fourth-generation cephalosporins, aminoglycosides such as amikacin, penicillins such as AMC, TIC, PRL, TZP, and SXT, and ertapenem.

We have only 14% resistance to imipenem in groups 2 and 4. in Addition to group 4, we note 14% resistance to CIP. Motioning that FOX has a 100% sensitivity in all groups.



	1	2	3	4
ETP	100%	100%	100%	100%
IPM	0%	14.30%	0%	14.30%
FOX	0%	0%	0%	0%
CTX	100%	100%	100%	100%
AMC	100%	100%	100%	100%
FEP	100%	100%	100%	100%
AK	100%	100%	100%	100%
CFR	100%	100%	100%	100%
CAZ	100%	100%	100%	100%
CFM	100%	100%	100%	100%
PTZ	100%	100%	100%	100%
PRL	100%	100%	100%	100%
TIC	100%	100%	100%	100%
SXT	100%	100%	100%	100%
CIP	0%	0%	0%	14.30%

Figure 27: Resistance profile of E.coli.

The figure depicts the distribution of antibiotics and their percentage of resistance concerning each group shown in the graph and table. As shown, we have resistance to most cephalosporins except for FOX, aminoglycosides such as amikacin, penicillins such as AMC, TIC, PRL, TZP, and SXT. We have 100% resistance to ertapenem but only 14% resistance to imipenem in groups 2 and 4, and only 14% resistance to CIP in group 4.

2. Serratia resistance profile

The results demonstrate a continuation of the previous results concerning the selection of Serratia using the oxytetracycline administration. The conduction of the sensibility profile shows a resistance profile identical to that previously. As we see before, except AK, SXT, and CIP, we have resistance to most antibiotics.

3. Clinical monitoring parameters

Every day, we examined the eating habits and reactions of each of our groups using appropriate tools.

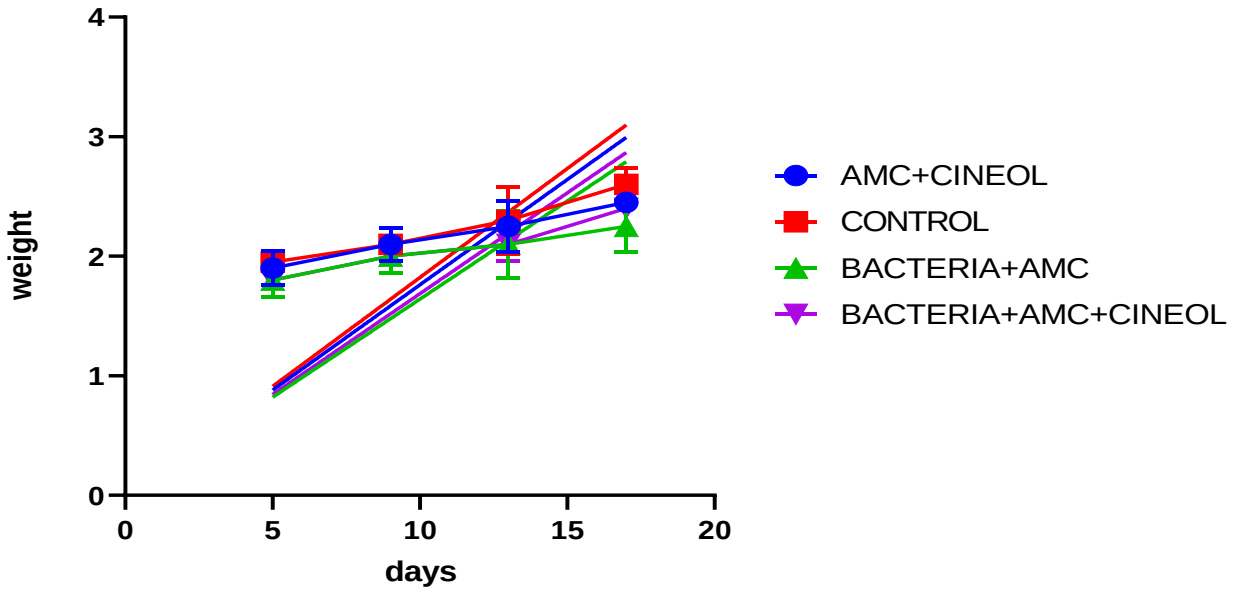
We noted that our rabbits' septic parameters were stable throughout the 17-day course of the inoculum administration.

The weight surveillance:

Remembering that the measurement weight was proceeding in parallel with the samples, we were able to have four measurements during this period of study. As Figure 8 shows, Our groups almost have an average standard deviation (SD) range between 2.03 +/- 0.18kg and 2.2 +/- 0.28kg (all the details is in figure 28)

The continued food administration allows the rabbits to gain weight as long as this evolution is justified in time and the amount of food administered.

A



B

	AMC+CINEOL	CONTROL	BACTERIA+AMC	BACTERIA+AMC+CINEOL
Number of values	4	4	4	4
Minimum	1,900	1,950	1,800	1,800
Maximum	2,450	2,600	2,250	2,400
Range	0,5500	0,6500	0,4500	0,6000
Mean	2,175	2,238	2,038	2,075
Std. Deviation	0,2327	0,2810	0,1887	0,2500
Std. Error of Mean	0,1164	0,1405	0,09437	0,1250
Lower 95% CI of mean	1,805	1,790	1,737	1,677
Upper 95% CI of mean	2,545	2,685	2,338	2,473

Figure 28: The weight evolution during the first phase of the protocol.

A: curve of weight evolution The X-axis represents the number of days, and the Y-axis represents the weighted average. The curve has a linear progression. B: table displaying the minimum and maximum ranges, mean, standard deviation, and confidence intervals for each group. Our groups almost have an average standard deviation (SD) range between 2.03 +/- 0.18kg and 2.2 +/- 0.28kg.

III. Decolonization

Bacterial discharge is the critical point in our protocol. We perform multiple comparisons. The comparison between all the groups was made to ensure the efficacy of the treatment and increase the credibility of future studies, as this is still a preclinical study for further projection in clinical studies.

1. Descriptive results

We will explain the outcome by categorizing the progression of the results into stages based on the orderly succession of the results.

a. First party

We collect two specimens during this stage, on the fourth and eighth days. The first sample was on day four. In general, we observed a rise in the bacterial load, specifically for the group bacteria + AMC, which was 350. In addition, the other group had almost the same load, a little less in comparison to this group. On the 8th day, we noticed a continuation of day 4. The bacterial load reached a peak in groups. Bacteria+AMC group had the highest bacterial load with 450 UCF, followed by group 4, with a maximum bacterial load of 400, and group 1 with a bacterial load of 350, but the control group was stable.

b. Second-party

The second part of this decolonization step includes tree samples on the 13th, 17th, and 20th days.

Sine the 13th day, we noticed a significant decrease in this bacterial load in the treated group, group 1. With a bacterial load of less than 50 UCF and group 4, we saw a

significant but not satisfactory decrease of 200. Compared to our treated groups, the other group has not changed and has remained constant with a very high load of 450 for group 3 and 250 for group 2.

The treatment group's discharge progression differs significantly from the control group's. Since the 17th day, group 2's bacterial discharge has been overlapping with the goal, and the bacteria + AMC + cineol group has gradually regressed to an 80 charge. However, the other groups have remained stable, with a slight decrease of 200 for group 2 and 400 for group 3.

The final sample followed the suction of the previous results, with the groups treated with AMC + cineol and bacteria + AMC + cineol achieving the goal. In contrast, the other groups did not change much, despite a high bacterial load.

For better visualization, the distribution of this discharge, all the details are in figure 29.

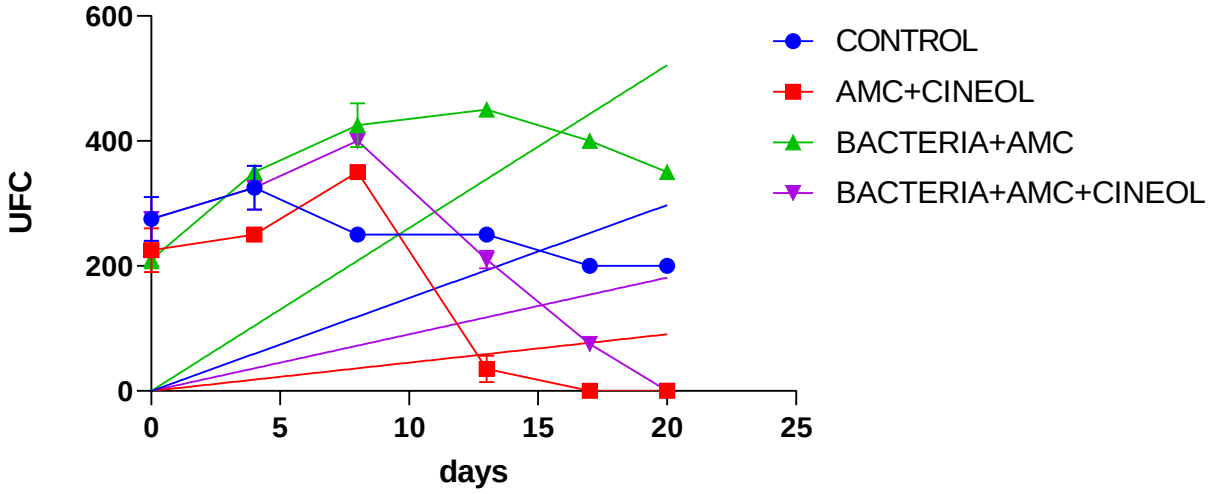


Figure 29: The figure depicts the X-axis days and the CFU bacterial load on the Y-axis.

We can divide the curve into two parts: the first part of days 0 to 8 in which we observe an increase in the load in all groups except the control group; this increase is depicted in figure 400 for the bacteria + AMC group and also the fourth group, and 350 for the control group. The second part shows a linear decreasing curve, particularly groups 1 and 4. Group 1 establishes an eradication until the 17th day, while Group 4 establishes an eradication until the 20th day. Other groups did not demonstrate a difference in bacterial load. Furthermore, it was significantly higher than the previous days.

2. Statistical analysis

In the statistical analysis of the results, we realized one-way ANOVA following Tukey's multiple comparisons test for all the groups and the sample carried out. We also realized Sidak's multiple comparisons test comparing day 0 to the last day of collection on day 20.

Similar to descriptive analysis, we divided the results of our statistical analysis into periods.

The first period's outcome for comparing the groups does not return significant on day 0. On day 4, all comparison calculations do not show any difference with that in front, but the comparison performed between the groups AMC + cineol vs. bacteria + AMC had a $p<0.0001$. Also, the group AMC + cineol vs. bacteria + AMC + cineol had a $p = 0.0027$, and the comparison made between the group control and all the other groups had a $p = 0.0001$.

The second period begins after the completion of the eighth day, and specifically from the 13th-day sample. On the 13th day, the comparison is significant with a $p<0.0001$ between all groups except the control group vs. bacteria + AMC + cineol. The remaining comparisons are statically significant, with $p<0.0001$ for days 17 and 20.

The Sidak test was used to demonstrate the evolution of the same group over time. The difference between days 0 and 20 is significant with $p<0.0001$ for two groups, groups 1 and 4, and $p=0.0383$ for the group control and $p=0.0009$ for the group bacteria + AMC. It means that the statistical results for the groups treated with our AMC + cineol combination are perfect.

All of these analytic results are in detail in the tables below (table 5 and 6).

Table 5: The results of statistical analysis of the bacterial load in all groups.

One-way ANOVA was conducted following Tukey's multiple comparisons test in the quantitative analysis to evaluate the significant difference levels between all groups on days 0, 4, 8, 17, and 20. The outcome shows a significant statical difference between all the groups with a P<0.05 begins on day 13 and becoming <0.0001 on day 20.

Tukey's multiple comparisons tests	Mean Diff,	95,00% CI of the diff,	Significant?	Summary	Adjusted P-Value
Days 0					
CONTROL vs. AMC+CINEOL	50,00	-1,609 to 101,6	No	ns	0,0600
CONTROL vs. BACTERIA+AMC	65,00	13,39 to 116,6	Yes	**	0,0099
CONTROL vs. BACTERIA+AMC+CINEOL	0,000	-51,61 to 51,61	No	ns	>0,9999
AMC+CINEOL vs. BACTERIA+AMC	15,00	-36,61 to 66,61	No	ns	0,8529
AMC+CINEOL vs. BACTERIA+AMC+CINEOL	-50,00	-101,6 to 1,609	No	ns	0,0600
BACTERIA+AMC vs. BACTERIA+AMC+CINEOL	-65,00	-116,6 to -13,39	Yes	**	0,0099
Days 4					
CONTROL vs. AMC+CINEOL	75,00	23,39 to 126,6	Yes	**	0,0027
CONTROL vs. BACTERIA+AMC	-25,00	-76,61 to 26,61	No	ns	0,5498
CONTROL vs. BACTERIA+AMC+CINEOL	0,000	-51,61 to 51,61	No	ns	>0,9999
AMC+CINEOL vs. BACTERIA+AMC	-100,0	-151,6 to -48,39	Yes	****	<0,0001
AMC+CINEOL vs. BACTERIA+AMC+CINEOL	-75,00	-126,6 to -23,39	Yes	**	0,0027
BACTERIA+AMC vs. BACTERIA+AMC+CINEOL	25,00	-26,61 to 76,61	No	ns	0,5498
Days 8					
CONTROL vs. AMC+CINEOL	-100,0	-151,6 to -48,39	Yes	****	<0,0001
CONTROL vs. BACTERIA+AMC	-175,0	-226,6 to -123,4	Yes	****	<0,0001
CONTROL vs. BACTERIA+AMC+CINEOL	-150,0	-201,6 to -98,39	Yes	****	<0,0001
AMC+CINEOL vs. BACTERIA+AMC	-75,00	-126,6 to -23,39	Yes	**	0,0027
AMC+CINEOL vs. BACTERIA+AMC+CINEOL	-50,00	-101,6 to 1,609	No	ns	0,0600
BACTERIA+AMC vs. BACTERIA+AMC+CINEOL	25,00	-26,61 to 76,61	No	ns	0,5498
Days 13					
CONTROL vs. AMC+CINEOL	215,0	163,4 to 266,6	Yes	****	<0,0001
CONTROL vs. BACTERIA+AMC	-200,0	-251,6 to -148,4	Yes	****	<0,0001
CONTROL vs. BACTERIA+AMC+CINEOL	40,00	-11,61 to 91,61	No	ns	0,1699
AMC+CINEOL vs. BACTERIA+AMC	-415,0	-466,6 to -363,4	Yes	****	<0,0001
AMC+CINEOL vs. BACTERIA+AMC+CINEOL	-175,0	-226,6 to -123,4	Yes	****	<0,0001
BACTERIA+AMC vs. BACTERIA+AMC+CINEOL	240,0	188,4 to 291,6	Yes	****	<0,0001
Days 17					
CONTROL vs. AMC+CINEOL	200,0	148,4 to 251,6	Yes	****	<0,0001
CONTROL vs. BACTERIA+AMC	-200,0	-251,6 to -148,4	Yes	****	<0,0001
CONTROL vs. BACTERIA+AMC+CINEOL	125,0	73,39 to 176,6	Yes	****	<0,0001
AMC+CINEOL vs. BACTERIA+AMC	-400,0	-451,6 to -348,4	Yes	****	<0,0001
AMC+CINEOL vs. BACTERIA+AMC+CINEOL	-75,00	-126,6 to -23,39	Yes	**	0,0027
BACTERIA+AMC vs. BACTERIA+AMC+CINEOL	325,0	273,4 to 376,6	Yes	****	<0,0001
Days 20					
CONTROL vs. AMC+CINEOL	200,0	148,4 to 251,6	Yes	****	<0,0001
CONTROL vs. BACTERIA+AMC	-150,0	-201,6 to -98,39	Yes	****	<0,0001
CONTROL vs. BACTERIA+AMC+CINEOL	200,0	148,4 to 251,6	Yes	****	<0,0001
AMC+CINEOL vs. BACTERIA+AMC	-350,0	-401,6 to -298,4	Yes	****	<0,0001
AMC+CINEOL vs. BACTERIA+AMC+CINEOL	0,000	-51,61 to 51,61	No	ns	>0,9999
BACTERIA+AMC vs. BACTERIA+AMC+CINEOL	350,0	298,4 to 401,6	Yes	****	<0,0001

Table 6: The results of statistical analysis of the bacterial load in all the groups.

One-way ANOVA was conducted following Sidaks multiple comparisons test in the quantitative analysis to evaluate the significant difference levels between all groups between days 0 and 20. The outcome shows a significant difference between the days with P<0,0001 in the group treated with amc+cineol, for the other group, the group treated by the AMC the P=0,0009, and the control group we found the smallest P=0,0383.

Sidak's multiple comparisons tests	Mean Diff,	95,00% CI of the diff,	Significant?	Summary	Adjusted P-Value
Days 0 – days 20					
CONTROL	75,00	4,045 to 146,0	Yes	*	0,0383
AMC+CINEOL	225,0	154,0 to 296,0	Yes	****	<0,0001
BACTERIA+AMC	-140,0	-211,0 to -69,04	Yes	***	0,0009
BACTERIA+AMC+CINEOL	275,0	204,0 to 346,0	Yes	****	<0,0001

3. Clinical monitoring parameters

Every day, we examined the eating habits and reactions of each of our groups using appropriate tools.

We noted that our rabbits' septic parameters and weight were unstable throughout the 20-day course of the decolonization.

a. Weight surveillance

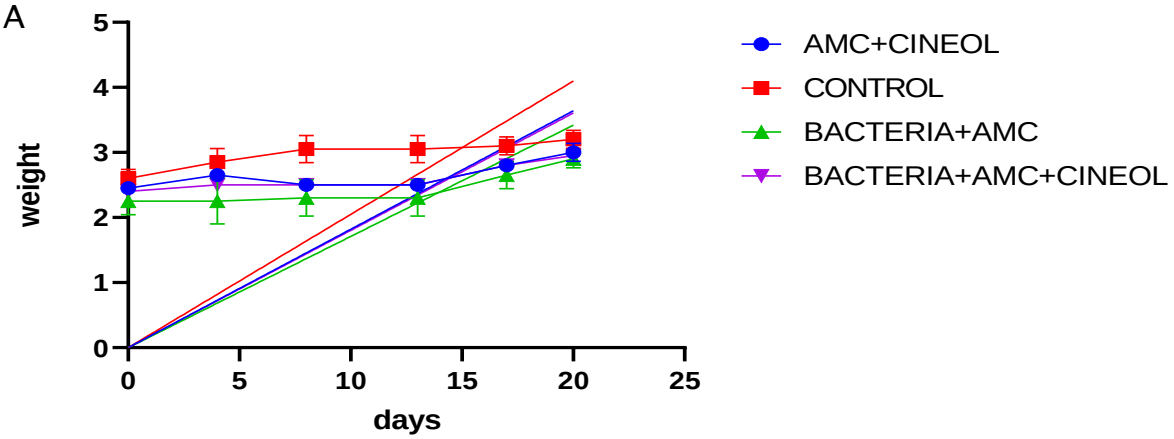
We have also divided the weight's evolution into two parts.

The first section covers the first week, beginning on day 0 and ending on day 8. Except for the control group, the weight evolution was halted during this time in all groups.

The second part, which lasted from the second week to the end of the protocol, followed the typical weight gain with a linear and increasing curve in all groups.

As shown in the figure, our group had different weights ranging from highest to lowest. The control group had a mean and SD of 2.975 +/- 0.2162, subsequently, the two groups treated with AMC+cineol, the group 1 and 4, almost the same average as 2.6 +/- 0.21, finally group 4 has the lowest average and SD 2.442 +/- 0.2710.
Continued food administration allows the rabbits to gain weight as long as this evolution is justified in terms of time and amount of food administered for this critical part of the protocol.
All these results are mentioned in detail in figure 30.

A



B

	AMC+CINEOL	CONTROL	BACTERIA+AMC	BACTERIA+AMC+CINEOL
Number of values	6	6	6	6
Minimum	2,450	2,600	2,250	2,400
Maximum	3,000	3,200	2,900	2,950
Range	0,5500	0,6000	0,6500	0,5500
Mean	2,650	2,975	2,442	2,608
Std. Deviation	0,2145	0,2162	0,2710	0,2154
Std. Error of Mean	0,08756	0,08827	0,1106	0,08796

Figure 30: The evolution of the weight during the decolonization phase of the protocol is depicted in graph part A and table part B.

A: curve of weight evolution The X-axis represents the number of days, and the Y-axis represents the weighted average. The curve is divided into two sections, one part from day 0 to day eight and the second from day 8 to day 20. The curve did not know a break for the group control, but for the other group, the break is remarkable in the first part, while for the second part, the evolution of the curve joined to the average, linear and increasing curve.

B: table displaying the weight minimum and maximum ranges, mean, standard deviation, and confidence intervals for each group. We have the highest average for the group control of 2.975, followed by the AMC + cineol 2.650 group. Hence, group 4 has an average of 2.608, almost the same as the previous group, and finally, group 3 has the lowest average of 2.442.

b. Fever monitoring

Temperature measurements taken on days 8, 13, 17, and 20 yielded different results depending on the group.

On day 8, the measurements show an increase in temperature in three groups, groups 1, 3, and 4 reaching 40°C for group 3 and more than 38,5 °C for the other group.

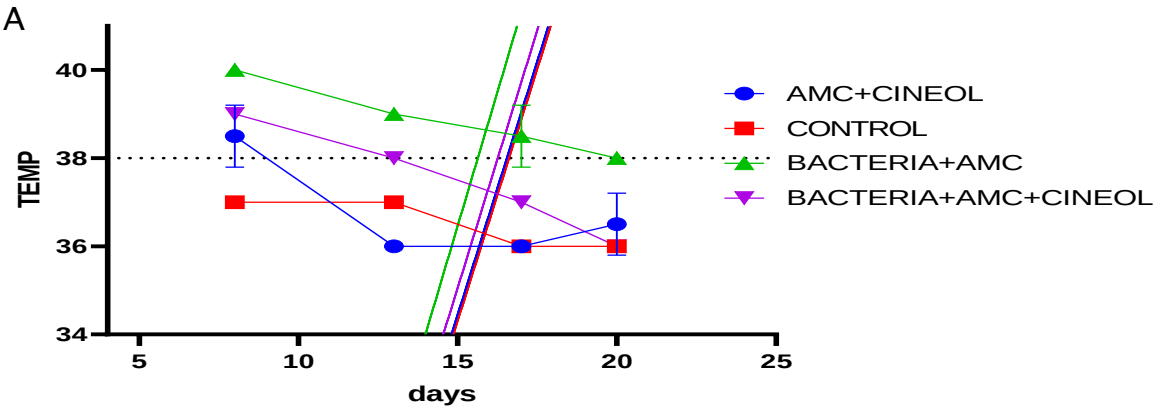
As the days pass, group 1 becomes apyretic on the 13th day, while groups 3 and 4 had temperatures above 38 °C.

Our entire group has been apyretic since day 17, except group 3, with a temperature above 38. The same result was observed on day 20's final day.

We noticed a steady decline in fever during the decolonization period, particularly in the group treated with AMC + cineol, which went from a febrile to an apyretic state in a matter of days, with an average temperature of around 37°C. In contrast, group 3 had an average of 38.88 +/- 0.8539, demonstrating the difference between these groups.

All these results are detailed in Figure 31.

A



B

	AMC+CINEOL	CONTROL	BACTERIA+AMC	BACTERIA+AMC+CINEOL
Number of values	4	4	4	4
Minimum	36,00	36,00	38,00	36,00
Maximum	38,50	37,00	40,00	39,00
Range	2,500	1,000	2,000	3,000
Mean	36,75	36,50	38,88	37,50
Std. Deviation	1,190	0,5774	0,8539	1,291
Std. Error of Mean	0,5951	0,2887	0,4270	0,6455
Lower 95% CI of mean	34,86	35,58	37,52	35,45
Upper 95% CI of mean	38,64	37,42	40,23	39,55

Figure 31: The temperature evolution from day 8 to day 20 With a temperature evolution curve, the x-axis represents the days.

The y axis represents the temperature. We made a mark separating the feverish groups from the non-feverish groups.

A: The curve segment demonstrates that the bacteria + AMC group maintains a temperature above 38°C on all days. A transition to the apyretic state was on the 13th day for group 1 and the 17th day for group 4. In contrast, the group control maintains an apyretic state throughout these days.

B: The table indicates temperature ranges, mean, standard deviation, and confidence intervals are shown in a table for each group. Except for group 3, which has an average of 38.88, none of the groups has a mean exceeding 37,5.

4. Last screen: ESBL

The final screening for the ESBL perfumed from day 0 to day 20 reveals many changes.

On day 4, the screening results showed the eradication of Serratia ESBL carriage in two groups, Amc+cineol and bacteria+AMC+cineol. However, Serratia was still present in the other groups. We also want to point out that our ESBL-E is present in all groups.

On day 8, we noticed a new strain that K.p detects in 50% of the bacteria+amc group, with the rest remaining unchanged from the previous day, except that Serratia was eliminated in group 4.

On day 13, there is a continuation of the results from day 8 with a K.p rate of 100% in group 3.

E.coli eradication began on day 17 for group 1, and we observed a rate of 100% KP in groups bacteria+AMC and control.

On the last day (day 20), we found the eradication of ESBL-E from group 1 and group 4, but the detection of KP strain was at 100% in all groups. The detection of Serratia strain continues in the two groups, the control group and the bacteria + AMC group; however, we note that the treatment was interrupted three days before the last sample.

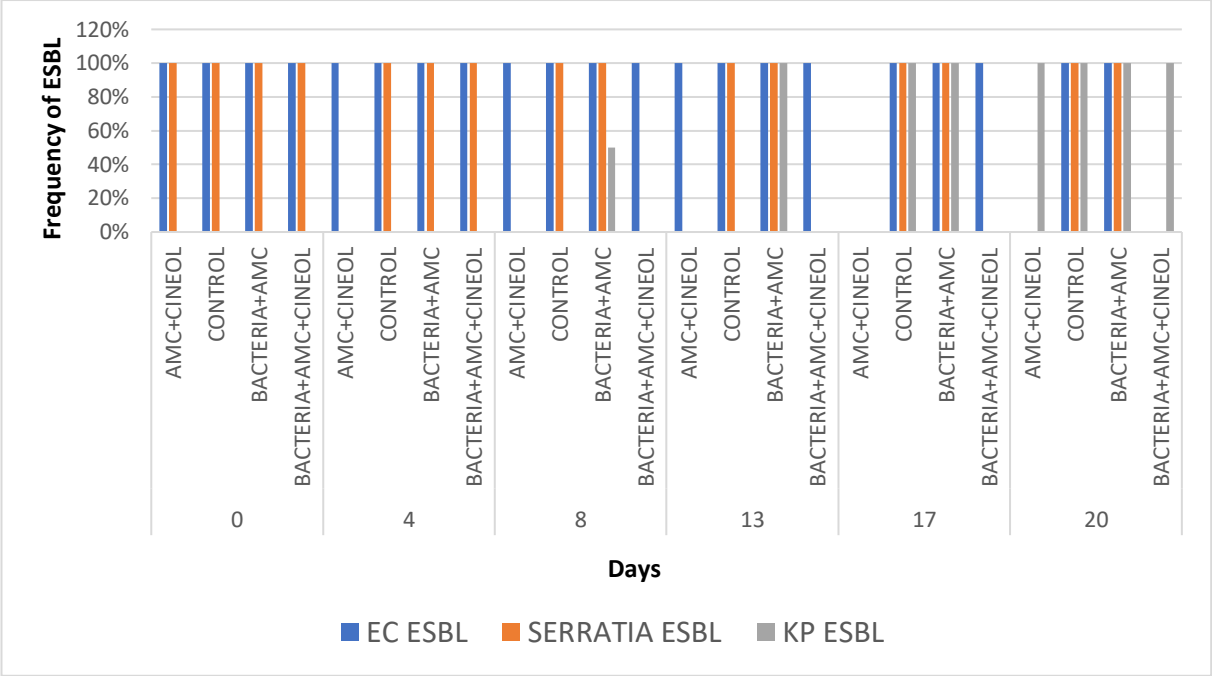


Figure 32: Screening for ESBL during the decolonization part of the protocol.

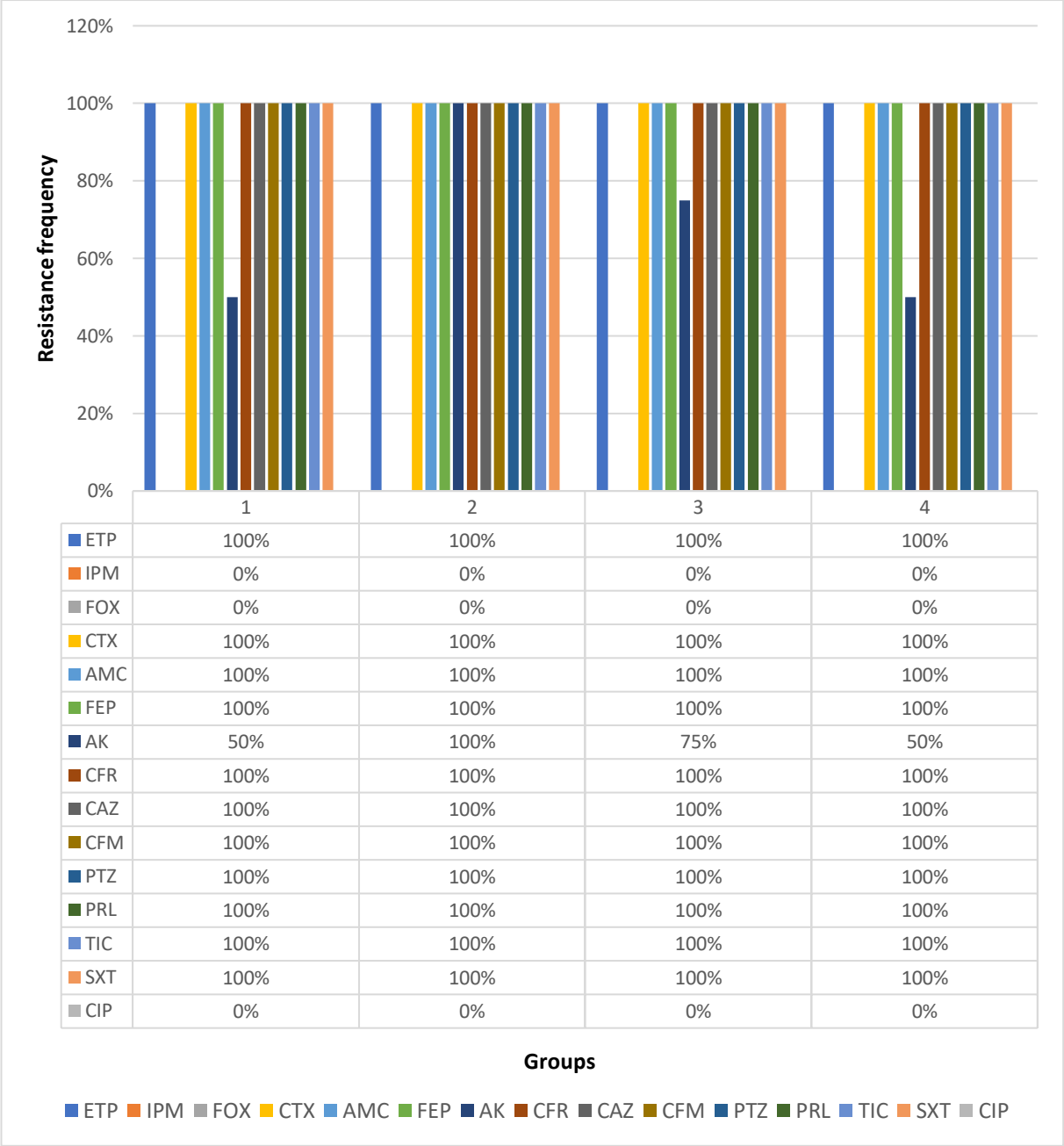
The distribution of resistance detected during the final stage of our protocol, decolonization with our treatment combination AMC + cineol, is depicted in graph A and table B. It depicts the frequency of the genre detected concerning the group and the days; we discovered that the eradication of Serratia is the result of the 8th day in groups 1 and 4 only and that the other group has a continuation of this kind; the eradication of ESBL-E began on day 17 in group 1 and the 20th day in group 4. On day 8, KP was found in group 3 and spread to all groups on day 20 during the final test.

5. E.coli Resistance profile from day 0 to day 13

Implementing the profile of sensibility in these samples from day 0 to day 13 made it possible to monitor and compare the ESBL-E state of resistance to the previous. 32 isolates were isolated in this period. The findings are consistent with previous findings. We have resistance to all cephalosporins except the fox, which had no resistance. Among the penicillins tested, we notice the AMC, TIC, PRL, TZP, SXT, and ERTapenem, in which all groups have a 100% resistance frequency.

The AK tested has 100% resistance in group 3, 75% resistance in group 3, and 50% resistance in groups 1 and 4. We found 0% resistance to imipenem and CIP.

All These results are detailed in Figure 33 below.



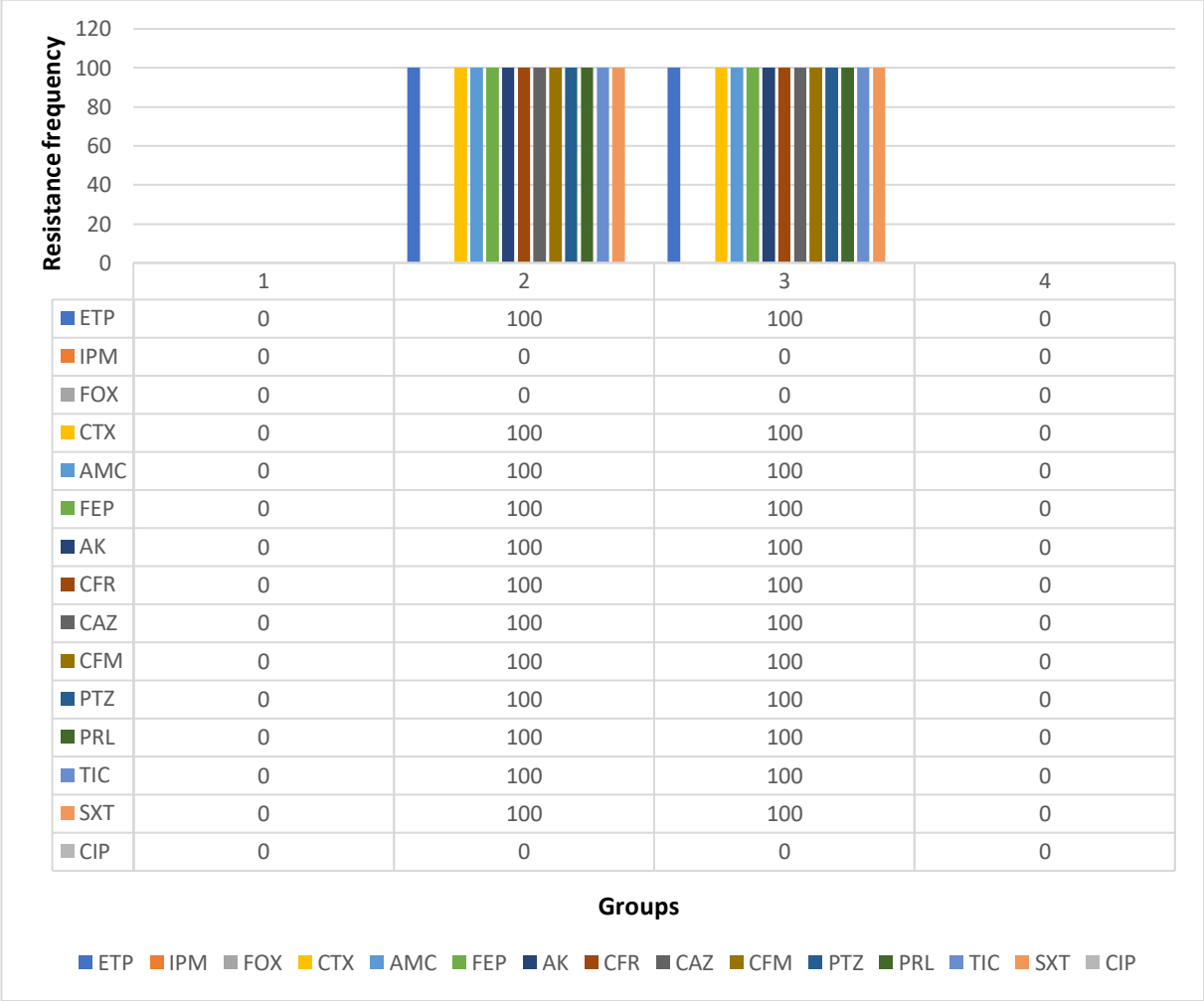
	1	2	3	4
ETP	100%	100%	100%	100%
IPM	0%	0%	0%	0%
FOX	0%	0%	0%	0%
CTX	100%	100%	100%	100%
AMC	100%	100%	100%	100%
FEP	100%	100%	100%	100%
AK	50%	100%	75%	50%
CFR	100%	100%	100%	100%
CAZ	100%	100%	100%	100%
CFM	100%	100%	100%	100%
PTZ	100%	100%	100%	100%
PRL	100%	100%	100%	100%
TIC	100%	100%	100%	100%
SXT	100%	100%	100%	100%
CIP	0%	0%	0%	0%

Figure 33: E.coli Resistance profile from day 0 to day 13.

It depicts the distribution of antibiotics and their percentage of resistance concerning each group. As shown, we have resistance to all cephalosporins except the fox, penicillin family, SXT, and ertapenem. However, for the AK, we have 100% resistance in group 3, 75% resistance in group 3, and 50% resistance in groups 1 and 4.

6. E.coli Resistance profile in Days 20

A profile of sensibility performed on the last day revealed that the groups treated with AMC + cineol underwent decolonization and even a change in resistance profile, becoming sensitive to all antibiotics tested.



	1	2	3	4
ETP	0	100	100	0
IPM	0	0	0	0
FOX	0	0	0	0
CTX	0	100	100	0
AMC	0	100	100	0
FEP	0	100	100	0
AK	0	100	100	0
CFR	0	100	100	0
CAZ	0	100	100	0
CFM	0	100	100	0
PTZ	0	100	100	0
PRL	0	100	100	0
TIC	0	100	100	0
SXT	0	100	100	0
CIP	0	0	0	0

Figure 34: Resistance profile of E.coli on day 20.

It depicts the distribution of antibiotics and their percentage of resistance concerning each group. As shown, in groups 2 and 3, we have resistance to all cephalosporins except the fox, penicillin family, SXT, ertapenem, and AK. However, for groups 1and 4, we have 0% of resistance for all antibiotics.

7. K.p resistance profile

The profile of sensibility performed for all KP detected from day 8 to day 20, which was 15isolats, reveals that this KP has a very high resistance profile, which is described as follows: we have 100% resistance for the entire cephalosporin family, as well as the penicillin family, including TIC, PRL, PTZ, ertapenem, and CIP.

SXT has a 14% resistance for group 3 and a sensitivity of 100% for the rest. AK has a resistance of 100% for groups 2 and 4, and the imipenem has the same. Allows us to describe it as 4Gcephalosporinase ESBL-KP.

	1	2	3	4
ETP	100%	100%	100%	100%
IPM	0%	100%	100%	0%
FOX	100%	100%	100%	100%
CTX	100%	100%	100%	100%
AMC	100%	100%	100%	100%
FEP	100%	100%	100%	100%
AK	0%	100%	100%	0%
CFR	100%	100%	100%	100%
CAZ	100%	100%	100%	100%
CFM	100%	100%	100%	100%
PTZ	100%	100%	100%	100%
PRL	100%	100%	100%	100%
TIC	100%	100%	100%	100%
SXT	0%	0%	14%	0%
CIP	100%	100%	100%	100%

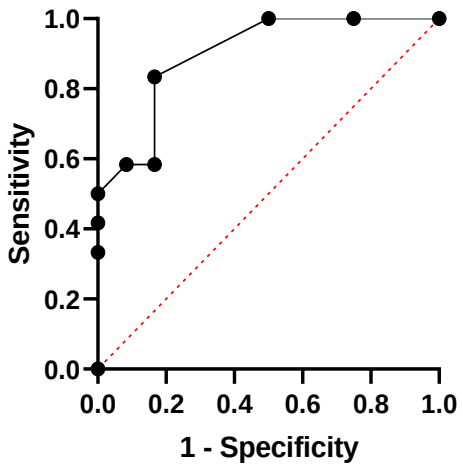
Figure 35: Resistance profile of K.p.

It depicts the distribution of antibiotics and the percentage of resistance in each group. We have resistance to all cephalosporins, penicillin family, CIP, and ertapenem, as demonstrated. For the AK, we have 100% resistance in groups 2 and 3, for the SXT, we have 14% resistance in group 3, and for imipenem, we have 100% resistance in groups 2 and 3.

IV. Sensitivity/specificity: ROC curve (receiver operating characteristic)

This test is performed to determine and compare the diagnostic performance of the screening test used, and thus as a quality criterion of screening test used in the protocol.

The test implicates two groups: the treated group, the AMC + cineol group, and for the control group, we chose the bacteria + AMC group. As shown in figure 34, the test has high sensitivity and specificity since the curve is located at the top and left and has high effectiveness with AUC = 0.8993 and P <0.0009.



The area under the ROC curve	
Area	0,8993
Std. Error	0,06196
95% confidence interval	0,7779 to 1,000
P value	0,0009
Data	
Controls (control)	12
Patients (treated)	12
Missing Controls	0
Missing Patients	0

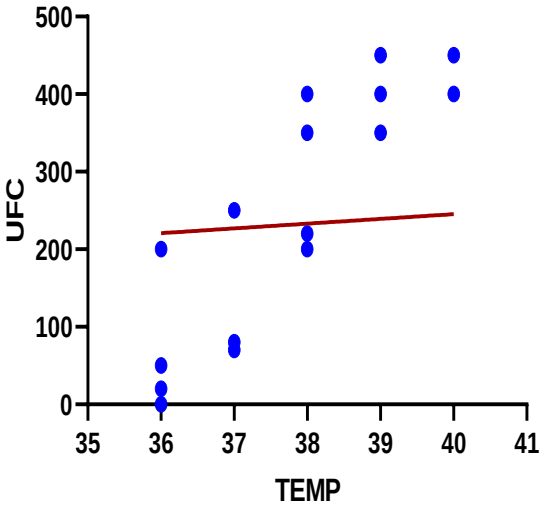
Figure 36: Illustrates the ROC curve and is located on the top and left with AUC = 0.8993 and P <0.0009.

V. Correlations

Correlations are a notion of a link that contradicts their independence. In this study, we correlate the bacterial load's eradication with several parameters: the number of colonies, fever, and weight gain, from the results described above.

1. Bacterial load vs. Fever

The protocol of this correlation was to take the bacterial load and the fever degree from all periods of decolonization, which took 20 days. The causality between bacterial load and fever reveals a highly positive significant correlation ($r = 0.8664$, $p<0.0001$). As an outcome, the variation in the number of colonies can explain 75,07% of the variation in temperature and vice versa.

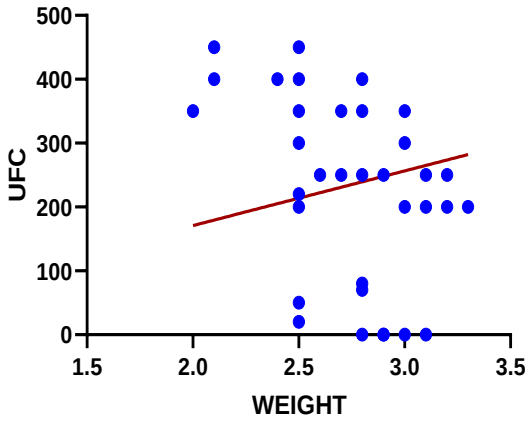


	TEMP Vs. UFC
Pearson r	
R	0,8664
95% confidence interval	0,7419 to 0,9332
R squared	0,7507
P-value	
P (two-tailed)	<0,0001
P-value summary	****
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	32

Figure 37: Correlation of the UFC and temperature with R=0.8664, R=0,7507 squared, and P<0.0001.

2. Bacterial load vs. Weight

We pursued another implication. The protocol was to take the bacterial load and the weight from the same period (the decolonization period). The correlation between weight and the number of colonies was inverse (r=-0.4649 p=0.0025). The variation in colony number can explain 21% of the variation in weight and vice versa.

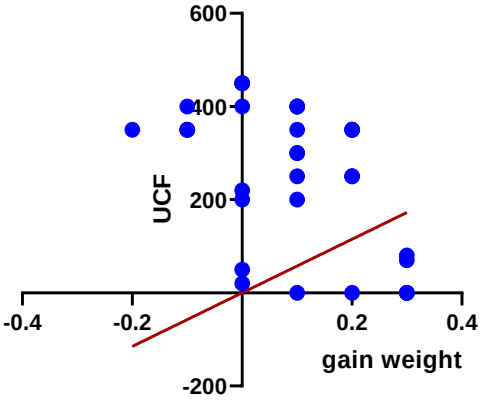


	Weight vs. UFC.
Pearson r	
R	-0,4649
95% confidence interval	-0,6782 to -0,1793
R squared	0,2161
P-value	
P (two-tailed)	0,0025
P-value summary	**
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	40

Figure 38: Correlation of the UFC and weight with R=-0,4649, Rsquared=0,2161,and P<0.0025.

3. Bacterial load vs. a Weight gain

Furthermore, the protocol was to take the bacterial load and the weight gain (also from the same period). As an outcome, the correlation between weight gain and the number of colonies was negative (r = −0.5089 p=0.0008). The variation in colony number can explain 26% of the variation in body weight and vice versa.

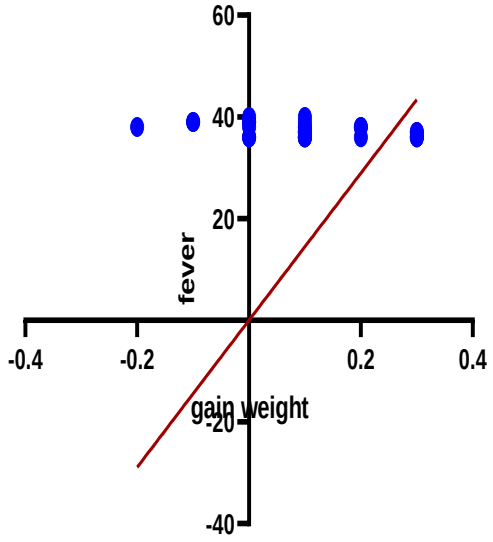


	gain weight Vs. UCF
Pearson r	
r	-0,5089
95% confidence interval	-0,7082 to -0,2346
R squared	0,2590
P-value	
P (two-tailed)	0,0008
P-value summary	***
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	40

Figure 39: Correlation of the UFC and weight gain with R=−0,5089, Rsquared=0,2590 and P<0.0025.

4. Fever vs. a weight gain

Finally, the protocol is to establish a correlation between fever and weight gain. The results are also negative (r=-0.4316 p=0.0136), and nearly 19% of the variation in gain weight can explain by the variation in fever and vice versa.



	gain weight Vs. fever
Pearson r	
r	-0,4316
95% confidence interval	-0,6783 to -0,09765
R squared	0,1863
P-value	
P (two-tailed)	0,0136
P-value summary	*
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	32

Figure 40: Correlation between fever and weight gain with r=-0,4316, Rsquared=0,1863, and P=0,0136.

VI. Eradication

The treatment response of the groups was quantified by Calculating the cumulative eradication rate for each screening performed up to the 20th day for the E.coli strain.

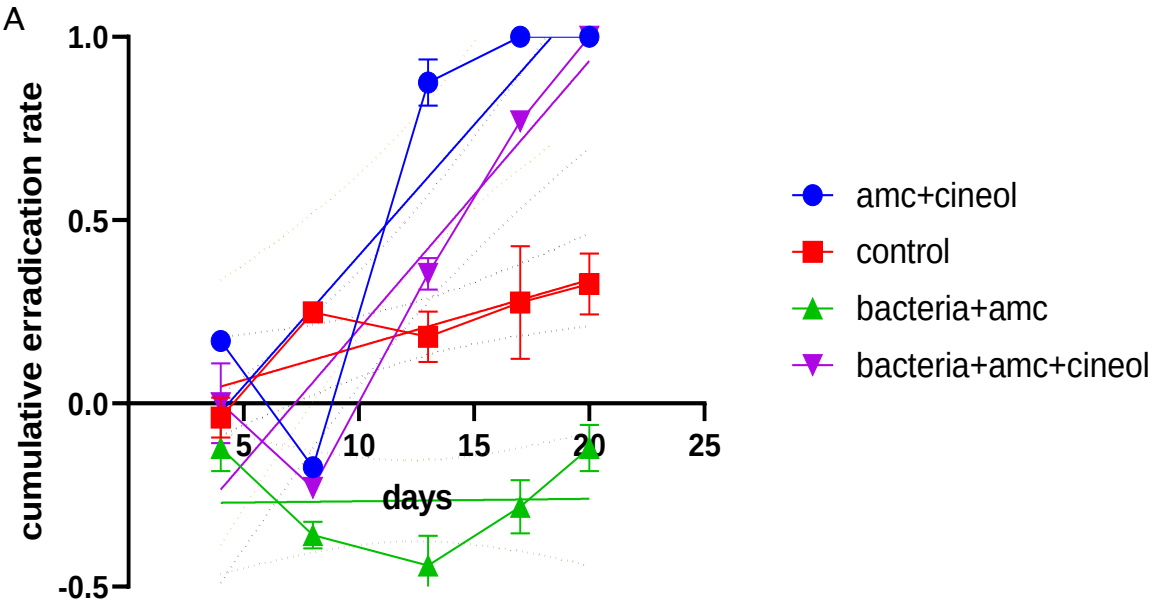
Except for group control, the cumulative eradication of all groups was negative during the first week.

On the 13th day, there is a cumulative eradication of nearly 80% of the AMC + cineol group and 35% of the bacteria + AMC + cineol group, which increases to 100% on the 17th day for the AMC + cineol group and 75% for the bacteria + AMC + cineol group, reaching 100% on the 20th day.

The control group had a cumulative eradication of 33%. However, the bacteria + AMC group was the only group that had a negative cumulative eradication, resulting in a cumulative eradication, less than -15%. However, before the cumulative eradication reached its ceiling on the 13th day, it was more than -40%. These results are described in detail in figure 40, part A.

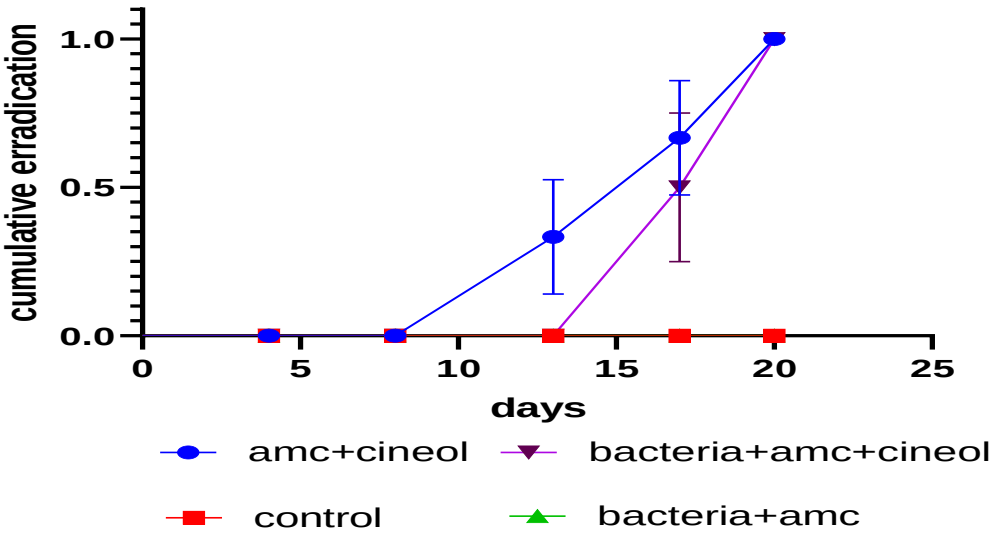
In part B, we have objective this eradication by Cumulative eradication curves for all the groups. The outcome finds that the log-rank test has a $P < 0.0022$.

A



	amc+cineol	control	bacteria+amc	bacteria+amc+cineol
Number of values	5	5	5	5
Minimum	-0,1740	-0,03846	-0,4423	-0,2308
Maximum	1,000	0,3256	-0,1218	1,000
Range	1,174	0,3641	0,3205	1,231
Mean	0,5742	0,1987	-0,2654	0,3785
Std. Deviation	0,5423	0,1424	0,1428	0,5133
Std. Error of Mean	0,2425	0,06366	0,06386	0,2296
Lower 95% CI of mean	-0,09913	0,02196	-0,4427	-0,2589
Upper 95% CI of mean	1,248	0,3755	-0,08807	1,016

A



Comparison of cumulative eradication Curves	
Log-rank (Mantel-Cox) test (recommended)	
Chi square	14,60
df	3
P value	0,0022
P-value summary	**
Are the survival curves sig different?	Yes

B

Figure 40: The curve shows days on the X-axis and cumulative eradicate on the Y-axis.

A: the two groups, 1 and 4, reached 100% on the 20th day. The control group had a cumulative eradication of 33%. The bacteria + AMC group was the only group with a negative cumulative eradication, resulting in a cumulative eradication of less than -15% .B: The result of the Comparison of cumulative eradication curves shows that the log-rank test has a P <0.0022.

Discussion

In this preclinical work using animal models, we attempted to decolonize the digestive tract of multiresistant bacteria using a combination of amoxicillin-clavulanic acid and cineol after achieving complete colonization by multiresistant bacteria type ESBL. The bacterial load and antimicrobial sensitivity profile were used to assess this.

Initial screening of animal colonization revealed no multiresistant bacteria in any group. However, the use of treatments based on oxytetracycline for 12 days has made it possible to select multidrug-resistant bacteria. According to Kony et al. [186], In this study, we found Serratia marcescens with a carbapenem, 3G, and 4G cephalosporin-resistant (100%), and none of our group members had a sign of infection.

These results could have other reasons. The emergence of the Serratia may follow the hypothesis that the source of this strain is food. Since we performed a food analysis and we found it contaminated with the same resistant bacteria. We should also mention that we chose to decolonize E. coli ESBL. It is the common pathogen associated with urinary and pulmonary infections and community and nosocomial infections. For this, the colonization period has lasted more than two weeks, with a charge of 10^5 E.coli ESBL.

The founding of this E.coli ESBL was a charge of 2 UFC in days fifth into 300 10^8/g UCF on the seventeenth days with no infection sign. This result was justified by Jeneffier and al[199], who described more than 10^8 CFU / g with a Bacterial dose of 5 x 10^8 CFU per 90 g of rabbit bodyweight used in one day.

Also, according to Panda and his team[200], except for the control group, each animal gavaged with 10^9 CFU of E. coli developed infection on day six after infection with a high bacterial titer 10^7 CFU.

Decolonization was the goal of our study through the administration of AMC+ cineol. This product was the objective of multiple studies. The intervention patent, published on 12/29/2017 by Morocco's office of industrial and commercial property [201], covers the performance of randomized clinical trials. On 23 patients with resistant bacterial infections over seven days, with the conventional formula of 500 mg amoxicillin, 62.5 mg clavulanic acid, and 100 mg cineol per 3 g of powder (the same formula used in the protocol). E.coli, Kp, and pseudomonas were isolated. The findings indicate that, except in one case, the treatment was effective. Additionally, an unusual patient was engaged in this trial because it entailed the healing of this patient who appeared with a 20-year-old urinary infection that had previously been thought resistant to all antibiotics suggest that this product could eradicate patients carrying MDR bacteria.

Our study shows that the groups receiving this product reach 100% of eradication, the groups 1 and 4, and other groups control and AMC no eradication has been perceived. The comparisons accomplished for all the groups on day 20 demonstrate that groups; AMC+CINEOL and BACTERIA+AMC+CINEOL compared with the groups' CONTROL and BACTERIA+AMC show a significant difference with $P<0,0001$.

The clinical parameters followed throughout this study were fever and weight.

To summarize, weight gain followed the growth curve throughout all research periods, except for the decolonization phase, when there was a noticeable interruption in the growth curve; weight stagnation for more than two weeks in all groups except the control.

In addition, the septic parameter, which is fever, we had a temperature above 38C for all groups except the control. This fever varied from group to group and from day to day, and in the end, it remained at 38C in only the AMC treated group.

The consistency of the results described above allowed us to correlate this eradication (bacterial load) with these parameters.

The correlation: bacterial load versus fever shows a highly significant correlation ($r=0.8664$, $p<0.0001$). These findings demonstrate that high fever is associated with a high bacterial load, are supported by a study conducted by SCARPACE and his team[202], which discovered that non-lethal doses of E. coli $1x10^6$ to $1x10^8$ colony forming units could cause a fever for more than 8 hours. In contrast, a 1×10^9 colony-forming units dose resulted in progressive hypothermia culminating in death.

Furthermore, fever directly impacts the decolonization process because colonies account for 75% of temperature variation. As a result, the increase in bacterial load induces fever, and our rabbit could be infected. However, because blood tests such as blood cultures, blood count, CRP, and procalcitonin were not done, we cannot claim certainly that our rabbits were infected.

During the first week of therapy, The colony number was significantly increased compared to the following days. As previously described, fever is attributed to this increase. According to a review published by Holzheimer et al. [203], antibiotics induced Endotoxin release and clinical sepsis. Other factors can be implicated. The medication delivered can create an endotoxic shock, allowing E. coli to release the endotoxic, creating an inflammation reaction, fever as a clinical response. Ceftazidime treatment has a greater rate of endotoxin release in E. coli. More research is needed to understand the specific mechanism of the product amc+cineol. Again, multiple studies show that this inflammatory response is linked to a lower mortality rate in septic shock. Failure to develop a fever during the first 24 hours after the

commencement of bacteremia is related to a significantly increased risk of shock and death, according to Kreger[204].

The other correlation done was the bacterial load versus the weight and the weight gain. The correlation between weight and the number of colonies we objectified was negative (r =−0.4649 p=0.0025). This finding explains why the bacterial load detected has negatively impacted weight. It may be related to weight stagnation in these groups, particularly in the first week, since we noticed a lack of appetite in all the treatment groups. The preceding result is supported by the negative correlation between weight gain and colony number (r = −0.5089, p=0.0008).

These findings imply that the parameters that correlate between them tell the number of colonies, fever, and weight gain. All of these parameters influence each other in varying degrees. Furthermore, by classifying them, we have that the highest correlation is colony number vs. fever. The following correlation realizes between colony number and weight returned more diminutive than achieved between colony number and weight gain, saying that the weight gain is more related to the colony number than the weight alone. Finally, this weight gain is correlated with fever, but it is the weakest correlation.

The results of correlation weight gain versus temperature (r=−0,4316, and P=0,0136) are supported by Abu et al. [205], Effet of heat stress on reproduction and survival rate of Wistar rats, the weight of the animal reduced drastically as the temperature increase.

The result correlation realized between the gain weight and the bacterial load obtained are appropriate and linked with the results obtained by Tulstrup and his team[206]. Pups born from both amoxicillin and vancomycin-treated dams gain less weight than

controls, and This was concordant with lower feed intake. Their results demonstrate that early-life exposure to an antibiotic-perturbed low-diversity microbiota is sufficient to cause changes in body weight persisting into adulthood.

The response to treatment of the groups treated with cineol + AMC was substantiated by the cumulative eradication rate, reaching a rate of 100% is in the two groups treated with AMC+cineol. Also, the outcome of cumulative eradication curves for all the groups with the log-rank test has a $P < 0.0022$.

The duration of treatment, which lasted two weeks, is justified by the literature cited below, that the eradication of patients carrying multiresistant bacteria is made by doubling the duration of treatment, reaching up to 28 days in some cases, but the average was 14 days.

The question is if we continue to administrate this inoculum, will there be decolonization?

This new experimental study discusses and is not found in the literature. Formulating an approach could justify this study. By linking this experience through the hospital service and stimulating a scenario, particularly a continuous source of contamination, from the personal care with multiple manipulations, also according to Mody and al[207], title Multidrug-resistant Organisms in Hospitals: What Is on Patient Hands and in Their Rooms? Hand contamination patient is a source of MDRO and correlates with contamination on high-touch room surface, can be a source of colonization/infections with the multiresistant bacteria. So the idea here is to have decolonization despite continuous administration of bacteria multiresistant orally. The results were generally good since we ended up with 100% eradication but delayed compared to the other group, who received the product after the colonization.

This preclinical study produced extraordinary results, including a 100% eradication of the 4Gcephalosporianse E.coli. The results are satisfactory compared to other studies; specifically, a guideline summarizes all the protocols used to combat the colonization of multidrug-resistant Gram-negative bacteria carriers developed by Tacconelli et al. [208].

A study realized by Rieg and all[209], the decolonization using the colistin against ESBL-E have eradication of 19/45 patients, (42 %, 7/18 low-dose[4 × 1 million units] colistin, 3/12 high-dose [4 × 2 million units] colistin, 9/15 rifaximin [2 × 400 mg]), for a treatment period of 14 days.

Paterson and team[210] report on nine interventions without control using norfloxacin-based treatment (400 mg) twice daily, the result of 100% eradication rate at day 5 of treatment, and this rate decreased by days, reaching 89% in 14days and 44% in 28 days.

Another study leads to different rates of eradication. The study carried out by Oostdijk and all[211] report on 77 interventions without control using Colistin (2 MIU) + tobramycin (80 mg) + cefotaxime (1 g) four times daily targeting as 3GCephRE bacteria resulting in eradication of 73%. The treatment duration was up to ICU discharge

Gutierrez-Urbon and all[212] reoprt on 6 intervention without control using colistin (1% solution, 1 mL / kg) + amikacin (3.2% solution, 1 mL / kg) four times daily targeting as 3GCephRE bacteria resulting in 0% erradication, treatment duration was 5 days.

Abecasis and all[213] report on 39 interventions without control using colistin + tobramycin + cefotaxime targeting as 3GCephRE bacteria resulting in 77% eradication.

The previous two studies were conducted in a pediatric intensive care unit.

Troché and all[214]; reported on six interventions without a control using two of colistin sulfate (1.5 MIU), neomycin (500 mg), or erythromycin (500 mg) four times daily targeting 3GCephRE bacteria, resulting in 46% eradication; the treatment duration was not reported.

We can cite some limitations of our study. The duration of follow-up for our group since our last day of sampling was the 20th day, and the fact that treatment was halted three days prior, but these are minor points given that we had achieved two consecutive negative results for successive sampling, implying that this 4Gcephalosporinase has been eradicated.

Statistically, the number of cages available to us: 8 divided between 4 groups, could have compromised the statistical significance of the results. Hence the limited number could help to see 100% eradication.

It would be better to follow this eradication by performing PCR to classify the carbapenem resistance.

Table7: resuming the eradication rate of the different protocols cited before and our protocol.

Author, year of publication	Target bacteria	Sample size	Time-point	protocol	Eradication rate
Rieg, 2015[209]	3GCephRE ESBL producer	Intervention 45; control not applicable	14 days after EoT	Colistin standard dose (1 MIU) or high dose (2 MIU) qid or rifaximin	Colistin SD 39%; colistin HD 25%; rifaximin 60%
Paterson, 2001	3GCephRE ESBL producer	Intervention 9; control not applicable	End of treat 14 days after EoT 28 days after EoT	Norfloxacin (400 mg) twice daily	100% 89% 44%
Oostdijk, 2012 [211]	3GCephRE	Intervention 77; control not applicable	NA	Colistin (2 MIU)+ tobramycin(80 mg)+ cefotaxime (1 g) four time daily	73%
Gutierrez-Urbon, 2015 [212]	3GCephRE ESBL producer	Intervention 6; control not applicable	End of treatment	colistin (solution 1%, 1 mL/kg)+ amikacin (solution 3.2%, 1 mL/kg) four time daily	0%

Abecasis, 2011 [213]	3GCephRE ESBL producer	Intervention 39; control not applicable	End of treatment	Colistin + tobramycin + cefotaxime	77%
Troche, 2005 [214]	3GCephRE ESBL producer	Intervention 37; control not applicable	End of treatment	colistin sulphate (1.5 MIU), neomycin (500 mg), or erythromycin (500 mg) four times daily	46%
Our study	4GESBL-E	Two groups, each 5 rabbits, and two other groups, which one the control group.	End of treatment	Cineol+amc 80 mg|kg twice daily	100%

Serratia eradication which was not our primary goal in this preclinical study, aimed primarily at eradicating 4GESBL-E.coli. The Serratia is classified before as a carbapenemase and 4Gcephalosporinase high level. 100% o eradication was found on the fourth day in the groups treated with the cineol + AMC. This eradication can be better evaluated by initiating other experimental studies, for multiple reasons; especially when compared to other studies such as Tascini and all[215] using Gentamicin (80 mg), four times daily for more than seven days, variable (mean, 16 days) with 50 Intervention without control taring CRE resulting in an eradication rate of 68%, which remains a low rate compared to our study. Also, this Serratia found was implicated in multiple drastic endemics in hospitals.

The K.p founded at the end of the decolonization period are classified as ESBL 4G cephaloporinase and carbapenemase. The spread of K.p in all groups is debatable, but not concerning for several reasons: the succession of the contamination was described first in the group treated only by AMC, then the control group, and thus in groups who did not receive our renowned treatment, and discovered in all groups the days following treatment discontinuation. For that, we can attribute to the proximity between the groups, which facilitated the contamination. This contamination is supported by the fact that contact precautions are essential measure, all the details are mentioned in the bibliography section, and decolonization is only par. Also, this hypothesis is supported by the fact that hospitalized patients are frequently colonized with microorganisms acquired from the hospital environment. Up to 75% of critically ill patients will be colonized within 48 hours. Podschun et al. [216], Asensio et al. [217], report that prior antibiotic use and the use of invasive plastic devices such as bladder catheters, endotracheal tubes, and intravenous catheters are significant risk factors for infections.

General conclusion and recommendations

This study aims to see how AMC + cineol affects multiresistant bacteria decolonization of the digestive system. The findings are fascinating and have never been published in the scientific literature previously.

We try to accomplish the goal of selecting and the emergence of multiresistant bacteria using antibiotics and inducing colonization. Resulting in 100% of colonization in all groups by the strain Serratia ESBL 4G cephalosporinase, and E.coli ESBL 4G cephalosporinase.

The decolonization of the pathogen E.coli 4G cephalosporinase was attempted with 100% eradication after two weeks of treatment with the AMC+cineol product.

We are also approaching eradication because continued administration allows us to eradicate 100% of resistant bacteria.

The clinical parameters examined in conjunction with this decolonization led to the conclusion that this treatment assisted in eradicating/treating these rabbits.

As a recommendation, it would be interesting:

For eradication, we recommend that treatment last no longer than 14 days.

We advise that we follow all hospital preventative measures, including systematic screening and the adoption of hygiene measures ranging from standard precaution to contact precaution, which is critical in preventing the spread of multiresistant bacteria.

We also advocate doing a longitudinal randomized clinical trial on a large screening population to determine the eradication rate and the duration, with follow-up intervals ranging from one month to more than one year.

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Abstract

The decolonization of ESBL-E carriers as a prevention strategy seems warranted, given the risk of subsequent invasive infection in ESBL-E carriers, the fact that carriers are a potential source of cross-transmission, the potential for chronic carriage, and the possibility of horizontal gene transfer conferring resistance to other bacteria in the intestinal tract. Only a few studies have examined the effect of decolonization attempts on ESBL-E carriage. Also, a few studies have been conducted to study the efficacy of a systematic ESBL-E eradication strategy. Therefore the main objective of this study is to decolonize the digestif tract using a new formulation, AMC boosting by cineol, as a promising treatment to eradicate or suppress MDR bacteria carriers.

Our work is a preclinical study conducted on an animal model, rabbits, spread over one year carried out at the Faculty of Medicine and Pharmacy and Dental Medicine Fez. The current study was performed in steps. First, the selection in the intestinal gut a high and MDR bacteria. The essential step was to assess the decolonization process. For this purpose, we divided our groups as follows: group one received AMC+cineol, group two was the control group, group three received bacteria+AMC, and group four received bacteria+amc+ cineol. Throughout all of these steps, we measured two parameters: weight and fever, allowing us to study the correlation between decolonization (bacterial load) and other factors such as fever and weight gain. All fecal samples were taken to the faculty's microbiology and molecular biology laboratory, where bacterial identification and antibiotics sensitivity profiles were performed following international guidelines. Prism 8 software was used for statistical analysis.

The initial screening of rabbits reveals that none of the isolates tested were highly multiresistant bacteria in the intestinal gut. In all groups, colonization induction results in 100% colonization by ESBL Serratia sp. and E. coli. Additionally, in all groups, the bacterial load of this E. coli was achieved the desired charge. The decolonization of the pathogen HDR bacteria was attempted with 100% eradication after two weeks of treatment with the AMC+cineol product, while no decolonization is expressed in the other groups. The comparisons of bacterial loads for all groups show that groups; AMC+CINEOL and BACTERIA+AMC+CINEOL have a significant difference with $P<0,0001$ compared to the groups' CONTROL and BACTERIA+AMC.

The correlation study showed the highest correlation between colony number and fever ($r=0.8664$, $p<0.0001$). Followed by colony number and weight ($r=-0.4649$ $p=0.0025$) and finally, between colony number and weight gain ($r = -0.5089$ $p=0.0008$), we can say that the weight gain is more related to the colony number than the weight alone.

The intestinal decolonization of rabbits by AMC + CINEOL showed complete eradication of HBR in the feces collected. This result is promising when using AMC boosting by cineol either as curative or prophylaxis treatment or a decontamination treatment in hospitalized patients to prevent or suppress the reservoirs of MDR bacteria in the intestinal gut.

Keywords: Selection pressure, Colonization, High resistant bacteria, Multidrug resistant, Decolonization, Eradication, Rabbits, Essential oil, Boosting antibiotic, MDRO management, Clinical parameters, Correlation, ESBL-E.coli.

Résumé

La décolonisation des porteurs de BLSE-E comme stratégie de prévention semble justifiée, étant donné le risque d'infection invasive ultérieure chez les porteurs de BLSE-E, le fait que les porteurs sont une source potentielle de transmission croisée, le potentiel de portage chronique et la possibilité de transfert horizontal de gènes conférant une résistance à d'autres bactéries dans le tractus intestinal. Seules quelques études ont examiné l'effet des tentatives de décolonisation sur le portage des BLSE-E. De même, peu d'études ont été menées pour étudier l'efficacité d'une stratégie d'éradication systématique des BLSE-E. Par conséquent, l'objectif principal de cette étude est de décoloniser le tractus digestif en utilisant une nouvelle formulation, AMC boostée par le cinéol, comme traitement prometteur pour éradiquer ou supprimer les porteurs de bactéries MDR.

Notre travail est une étude préclinique menée sur un modèle animal, les lapins, étalée sur une année réalisée à la Faculté de Médecine et de Pharmacie et de Médecine Dentaire de Fès. L'étude actuelle a été réalisée en plusieurs étapes. Tout d'abord, la sélection dans l'intestin d'une bactérie MDR et élevée. L'étape essentielle était d'évaluer le processus de décolonisation. Pour cela, nous avons divisé nos groupes comme suit : le groupe un a reçu AMC+cinéol, le groupe deux était le groupe témoin, le groupe trois a reçu bactéries+AMC, et le groupe quatre a reçu bactéries+amc+cinéol. Tout au long de ces étapes, nous avons mesuré deux paramètres : le poids et la fièvre, ce qui nous a permis d'étudier la corrélation entre la décolonisation (charge bactérienne) et d'autres facteurs tels que la fièvre et la prise de poids. Tous les échantillons fécaux ont été apportés au laboratoire de

microbiologie et de biologie moléculaire de la faculté, où l'identification bactérienne et les profils de sensibilité aux antibiotiques ont été réalisés conformément aux directives internationales. Le logiciel Prism 8 a été utilisé pour l'analyse statistique.

Le dépistage initial des lapins révèle qu'aucun des isolats testés n'était une bactérie hautement résistante dans l'intestin. Dans tous les groupes, l'induction de la colonisation entraîne une colonisation à 100 % par les BLSE Serratia sp. et E. coli. De plus, dans tous les groupes, la charge bactérienne de cet E. coli a atteint la charge souhaitée. La décolonisation du BHR E.coli a été achevé avec une éradication de 100% après deux semaines de traitement avec le produit AMC+cinéol, alors qu'aucune décolonisation n'est exprimée dans les autres groupes. Les comparaisons des charges bactériennes pour tous les groupes montrent que les groupes AMC+Cinéol et BACTERIA+AMC+Cinéol présentent une différence significative avec $P<0,0001$ par rapport aux groupes CONTROL et BACTERIA+AMC.

L'étude de corrélation a montré la plus forte corrélation entre le nombre de colonies et la fièvre ($r=0.8664$, $p<0.0001$). Suivie par le nombre de colonies et le poids ($r=-0.4649$, $p=0.0025$) et enfin, entre le nombre de colonies et le gain de poids ($r = -0.5089$, $p=0.0008$), nous pouvons dire que le gain de poids est plus lié au nombre de colonies qu'au poids seul.

La décolonisation intestinale des lapins par AMC + CINEOL a montré une éradication complète de BHR E.coli dans les fèces collectées. Ce résultat est prometteur pour l'utilisation de l'AMC renforcé par le cinéol comme traitement curatif ou prophylactique ou encore comme traitement de décontamination chez les patients hospitalisés pour prévenir ou supprimer les réservoirs de bactéries MDR dans l'intestin.

Mots clés : Pression de sélection, Colonisation, Bactéries hautement résistantes, Bactéries multi-résistantes, Décolonisation, Eradication, Lapins, Huile essentielle, Antibiotique booster, Gestion MDRO, Parametre clinique, correlation,BLSE-E.coli.

ملخص

يبدو أن إنهاء الاستيطان لحاملي ESBL-E كاستراتيجية وقائية له ما يبرره، نظرًا لخطر اجتياح العدوى لاحقًا، وحقيقة أن الناقلين هم مصدر محتمل للانتقال المتقاطع، وإمكانية النقل المزمن، وإمكانية نقل الجينات الأفقي الذي يمنح المقاومة للبكتيريا الأخرى في الأمعاء. فقط عدد قليل من الدراسات قاموا بفحص تأثير محاولات إنهاء الاستيطان لحاملي ESBL-E . أيضًا، تم إجراء عدد قليل من الدراسات لدراسة فعالية استراتيجية منهجية لاستئصال .لذلك فإن الهدف الرئيسي من هذه الدراسة هو إزالة استعمار الجهاز الهضمي باستخدام تركيبة جديدة أموكسيسيلين/حمض الكلافولانيك معزز بواسطة السينول، كعلاج واعد لفتك أو قمع هذه البكتيريا .

عملنا عبارة عن دراسة قبل السريرية أجريت على نموذج حيواني، الأرانب، موزعة على عام واحد نفذت في كلية الطب والصيدلة وطب الأسنان بفاس. تم إجراء الدراسة الحالية على خطوات أهمها. أولاً، انتقاء البكتيريا متعددة المقاومة في الأمعاء عن طريق المضادات الحيوية. والخطوة الأساسية كانت تقييم عملية إنهاء الاستيطان، لهذا الغرض، قمنا بتقسيم مجموعاتنا على النحو التالي: المجموعة الأولى تلقت أموكسيسيلين/حمض الكلافولانيك و السينول ، المجموعة الثانية كانت المجموعة الضابطة ، المجموعة الثالثة تلقت البكتيريا و أموكسيسيلين/حمض الكلافولانيك ، المجموعة الرابعة تلقت البكتيريا و أموكسيسيلين/حمض الكلافولانيك و السينول .خلال كل هذه الخطوات، قمنا بقياس عاملين: الوزن ودرجة الحرارة، مما سمح لنا بدراسة العلاقة بين إنهاء الاستيطان (الحمل البكتيري) وعوامل أخرى مثل درجة الحرارة وزيادة الوزن. تم أخذ جميع عينات البراز إلى مختبر الأحياء الدقيقة والبيولوجيا الجزيئية بالكلية، حيث تم إجراء تحديد البكتيريا وحساسية المضادات الحيوية وفقًا للإرشادات الدولية. تم استخدام برنامج البريزم ٨ للتحليل الإحصائي.

كشف الفحص الأولي للأرانب أن أيا من العازلات التي تم فحصها لم تكن بكتيريا عالية المقاومة في الأمعاء المعوية. في جميع المجموعات ، نتج عن تحريض الاستيطان استيطان بنسبة 100٪ بواسطة ESBL Serratia sp. و E. coli.

بالإضافة إلى ذلك ، في جميع المجموعات ، تم تحقيق الحمل البكتيري لهذه E. coli. بالشحنة المطلوبة. تمت محاولة إزالة الاستيطان من بكتيريا E. coli الممرضة مع الاستئصال بنسبة 100٪ بعد أسبوعين من العلاج بمنتج أموكسيسيلين/حمض الكلافولانيك و السينول ، بينما لم يتم التعبير عن إزالة الاستيطان في المجموعات الأخرى. تظهر مقارنات الأحمال البكتيرية لجميع المجموعات أن المجموعتين ؛ أموكسيسيلين/حمض الكلافولانيك و السينول و البكتيريا أموكسيسيلين/حمض الكلافولانيك و السينول لهما فرق كبير مع $P<0،0001$ مقارنة بمجموعة التحكم و البكتيريا أموكسيسيلين/حمض الكلافولانيك .

أظهرت دراسة الارتباط أن أعلى ارتباط هو بين الحمل البكتيري والحمى ($r = 0.8664$، $p < 0.0001$). متبوعًا بالحمل البكتيري والوزن ($r = -0.4649$ $p = 0.0025$) وأخيراً ، بين الحمل البكتيري وزيادة الوزن ($r = -0.5089$، $p = 0.0008$) ، يمكننا القول أن زيادة الوزن مرتبطة بعدد الحمل البكتيري أكثر من الوزن وحده.

أظهر إزالة الاستيطان المعوي للأرانب بواسطة أموكسيسيلين/حمض الكلافولانيك الفتك التام للبكتيريا متعددة المقاومة E. coli في البراز الذي تم جمعه. هذه النتيجة واعدة أظهرت انه يمكن استخدام أموكسيسيلين/حمض الكلافولانيك والسينول كعلاج واعد للمرضى لفتك أو قمع هذه البكتيريا متعددة المقاومة في الأمعاء.

الكلمات الأساسية : ضغط الاختيار، حاملي البكتريا، البكتيريا عالية المقاومة، البكتيريا متعددة المقاومة، الأرانب، فتك، القضاء على الاستيطان، تدبير البكتيريا متعددة المقاومة، الزيوت العطرية، معزز المضادات الحيوية، ESBL-E.coli.الترابط، المعايير السريرية.

جامعة سيدي محمد بن عبد الله - فاس
ⵜⴰⵙⴷⴰⵡⵉⵜ ⵙⵉⴷⵉ ⵎⵓⵃⴰⵎⵎⴷ ⴱⵏ ⵄⴱⴷⵍⵍⴰⵀ ⴳ ⴼⴰⵙ
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كلية الطب والصيدلة وطب الأسنان
ⵜⴰⵖⵔⵓⵎⵜ ⵏ ⵜⵉⴱⴱⵉⵜ ⴷ ⵜⵉⵎⵓⵔⵉⵜ ⴷ ⵜⵉⴱⴱⵉⵜ ⵏ ⵜⵣⴷⵓⵖⵜ
FACULTÉ DE MÉDECINE, DE PHARMACIE ET DE MÉDECINE DENTAIRE

سنة 2021 أطروحة رقم : 299/21

دراسة استباقية بهدف القضاء على الاستيطان المعوي لحاملي البكتيريا الجرام السالبة المتعددة المقاومة. (دراسة قبل سريرية)

الأطروحة

قدمت ونوقشت علانية يوم 26/11/2021

من طرف

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المزداد في 05/02/1994 بفاس

لنيل شهادة الدكتوراه في الطب

الكلمات الأساسية:

ضغط الاختيار، حاملي البكتريا، البكتيريا عالية المقاومة، البكتيريا متعددة المقاومة، الأرانب، فتك، القضاء على الاستيطان، تدبير البكتيريا متعددة المقاومة، الزيوت العطرية، معزز المضادات الحيوية، الترابط، المعايير السريرية ، ESBL-E.coli.

اللجنة

الرئيس	السيد محمد شكيب بنجلون..... أستاذ في علم أمراض الرئة والسل.....
المشرفون	السيد عدنان رمال..... أستاذ في علم الأحياء الدقيقة و علم الأدوية.....
	السيدة أومختار بشرى..... أستاذة في علم الأحياء الدقيقة.....
أعضاء	السيد طارق صقلي حسيني ..... أستاذ في علم أمراض الكلي.....
	السيدة حمامي فوزية..... أستاذة في طب الأطفال.....
	السيد بوكطة ابراهيم..... أستاذ في التخدير والإنعاش..... ...