UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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Dept. of Biotechnology

STUDY OF THE DISTRIBUTION OF ANTIMICROBIAL RESISTANCE IN ORGANIC CROPS AND THEIR ENVIRONMENT

Master's Thesis

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(ETSIAMN)

STUDY OF THE DISTRIBUTION OF ANTIMICROBIAL RESISTANCE IN ORGANIC CROPS AND THEIR ENVIRONMENT

MASTER'S THESIS IN BIOMEDICAL BIOTECHNOLOGY

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Título: Estudio de la distribución de la resistencia a los antimicrobianos en los cultivos ecológicos y su entorno

Títol: Estudi de la distribució de la resistència als antimicrobians en els cultius ecològics i el seu entorn

Resumen:

Los agentes que matan microorganismos o inhiben su crecimiento se conocen como antimicrobianos. Los antimicrobianos se han utilizado de muchas formas diferentes durante miles de años. Son una herramienta crucial en la lucha contra las enfermedades infecciosas. Sin embargo, la resistencia de las bacterias patógenas a estos antimicrobianos es uno de los principales problemas de salud pública mundial. Dentro de estos patógenos, las bacterias Gram-negativas son consideradas de importancia crítica por la Organización Mundial de la Salud (OMS).

Además, la creciente tendencia a consumir verduras crudas y hojas verdes frescas para evitar una dieta rica en grasas y las consiguientes enfermedades cardiovasculares (ECV) y metabólicas agrava aún más la situación.

En este trabajo de investigación se tomaron y evaluaron muestras de verduras ecológicas como coles, fresas, lechugas, agua y muestras de suelo para determinar su papel en la transmisión y propagación de bacterias resistentes a los antibióticos y sus genes en el medio ambiente.

Los aislados sospechosos de ser resistentes a betalactámicos, carbapenems y quinolonas se identificaron mediante diferentes pruebas bioquímicas (oxidasa-negativa/catalasa-positiva/Gram-negativa/API). Posteriormente, se determinaron sus perfiles de resistencia mediante el método del antibiograma en placa de disco. Siendo las más frecuentes las resistencias a β-lactámicos (penicilinas, cefalosporinas de 3ª generación y carbapenemes) .

Se estudió la presencia de genes de resistencia a betalactámicos (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CMY2}$), a carbapenemes (*bla*$_{KPC}$, *bla*$_{OXA}$, *bla*$_{IMP}$ y *bla*$_{VIM}$) y a quinolonas (*qnrA*, *qnrB*, *qnrS*) mediante PCR y electroforesis en gel.

Nuestra investigación demostró la presencia del mayor número de genes resistentes a carbapenemes (64,7%), seguidos de genes resistentes a betalactámicos (58,8%) y quinolonas (29,4%).

La resistencia a los antimicrobianos es un problema sanitario mundial que tiene implicaciones de gran alcance y afecta a varios aspectos del desarrollo sostenible. Los Objetivos de Desarrollo Sostenible 2 (Hambre cero), 3 (Buena salud y bienestar), 6 (Agua limpia y saneamiento), 11 (Ciudades y comunidades sostenibles), 12 (Consumo y producción responsables) y 15 (Vida en la tierra), establecidos por las Naciones Unidas, son de especial consideración cuando se trata de la presencia de bacterias resistentes a los antibióticos en alimentos orgánicos, suelo y agua.

La identificación de genes resistentes a los antibióticos en los alimentos ecológicos subraya la urgencia de un enfoque multifacético para combatir la resistencia a los antimicrobianos. Los esfuerzos de salud pública deben centrarse tanto en la prevención como en el tratamiento, haciendo hincapié en el uso responsable de antibióticos, la vacunación y las prácticas de control de infecciones. Estas acciones son cruciales para salvaguardar la salud de las personas y las comunidades en todo el mundo. Hacer frente a la resistencia antimicrobiana es crucial para un futuro más sano y sostenible

Palabras clave: *Resistencia a los antimicrobianos, agricultura ecológica, pruebas bioquímicas, antibiogramas, betalactámicos, carbapenems, quinolonas, genes de resistencia*

Title: Study of the distribution of antimicrobial resistance in organic crops and their environment.

Abstract:

Agents that kill microorganisms or inhibit their growth are known as antimicrobials. Antimicrobials have been used in many different forms for thousands of years. They are a crucial tool in fighting infectious diseases. However, resistance posed by pathogenic bacteria to these antimicrobials is one of top global public health issues. Within these pathogens, Gram negative bacteria are considered to be of critical importance by the World Health Organisation (WHO).

Moreover, the rising trend of eating raw vegetables and fresh green leaves in order to avoid a high-fat diet and the subsequent cardiovascular diseases (CVDs) and metabolic diseases further aggravates the situation.

In this research work, samples from organic vegetables such as cabbage, strawberries, lettuce, water, and soil samples were taken and evaluated to determine their role in the transmission and spreading of antibiotic-resistant bacteria and their genes in the environment.

Isolates suspected of being resistant to beta-lactam, carbapenem, and quinolones were identified through different biochemical tests(oxidase-negative/catalase-positive/Gram-negative/API). Later, their resistance profiles were determined through the disk-plate antibiogram method. Resistance to β-lactams (penicillins, third generation cephalosporins and carbapenems) were the most frequent.

The presence of Beta-lactam resistance genes (bla_{TEM}, bla_{SHV}, bla_{CMY2}), carbapenem resistance genes (bla_{KPC}, bla_{OXA}, bla_{IMP} *and* bla_{VIM}) and quinolones resistant genes (*qnrA, qnrB, qnrS*) was studied through PCR and gel electrophoresis.

Our research showed the presence of the highest number of carbapenem-resistant genes (64.7%), followed by β-lactam (58.8%) and quinolones resistant genes (29.4%).

Antimicrobial resistance is a global health concern that has far-reaching implications, touching upon various aspects of sustainable development. Sustainable Development Goals 2 (Zero hunger), 3 (Good health and well-being), 6 (Clean water and sanitation), 11 (Sustainable cities and communities), 12(Responsible consumption and production) and 15 (Life on land) are established by the United Nations and are of special consideration when it comes to the presence of antibiotic resistant bacteria in organic food, soil and water.

The identification of antibiotic resistant genes in organic food underscores the urgency of a multifaceted approach to combat antimicrobial resistance. Public health efforts should focus on both prevention and treatment, emphasising responsible antibiotic use, vaccination, and infection control practices. These actions are crucial to safeguarding the health of individuals and communities worldwide. Addressing antimicrobial resistance is crucial for a healthier and more sustainable future.

Key words: *Antimicrobial resistance, organic farming, biochemical tests, antibiograms, b-lactam, carbapenem, quinolones, genes of resistance*

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List of abbreviations

AMC Amoxicillin

AMP Ampicillin

AMR Antimicrobial resistance

ARG Antibiotic resistance genes

API Analytical Profile Index

BPW Buffered peptone water

C Chloramphenicol

CAECV Ecologic Agriculture Committee from Valencian Community

CAZ Ceftazidime

CDC Centres for Disease Control and Prevention

CIP Ciprofloxacino

CLSI Clinical and Laboratory Standards Institute

CN Gentamicin

CRO Ceftriaxone

CTX Cefotaxime

CV Valencian Community

EC European Commission

ECDC European Centers for Disease Control and Prevention

EFSA European Food Safety Authority

ESBL Extended-spectrum b-lactamases

EU European Union

IMP Imipenem

IND Indol

LEV Levofloxacin

MCA MacConckey Agar

MEM Meropenem

MH Mueller-Hinton Agar

NA Nalidixic acid

ODC Decarboxilase ornithin

OHHLEP One Health High Level Expert Panel

WHO World Health Organization

PBP Penicillin binding proteins

PCA Plate Count agar

SC Agar mSuperCARBA

SCF Scientific Committee on Food

TDA Deaminase tryptophan

TE Tetracycline

TSB Tryptic soy broth

URE Urease

VP Voges-Proskauer

1. INTRODUCTION

Antibiotics are one of the wonder discoveries of the 20th century (Davies and Davies., 2010). Human beings have used them in large amounts, not only in medical contexts but also as growth factors in agriculture and livestock as they are a cost-effective tool for improving feed efficiency and preventing disease in agricultural animals. They have revolutionised medicine in many respects and countless lives have been saved. Antibiotics have indeed been a turning point in human history.

There is no denying that Antibiotics play a crucial role in modern medicine. They are medications that are used to treat bacterial infections by killing or inhibiting the bacterial growth. The first antibiotic, Salvarsan or Arsphenamine, was discovered in 1910 which was the first effective treatment for syphilis and human African trypanosomiasis. The production of antibiotics was then boosted by the discovery of penicillin by Fleming in 1928 (Zumbado Morales *et al*., 2022). Prior to the discovery of antibiotics, bacterial infections were a major cause of illness and death. Pathogenic micro-organisms including bacteria, viruses, and fungi, are of special concern in hospitals as they adversely affect the optimal functioning of medical devices, drugs, surgical equipment, dental restorations, and bone cements (Jiao *et al*., 2017). About 700 bacterial species have been identified in the oral microbiome, *Enterococcus faecalis* being one of them. It has been found to be a causative agent of oral infectious processes such as pulp necrosis, exposed canals in the oral cavity and persistent apical periodontitis (Martinez *et al*., 2014). Antibiotics provided a means to target and eliminate bacteria, making it possible to treat a wide range of infections. It has also played a significant role in advancing surgical procedures. *A. baumannii* is one of the most important species and is responsible for a significant proportion of nosocomial infections, including urinary tract infections, endocarditis, surgical-site infections, meningitis, septicemia, and ventilator-associated pneumonia among patients in intensive care units. *A. baumannii* has more recently become a cause for major concern in clinical practice due to its high level of antimicrobial resistance. The risk of infection during surgeries was reduced a great deal with the use of antibiotics, allowing for more complex and life-saving operations (Khosravi *et al*., 2105).

Furthermore, it has had a profound impact on life expectancy worldwide. Antibiotics have saved countless lives by effectively treating bacterial infections, especially those of infants, children, and the elderly who are more vulnerable to infections (Desa and Trevenen, 1984). They have also helped in the eradication and control of diseases like cholera and diphtheria as well as reducing maternal mortality rates associated with infections during childbirth (Adedeji W.A, 2016).

In addition to improving human health and physical well-being, they have revolutionised agricultural practices. They are used in livestock and poultry farming to prevent and treat bacterial infections, leading to improved animal health and increased food production. Antibiotics have several applications in animals. They are used to treat active infections, prevent infections, and even as growth promoters. In the 1940s, it was discovered that giving broad-spectrum antibiotics to pigs and chickens enhanced their growth and meat production (Megan Cully, 2014).

However, the successful use of this therapeutic agent is compromised by the development of resistance to it. The appearance of resistant strains in hospitals, communities, and in the environment due to their overuse or misuse is the real wonder and the major health crisis nowadays. Over time, bacteria can evolve and become resistant to the effects of antibiotics, making infections harder to treat. The development of resistance is a normal evolutionary process for microorganisms, but it is

accelerated by the selective pressure exerted by widespread use of antibacterial drugs (WHO, 2014). The misuse and overuse of antibiotics in plants, animals and humans have accelerated the emergence of antibiotic-resistant bacteria, posing a significant global health threat. Liu and Pop (2009) demonstrated in one of their studies that currently, ARDB contains resistance information for more than 13,293 genes, 377 types, 257 antibiotics, 632 genomes, 933 species and 124 genera. Antibiotic resistance has been declared one of the three most important public health threats of the 21st century (WHO, 2014).

1.1. SOCIAL AND ECONOMICAL IMPACT OF ANTIMICROBIAL RESISTANT BACTERIA/ GENES (ARB/ARGs)

1.1.1. Deaths caused by ARB/ARGs

Microbial infection is a major challenge to human health worldwide. It is one of the major health crises and causes approximately 700,000 deaths each year (Mahmud *et al*., 2018). In 2013 only, it caused 9.2 million deaths due to the biofilm formation of resistant bacteria. These pathogenic microorganisms are of great concern because they are not only a threat to human life but also adversely affect the functioning of medical devices, drugs, or other surgical equipment (Avila *et al*., 2018). The number of annual deaths, reported by the U.S. Centre for Disease Control and Prevention, are up to 23,000 (Jiao *et al*.,2017). In fact, according to the Centres for Disease Control and Prevention (CDC) (CDC, 2019), antibiotic resistance is one of the world's most serious public health issues, making diseases more difficult and expensive to treat, those were previously easily treatable.

According to estimates from the European Union/European Economic Area (EU/EEA) alone, every year more than 670, 000 infections are caused by bacteria resistant to antibiotics, and about 33, 000 people die as a direct result of these infections, making antimicrobial resistance (AMR) a major public health concern in the WHO European Region (Antimicrobial resistance surveillance in EU, 2022). In Spain, according to the Spanish Ministry of Health, almost 3000 people die every year due to infections caused by antibiotic-resistant bacteria (PRAN-MA, 2019; Amato *et al*., 2021).

Systematic analyses of the scientific literature demonstrate that patients infected with resistant bacteria have a higher risk of poorer clinical outcomes and mortality compared to those infected with non-resistant microbes, according to the World Health Organization (WHO, 2014). Due to a lack of sufficient evidence, it is currently unclear what the expected societal impact and economic implication will be if the only viable therapy for an illness is totally lost as a result of antibiotic resistance. In 2013, the US Centers for Disease Control (CDC) proclaimed that the world is getting closer to a post-antibiotic era in which widespread, treatable diseases are developing into deadly illnesses which was further reiterated by the World Health Organization in its report on global monitoring of antimicrobial resistance (WHO,2014). The estimated number of deaths caused annually by ARB in the United States of America and in the European Union, are calculated as 23,000 and 25,000, respectively (https://www.cdc.gov/drugresistance/, ECDC/EMEA, 2009). However, it is unknown yet whether the resistance was caused by contaminated food. The relevance of the presence of ARB and ARGs in food in these figures is currently unknown (Cerqueira *et al.,* 2019). According to Jiao *et al*., (2017), the World Health Organization has appealed to researchers worldwide to prioritise their efforts toward concerted efforts in combating the spread of antimicrobial-resistant microorganisms.

1.1.2. Economic impact of antibiotic resistant bacteria ARBs/ARGs

Antibiotic resistance is a multinational health crisis. Not only is it causing loss to humans but also has an important and huge economic impact. A finding by (PRAN-MA, 2019; Amato *et al*., 2021) shows that resistant bacteria cause more deaths as compared to sensitive ones. Only in the US, the estimated cost of diseases caused by resistant bacteria is around 20 billion dollars per year. According to another report, antibiotic resistance is estimated to cause around 300 million premature deaths by 2050, with a loss of up to $100 trillion (£64 trillion) to the global economy. Furthermore, the world will have to depend on the less effective, intrusive, and time-consuming procedures including debridement, disinfection, amputation, and isolation if the surge in AMR continues. Human civilization will be significantly impacted by the rising prevalence of fatal and disabling diseases (Michael *et al*., 2014).

Some of the significant human pathogens, such as *Escherichia coli* and *Klebsiella pneumoniae*, can cause serious urinary tract and bloodstream infections and have already become resistant to 3rd generation cephalosporins and fluoroquinolones. *E. coli* constitute the normal intestinal microbiota. However, it is associated with intra-abdominal infections such as peritonitis and causes meningitis in newborns. It is also one of the leading causative agents of food-borne diseases. Normally, the infection is through an infected person but sometimes it could be transmitted from infected animals through the food chain. Carbapenem is the only solution to treat them. Nevertheless, in some cases they have even become resistant to carbapenems. The resistance to carbapenems is mainly mediated by **metallo-beta lactamases** which confers resistance to all available beta-lactam antimicrobial drugs (WHO, 2014).

Another rarely found human pathogen is *Sphingobacterium multivorum*, initially detected in sheep dung and can be found in soils as well. *Sphingobacterium multivorum* is a saprophytic bacterium that has been shown to transfer plasmids from nitrifying bacteria. They are tiny, pleomorphic, Gram-negative bacilli that contain sphingolipids and are closely linked to *Flavobacterium* species. They form the *Flavobacterium-Cytophaga-Sphingobacterium* complex, which is identified by the presence of homospermidine as a shared chemotaxonomic marker. The nearest genus to *Sphingobacterium* is *Pedobacter*, with which it would form a distinct branch of the V rRNA superfamily: the family *Sphingo -bacteriaceae*. *Flexibacter*, *Weeksella*, *Cytophaga*, and *Moraxella anatipestifer* are other species classified in the superfamily V rRNA with *Sphingobacterium* (Anales de medicina interna, 2001).

Flemming in 1945, said that “It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them”. Underdosage of antibiotics expose the microbes to non-lethal quantities of the drug, hence making them resistant. Therefore, the correct dose of an antibiotic is highly significant. When treating an antibiotic-resistant pathogen, the clinical situation and the range of available treatments may affect how susceptibility patterns are interpreted. For instance, the level of gentamicin found in the urine may be high enough to cure a lower urinary tract infection carried on by gentamicin-resistant bacteria. For *Streptococcus pneumoniae*, several penicillin breakpoints have been defined based on whether the isolate is causing meningitis vs other forms of infections, taking into account the amounts of the medication that actually reach the cerebral fluid. Furthermore, the size of the bacterial inoculum might affect an organism's in vivo sensitivity to a certain antibiotic. For instance, data have suggested that certain cephalosporins, including cefazolin, may not be effective in the setting of high inoculum deep seated infections caused by cephalosporin susceptible *S. aureus* (Michael *et al*., 2014)

1.2. SURVEILLANCE OF ARB/ARGs ACROSS EU

Surveillance or monitoring studies are required to prevent the spread of antibiotic-resistant microorganisms and to understand the distribution, prevalence, and temporal variations in antibiotic resistance. These studies offer data on the distribution and changes in the trend of resistance rates throughout time. In order to protect public health, ensure effective treatment, promote infection control procedures, encourage the right use of antibiotics, and facilitate a worldwide response to address this pressing issue, surveillance of antibiotic resistance is critically important.

Furthermore, the information they give is essential for developing resistance reduction programs as well as measuring the efficiency of the actions implemented. By monitoring resistance patterns and collecting data, health authorities can make informed decisions and take proactive measures to preserve the effectiveness of antibiotics and safeguard public health.

For effective monitoring of ABR, several regional surveillance zones, including WHO African Region, WHO American Region, WHO Eastern Mediterranean Region, WHO European Region, WHO South-East Asia Region and WHO Western Pacific Region have been created to contribute to the improvement of surveillance of ABR (WHO, 2014). Another vigilance program operating for the European Union is the "European Antimicrobial Resistance Surveillance Network (EARS-Net)" (ECDC, n.d.), which monitors for resistance in isolates from invasive clinical samples. One of the system's goals is to collect data on antibiotic resistance, analysing antimicrobial resistance temporal patterns, advising policymakers on antimicrobial resistance and antibiotic resistance challenges, and so on. Furthermore, by offering standardised techniques, these platforms enable comparison of findings across European nations.

In addition to clinical isolate programs, there are continuing monitoring programs intended to determine if resistance reported in infections in humans developed first in animals and subsequently was transferred to people (Frye and Jackson *et al*., 2013). These investigations are aimed at identifying the occurrence of resistance in isolates from food animals and humans. In Europe, the European Food Safety Authority (EFSA) and the European Centre for Disease Prevention and Control (ECDC) work together to ensure food safety. ECDC (2019) publishes an annual report on the prevalence and progress of antibiotic resistance in zoonotic and indicator bacteria in animals, people, and food.

AMR percentages for the bacterial species-antimicrobial group combinations that are being monitored overall in the EU/EEA remain high, with **carbapenem resistance** in *Escherichia coli* and *Klebsiella pneumoniae* and **vancomycin resistance** in *Enterococcus faecium* showing a notable increase between 2016 and 2020. *Enterobacteriaceae* that produce extended-spectrum b-lactamases (ESBLs) are *Escherichia coli* and *Klebsiella pneumonia*. These enzymes give resistance to extended-spectrum cephalosporins and other b-lactam antibiotics, reducing the number of treatments available for infections caused by these bacteria in the urinary tract, bloodstream, and other areas of the body.

It is concerning that several nations in the European Region have high rates of carbapenem and third-generation cephalosporin-resistant *K. pneumoniae,* as well as carbapenem-resistant *Acinetobacter* species and *Pseudomonas aeruginosa*. Greater efforts and investments are needed in the WHO European Region to effectively combat AMR (AMR Surveillance in EU 2022). Control and preventive measures against bacteria must be taken in order to combat ABR. These measures include access to

clean water, improved hygienic conditions and encouraging the use of (preventive) vaccines (WHO, 2014).

1.2.1. *Escherichia coli:*

According to the surveillance report by AMR in EU (2022), Community-acquired bloodstream infections and urinary tract infections are most frequently caused by *E. coli*. In the WHO European Region in 2020, fluoroquinolone resistance was typically lower in the north and west and greater in the south and east.

Norway, one of the 40 countries/areas that reported data on this microorganism, has an AMR level < 10%. A rate of 25% or above was recorded by 20 nations or regions (50%) overall. Three (8%) nations (North Macedonia, the Russian Federation, and Turkey) have AMR levels of 50% or higher. Austria, Belgium, Denmark, Estonia, Finland, France, the Netherlands, Norway, Sweden, and Switzerland reported the lowest percentages of third-generation cephalosporin resistance in *E. coli* in 2020 (5% to less than 10%), whereas AMR percentages equal to or above 50% were seen in five (13%) countries (Belarus, North Macedonia, the Russian Federation, Turkey, and Ukraine).

1.2.2. *Pseudomonas aeruginosa*:

P. aeruginosa is a frequent source of infection in hospitalised patients, particularly in individuals with weakened immune systems. This includes bloodstream infections, urinary tract infections, and hospital acquired pneumonia. It is difficult to manage in medical settings since it is inherently resistant to many antimicrobial treatments. The prevalence of carbapenem-resistant *P. aeruginosa* varies significantly within the WHO European Region.

Four (10%) of the 41 nations and regions that reported data on this microorganism in 2020 showed AMR percentages below 5% (Denmark, Finland, the Netherlands, and Sweden), whereas six (15%) reported percentages equal to or above 50% (Belarus, Bosnia and Herzegovina, Montenegro, the Republic of Moldova, Serbia, and Ukraine) (AMR Surveillance in EU, 2022).

1.2.3. *Acinetobacter baumannii:*

Bloodstream infections from central lines, ventilator-associated pneumonia, and postoperative wound infections are the most common infections caused by *Acinetobacter species*. This pathogen is also responsible for a variety of diseases, including urinary tract infections, skin and soft tissue infections, bacteraemia, pneumonia, osteomyelitis, and meningitis (Ababneh *et al.,*2022). Once established, *Acinetobacter spp*. can persist in the healthcare setting and are challenging to eliminate. Ireland, the Netherlands, and Norway had percentages of carbapenem-resistant *Acinetobacter spp.* below 1%, while 21 (55%) countries and areas, mostly in southern and eastern Europe, had percentages equal to or above 50%. These percentages varied widely within the region in 2020 (AMR Surveillance in EU, 2022).

Other multi drug resistant bacteria include Carbapenem-Resistant *Enterobacteriaceae* (CRE) and Methicillin-Resistant *Staphylococcus aureus* (MRSA). *Enterobacteriaceae* have acquired resistance to carbapenem antibiotics, which are often considered the last resort treatment for serious infections. CRE infections can be challenging to treat, particularly in healthcare settings, and can lead to high mortality rates (Garcia and Garcia, 2019). MRSA is a type of *Staphylococcus aureus* bacteria that has

developed resistance to multiple antibiotics, including methicillin and other β-lactam antibiotics. MRSA infections can range from mild skin and soft tissue infections to severe bloodstream infections, pneumonia, sepsis and death (Kourtis *et al*., 2019).

1.3. BETA-LACTAMASES AND CARBAPENEMASES

1.3.1. Extended Spectrum Beta-lactamases

ESBLs are enzymes that hydrolyse the majority of penicillin and cephalosporins, such as oxyimino-β-lactam compounds (cefuroxime, third- and fourth generation cephalosporins and aztreonam) but neither cephamycin nor carbapenems as stated by European Committee on antimicrobial susceptibility testing (EUCAST).

There are currently two major classification schemes for β-lactamases: the **Ambler molecular classification**, which divides β-lactamases into distinct molecular classes (A, B, C, and D) based on amino acid sequence, and the **Bush-Jacoby-Medeiros functional classification** scheme, which categorises β-lactamases based on similarities in substrate hydrolysis profiles and responses to various inhibitors (Bush and Bradford., 2019). Most ESBLs belong to the Ambler class A of β-lactamases and are inhibited by β-lactamase inhibitors (clavulanic acid, sulbactam and tazobactam) and by diazabicyclooctane (avibactam)(EUCAST).

1.3.1.1. Distribution and health-related importance:

Since 1983, when the first ESBL-producing strains were discovered, they have been found all over the world and currently, about 2,770 distinct naturally occurring β-lactamases have been identified (Bush and Bradford., 2019). This dispersion is the consequence of the horizontal transmission of ESBL genes on plasmids, the clonal growth of producer organisms, and, less frequently, their development from the start. CTX-M enzymes, which have been around since the early 2000s, are by far the most clinically significant types of ESBLs, followed by SHV and TEM-derived ESBLs.

ESBL generation has mostly been seen in *Enterobacteriaceae*, initially in hospital settings, then in nursing homes, and from 2000, in the general population (outpatients, healthy carriers, ill and healthy animals, food items). *E. coli* and *K. pneumoniae* are the ESBL-producing species that are most commonly found. All other clinically significant *Enterobacteriaceae species*, however, also frequently generate ESBLs. Large differences have been documented in several studies on the incidence of ESBL-positive isolates, which relies on a variety of variables including species, geographic location, hospital/ward, group of patients, and kind of infection. According to EARS-Net data from 2015, more than 25% or perhaps 50% of European nations had invasive *K. pneumoniae* isolates that were resistant to third generation cephalosporins. Based on regional ESBL test findings, the majority of these isolates were assumed to be ESBL producers, with the exception of Greece and Italy, which had a significant number of KPC-type carbapenemase-producing isolates.

Penicillins, cephalosporins, carbapenems, and monobactams are the four main groups of β-lactams. Each of these substances has a four-membered azetidinone ring in its structure. Even though the first three have undergone several revisions and been used in clinical settings, the FDA has only given its approval to one monobactam, aztreonam (Bush and Bradford., 2019).

1.3.2. Carbapenemases

According to European Committee on antimicrobial susceptibility testing (EUCAST), Carbapenemases are β-lactamases that hydrolyse numerous antibiotics, including penicillin, most cephalosporins, carbapenems, and monobactams (monobactams are not hydrolyzed by metallo-lactamases) (EUCAST., 2017).

1.3.2.1. Distribution and health-related importance:

It is believed that the problem of carbapenemases spreading throughout Europe began in the second half of the 1990s in numerous Mediterranean nations, where it was mostly seen in *Pseudomonas aeruginosa*. Greece suffered an outbreak of *Klebsiella pneumoniae* with Verona integron-encoded metallo-lactamase (VIM) in the early 2000s, followed by an outbreak of carbapenemase from *K. pneumoniae* (KPC). The OXA-48 carbapenemases are now the group of carbapenemases in Europe with the highest growth rate. Around 62 and 33% of invasive *K. pneumoniae* in Greece and Italy, respectively, are no longer sensitive to carbapenems. Compared to 6/38 in 2013, 13/38 countries reported an endemic condition or inter-regional spread of Carbapenemase-Producing *Enterobacteriaceae* (CPE) in 2015. Only three nations responded that they had not found a single instance of CPE. The New Delhi Metallo-lactamases (NDMs), which are extremely common in the Middle East and the Indian subcontinent and have frequently been imported into Europe, are another group of particularly troublesome carbapenemases. There are also instances of regional dissemination in some nations. The IMP-carbapenemases are also common in some parts of the world.

The Ambler class A carbapenemase KPC, the class B metallo--lactamases (MBLs) of the IMP-, NDM-, or VIM-type, and the class D OXA48-like oxacillinase are at present the most common carbapenemases among *Enterobacteriaceae* (Zurfluh *et al*., 2015). Since they are easily transmissible and may impart resistance to almost all β-lactams, carbapenemases are a cause of concern. Further, infections with Carbapenemase-Producing *Enterobacteriaceae* (CPE) are linked to high death rates, and carbapenemase-producing strains typically have mechanisms for resistance to a variety of antimicrobial drugs.

1.4. MECHANISMS OF ANTIBIOTIC RESISTANCE

Many bacterial pathogens like *Acinetobacter baumannii, Pseudomonas aeruginosa, Escherichia coli, Stenotrophomonas maltophilia, Citrobacter freundii, Pseudomonas oryzihabitans, Enterobacter cloacae, Pasteurella pneumotropica,* etc are believed to have been associated with epidemics and nosocomial (hospital linked) infections with a high mortality rate (Davies and Davies., 2010).“Survival of the fittest” is a consequence of an immense genetic plasticity of bacterial pathogens that trigger specific responses that result in mutational adaptations, acquisition of genetic material or alteration of gene expression producing resistance to virtually all antibiotics currently available in clinical practice (Munita and Arias., 2016).

Several mechanisms have been characterised through which bacteria become resistant to antibiotics. The following are two major genetic strategies by which bacteria acquire resistance against antibiotics.

(a) Mutations in gene(s), often associated with the mechanism of action of the compound.

(b) Acquisition of foreign DNA coding for resistance determinants through horizontal gene transfer (HGT).

1.4.1. Mutational resistance:

Generally, mutations resulting in antimicrobial resistance alter the antibiotic action via one of the following mechanisms, (i) the production of enzymes that digest/metabolise the antibiotic. (ii) efflux pumps that eliminate the drug from the cell. (iii) modifications to the cellular target of the antibiotic that prevent binding. (iv) activation of an alternate pathway that bypasses drug action. (v) particularly for Gram-negative bacteria, down-regulation or elimination of transmembrane porins through which drugs enter the cell (Vila and Cespedes., 2007).

1.4.1.1. Production of enzymes that digest/metabolise the antibiotic (Antibiotic inactivation):

There are three main enzymes that inactivate antibiotics such as β-lactamases, aminoglycoside-modifying enzymes, and chloramphenicol acetyltransferases (AACs).

β-lactamases: β-lactamases hydrolyse β-lactams that have ester and amide bond, e.g., penicillin, cephalosporins, monobactams, and carbapenems. About 300 β-lactamases are known till date. B-lactamases can be further sub-divided into four groups (*Kapoor et al.,* 2017).

Class A β-lactamases: They are also known as penicillinase and are susceptible to clavulanic acid. Two commonly found β-lactamases are (designated as) TEM-1, and SHV-1 that are found in members of *Enterobacteriaceae*. Penicillinases are characterised by little or no activity against cephalosporins (*Kapoor et al.,* 2017). Extended-Spectrum β-Lactamases originated from this group/class but due to change in substrate profile (amino acid substitution), they hydrolyse most cephalosporins. Nevertheless, ESBL are resistant to penicillin, third-generation cephalosporins (e.g., ceftazidime, cefotaxime, ceftriaxone), aztreonam, cefamandole, cefoperazone, but are sensitive to methoxy-cephalosporins, e.g., cephamycin and carbapenems and are inhibited by inhibitors of β-lactamases, e.g., clavulanic acid, sulbactam, or tazobactam (Jacoby and Muñoz., 2005).
Class B β-lactamases: These are metallo β-lactamases and require enzymes such as zinc or heavy metals for catalysis and their activity is inhibited by chelating agents. These classes of enzymes are resistant to inactivation by clavulanate, sulbactam, aztreonam, and carbapenems. E.g., New Delhi metallo-β-lactamase (Rasmussen and Bush., 1997).
Class C β-lactamases: They are also known as cephalosporinase and are produced by Gram-negative bacteria apart from *Salmonella* and *Klebsiella*. Class C hydrolyses cephalosporins including extended-spectrum cephalosporins, in comparison to class A β-lactamases, these have large cavities, and as a result, they can bind the bulky extended-spectrum penicillin. An example of this type is AmpC β-lactamases. This class of enzymes is resistant to all β-lactams except carbapenems. They are not inhibited by clavulanate [Crichlow *et al.,* 1999; Trepanier *et al.,*1999].
Class D β-lactamases: These are oxacillin hydrolysing enzymes. They are found commonly in *Enterobacteriaceae* and in *P. aeruginosa*. Oxacillin-hydrolysing enzymes confer resistance to penicillin,

cloxacillin, oxacillin, and methicillin. They are weakly inhibited by clavulanic acid but are inhibited by sodium chloride (Naas and Nordmann., 1999).

Aminoglycoside modifying enzymes (AGE's): AG are neutralised by specific enzymes: Phosphoryl-transferases, nucleotidyl-transferases or adenylyl-transferases, and AACs. These aminoglycoside-modifying enzymes (AMEs) reduce affinity of a modified molecule, impede binding to the 30S ribosomal subunit and provide extended-spectrum resistance to AG's and FQ. AMEs are identified in *S. aureus*, *E. faecalis*, and *S. pneumoniae* strains.

Chloramphenicol-acetyl-transferases: Few Gram-positive and Gram-negative bacteria and some of *Haemophilus influenzae* strains are resistant to chloramphenicol, and they have an enzyme chloramphenicol transacetylase that acetylates hydroxyl groups of chloramphenicol. Modified chloramphenicol is unable to bind to a ribosomal 50S subunit properly (Kapoor *et al*., 2017).

1.4.1.2. Efflux pump mediated resistance:

Efflux transporters are expressed in all living cells, protecting them from the toxic effects of organic chemicals. Over-expression of these efflux transporters on bacterial cells have always been associated with multi-drug resistance. In Gram-negative bacteria, the outer membrane limits the rate of antimicrobials entering the cell and the multidrug efflux pumps actively export multiple, structurally distinct classes of antimicrobials out of the bacteria. The antimicrobials expelled out of the cell must cross the low permeability outer membrane in order to enter again; therefore, the efflux pumps work synergistically with the low permeability of the outer membrane. An increased efflux of antibiotic from the bacterium produces a reduction in drug accumulation and an increment in the MIC. The most common antimicrobials expelled by the efflux pumps are macrolides, tetracyclines and quinolones (Poole, 2002).

1.4.1.3. Target site alteration genes:

Some bacteria acquire resistance by acquiring mutations in the genes that encode the target sites for antibiotics. These mutations can alter the structure or function of the target, making it less susceptible to the antibiotic's action. For example, mutations in genes encoding bacterial ribosomes can reduce the binding affinity of antibiotics like tetracycline or erythromycin.

1.4.1.4. Outer Membrane Proteins (OMPs):

Porins are proteins able to form channels allowing the transport of molecules across lipid bilayer membranes that show little permeability for hydrophilic solutes. They provide membranes with multiple functions. Porins can act as potential targets for adhesion to other cells and binding of bactericidal compounds to the surface of Gram-negative bacteria. Variations in their structure as a mechanism to escape from antibacterial pressure or regulation of porin expression in response to the presence of antibiotics are survival strategies that have been developed by many bacteria. Porins may play a significant role in mechanisms of resistance (Vila and cespedes., 2007).

1.4.2. Horizontal gene transfer:

Horizontal gene transfer (HGT) in bacteria refers to the process by which bacteria can transfer genetic material horizontally from one organism to another, rather than through vertical transmission from

parent to offspring. It allows bacteria to acquire new genetic traits, such as antibiotic resistance genes or virulence factors, from other bacteria in their environment. Most antimicrobial agents used in clinical practice are (or derived from) products naturally found in the environment (mostly soil).

Classically, bacteria acquire external genetic material through three main strategies:

1.4.2.1. Transformation (Incorporation of naked DNA):

In transformation, bacteria can take up free DNA fragments from the environment and incorporate them into their own genome. These DNA fragments can come from lysed bacterial cells or released as extracellular DNA by other organisms. If the acquired DNA contains functional genes, they can be expressed by the recipient bacteria and confer new traits. This is probably the simplest type of HGT, but only a handful of clinically relevant bacterial species are able to "naturally incorporate "naked DNA to develop resistance (Chen and Dubnau, 2004).

1.4.2.2. Conjugation (bacterial sex):

Conjugation involves the transfer of genetic material between bacteria through direct cell-to-cell contact. It requires the presence of a plasmid (a circular DNA molecule that can replicate independently from a donor bacterium, which is transferred to a recipient bacterium). The plasmid can carry genes for antibiotic resistance or other advantageous traits. Emergence of resistance in the hospital environment often involves conjugation and is likely to occur at high rates in the gastrointestinal tract of humans under antibiotic treatment. Conjugation can happen either directly through direct transfer from a chromosome to chromosome or through specialised mobile genetic elements (MGEs) as vehicles to share valuable genetic information. The most important MGEs are integrons, bacteriophages, plasmids and transposons, all of which play a crucial role in the development and dissemination of antimicrobial resistance among clinically relevant organisms (Munita and Arias., 2016).

Integrons: Integrons are ancient structures that mediated the evolution of bacteria by acquiring, storing, disposing, and resorting to the reading frameworks in gene cassettes. The term integron describes a large family of genetic elements, all of which are able to capture gene cassettes. Integrons play an important role in the distribution of antibiotic resistance, especially in Gram-negative pathogens. Integrons are site specific recombination systems capable of recruiting open reading frames in the form of mobile gene cassettes. It provides an efficient and rather simple mechanism for the addition of new genes into bacterial chromosomes, along with the necessary machinery to ensure their expression. Integrons are genetic systems that allow bacteria to capture and express gene cassettes and can be found as part of plasmids, chromosomes, and transposons. Integrons are formed by an *intI*gene, encoding an integrase that is a site-specific recombinase, an attachment site *attI,* and one of the two strong promoters (P) that drive the expression of inserted gene cassettes. Gene cassettes can be inserted one after the other into the integron insertion site, producing the formation of long arrangements of ARGs that can be transferred simultaneously among bacterial populations. This mobile genetic element can be usually found in clinical bacterial strains, possibly because most of the cassettes identified are associated with antibiotic resistance.

1.4.2.3. Transduction (phage mediated):

Transduction occurs when genetic material is transferred between bacteria by bacteriophages, which are viruses that infect bacteria. During the infection process, the bacteriophage can accidentally package bacterial DNA instead of its own genetic material. When this bacteriophage infects another bacterium, it injects the packaged bacterial DNA, which can then be integrated into the recipient´s genome. Transduction can lead to the transfer of both specific genes and random fragments of bacterial DNA.

Horizontal gene transfer does, in fact, play a significant role in bacterial evolution and adaptation by enabling bacteria to quickly pick up novel features that improve their survival and competitiveness in shifting settings. But it can also help transmit genes that make bacteria resistant to antibiotics, making it harder to treat bacterial infections. In order to combat antibiotic resistance and stop the spread of undesirable features among bacteria, it is crucial to comprehend the processes and dynamics of horizontal gene transfer [Munita and Arias., 2016; Sabbagh *et al.,* 2021].

1.5. CLASSES AND MODES OF ACTIONS OF ANTIBIOTICS

Antibiotic discovery, modes of action, and mechanisms of resistance have been productive research topics. An antibiotic is a natural, synthetic or semi-synthetic drug that at low concentrations exerts an action towards sensitive micro-organisms (selective toxicity) (Morales *et al*., 2022). Due to its complex chemical nature, biosynthetic pathways, evolution and biochemical mode of action, it has always been a challenge for pharmaceutical industries [Oesterhelt and Brunner., 2008; Sengupta *et al.,* 2013].

The pathogens which were once susceptible to antimicrobial drugs are now resistant causing the infections to grow instead of treating them. For example, common community acquired conditions, such as pneumonia was once treatable with penicillin but not anymore in many cases since the micro-organism has become resistant to it. Similarly, other conditions, such as cystitis, common neonatal infections, patients receiving cancer treatments, organ transplant etc are highly vulnerable to infections and treating them is becoming extremely difficult or even impossible. Moreover, the last completely new class of drug was developed two decades ago, during 1980, so it's extremely important to preserve the efficacy of already existing drugs by supporting alternative therapies like vaccines (WHO 2014).

Antibiotics can disrupt essential processes or structures in the bacterial cell. This either kills the bacterium or slows down bacterial growth. Depending on these effects an antibiotic is said to be bactericidal or bacteriostatic. A **bactericidal antibiotic** kills the bacteria while **the bacteriostatic antibiotics** stop bacterial growth without killing them. The human immune system is then needed to clear the infection.

There are several classes of antibiotics with different mechanisms of action and bacterial targets. They either attack the cell wall or membrane of the bacterial cell, disrupt the protein synthesis and nucleic acid synthesis. These targets are absent or structurally different in human and mammalian cells, which prevents antibiotics from harming human cells. However, in some cases they could have unpleasant side effects.

1.5.1. Antibiotics targeting cell wall:

Bacterial cells are surrounded by a cell wall made of peptidoglycan, which consists of long sugar polymers. The peptidoglycan undergoes cross-linking of the glycan strands by the action of trans-glycosidases, and the peptide chains extend from the sugars in the polymers and form cross links, one peptide to another. The D-alanyl-alanine portion of the peptide chain is cross linked by glycine residues in the presence of penicillin binding proteins (PBPs). This cross-linking strengthens the cell wall. **β-lactams** and the **glycopeptides** inhibit cell wall synthesis.

β-lactam antibiotics: The aim of antibiotics in this group is to target PBPs. They are characterised by a β-lactam ring which mimics the D-alanyl D-alanine portion of peptide chain that is normally bound by PBP. The PBP interacts with β-lactam rings and are not available for the synthesis of new peptidoglycan. The disruption of the peptidoglycan layer leads to the lysis of the bacterial cell (Džidić *et al*., 2008). Antibiotics, such as amoxicillin, ampicillin, cefotaxime, ceftazidime, ceftriaxone, carbapenems imipenem and meropenem are included in this group.

Glycopeptides: The glycopeptides bind to the D-alanyl D-alanine portion of the peptide side chain of the precursor peptidoglycan subunit. The large drug molecule vancomycin prevents binding of this D-alanyl subunit with the PBP, and hence inhibits cell wall synthesis.

1.5.2. Inhibitors of protein biosynthesis:

The bacterial DNA or genetic information is transcribed into an RNA molecule, known as messenger RNA. This messenger RNA is then translated into a specific protein by a macromolecular structure, called ribosomes. The bacterial 70S ribosome is composed of two ribonucleoprotein subunits, the 30S and 50S subunits. (Yoneyama and Katsumata; 2014). Antibiotics can be classified into different classes depending on which subunit is attacked by the antimicrobial (antibiotic).

1.5.2.1. Inhibitors of 30S subunit

Aminoglycosides: The positively charged aminoglycosides interact with the negatively charged outer membrane (OM) resulting in the formation of large pores, which allows the penetration of antimicrobials into the bacterial cell. In order to get to the ribosome, the antimicrobial have to pass through cytoplasm. This needs energy and oxygen. That is why this antimicrobial only works effectively in aerobic conditions and has poor activity against anaerobic bacteria. The efficiency is much higher when combined with antibiotics which inhibit cell wall synthesis, such as, β-lactams and glycopeptides as it allows greater penetration AG within the cell and low dosage. AGs interact with the 16S r-RNA of the 30S subunit near the A site through hydrogen bonds. They cause misreading and premature termination of translation of mRNA. (Kapoor *et al*., 2017)

Tetracyclines: Tetracyclines, such as tetracycline, chlortetracycline, doxycycline, or minocycline prevents binding of the tRNA to the A-site by binding to the conserved regions of the 16S rRNA (Yoneyama and Katsumata, 2006).

1.5.2.2. Inhibitors of 50S subunit

It can be divided further into chloramphenicol, macrolides and oxazolidinones which bind to the 23S rRNA of the 50S subunit causing impairment of protein synthesis.

1.5.3. Inhibitors of DNA replication:

Quinolones: The enzyme, bacterial DNA gyrase is responsible for cutting DNA and introducing negative supercoils. Hence preventing the excessive positive supercoiling of the strands when they separate to permit replication or transcription. The fluoroquinolones (FQ) inhibit this enzyme and the subsequent DNA replication.

This gyrase enzyme has two subunits, 2A subunits and 2B subunits. A-subunit carries out the nicking of DNA, B-subunit introduces negative supercoils, and then A-subunit reseals the strands. The FQ's bind to A-subunit with high affinity and interfere with its strand cutting and resealing function (Kapoor et al., 2017). Examples of quinolones are levofloxacin, ciprofloxacin and nalidixic acid.

1.6. ANTIBIOTIC RESISTANCE GENES AS EMERGING ENVIRONMENTAL CONTAMINANTS

Antibacterial resistance genes are considered as environmental pollutants because they persist in the environment or bacterial population even after the disappearance of the selection pressure [Martínez *et al*., 2003; Amato *et al*., 2021]. This resistance has a significant impact on the environment as not only the bacteria itself is resistant to antibiotics but also propagates the genes of resistance to the environment (Pruden *et al*., 2006). Because antibacterial resistance genes continue to exist in the environment or in bacterial populations long after the selection pressure has subsided, they are regarded as environmental pollutants [Martinez *et al*., 2003; Amato *et al*., 2021]. Due to the propagation of resistance genes as well as the existence of antibiotic-resistant bacteria, antibiotic contamination is important (Pruden *et al*., 2006). Even in unpolluted and/or remote places, microbial communities in natural habitats harbour ARB and ARGs [Allen *et al*., 2010; Bhullar *et al*., 2012]. The ARB and ARGs move across ecosystems in such a way that their presence in one environment has an impact on the other. This illustrates how ubiquitous antibiotic resistance is since Antibiotic Resistance Genes (ARGs) may be identified in many habitats outside of clinical settings. Overprescribing or other improper use/disposal of antibiotics in humans is generally considered to contribute to the problem. Avoparcin, an antibiotic growth-promoter used in poultry, was recently banned in Europe because of its association with the development of *vancomycin-resistant enterococci*. ARGs can be considered to be emerging "contaminants" for which mitigation strategies and understanding the dynamics of ARGs in different ecosystems are needed to prevent their widespread dissemination (Pruden *et al*., 2006).

1.6.1. Soil

Although antibiotics have improved human health, they have also created uncertainty in the ecological environment, particularly in the microbial community ecology (Chen *et al*., 2013). Most antibiotics administered in people or poultry are not completely absorbed. They are discharged into the soil environment with domestic wastewater discharge and animal manure, where they create an antibiotic-resistant environment and promote the occurrence and growth of resistant bacteria in the soil microbial community (Ding *et al*., 2019). Due to exposure to various microbial populations and frequent use of antibiotics in agriculture, soil serves as a substantial reservoir for ARGs. ARGs in soil

can come from environmental sources, animal dung, irrigation with wastewater, and agricultural activities. The abundance of ARB in soil microbiomes is increased by selective pressure on these environments caused by the inclusion of antibiotics from various agricultural techniques [Heuer *et al*., 2011; Chen *et al*., 2016; Hu *et al*., 2016]. Shi *et al*., (2020) have found 33 ARGs in all, further classified in 8 distinct main groups, including bla_{TEM}.

Extensive study has revealed that ARGs may be passed down the food chain by eating agricultural products containing resistant bacteria. The widespread presence of ARGs in the bacterial community can increase the variety of ARB and endanger human health with dangerous bacteria. The differences in ARGs in soil environments might be attributed to differences in antibiotic use, microbial community makeup, and soil characteristics. A teaspoon of healthy soil contains more living creatures than there are people on the planet. Protecting natural resources and biodiversity is vital for both human health and the health of our planet (FAO, 2023). Thus, understanding the origins and evolution of ARGs in various contexts is critical for developing effective measures to limit their spread.

1.6.2. Vegetables

Bacteria-contaminated food serves as a vehicle for transmitting ARGs to humans, potentially impacting human health. The presence of ARGs in the food chain can result from various factors, including antibiotic use in animal husbandry, agricultural practices, and cross contamination during food processing.

The presence of *A. baumannii* in food is seen as a severe issue, as contamination of the food chain with this bacterium may allow it to make its way into healthcare settings and therefore increasing the burden of nosocomial infections caused by this pathogen (Ababneh *et al*., 2022). Consumption of raw or fresh, low-processed vegetables can contribute to the spread of this bacteria in both community and hospital settings. *A. baumannii* can enter vegetables and fruits when they are growing in the soil, during harvesting, through organic fertilisers, from polluted irrigation water, and during transportation and handling (Machado and Moreira., 2019). Furthermore, vegetables and fruits have a high-water activity, which aids in the growth of pathogens such as *Acinetobacter species*. Lettuce samples contaminated with *Acinetobacter baumannii* have already been reported by [Karumathil *et al*., 2016; Carvalheira *et al*., 2017].

The detection of Carbapenemase-Producing *Enterobacteriaceae* (CPE) in the fresh produce has been reported to be the consequence of international trade. A study conducted by (Zurfluh *et al*., 2015) involving different nations, demonstrated that international production and trade of fresh vegetables constitute a possible route for the spread of carbapenemase-producing *Enterobacteriaceae* (*Klebsiella variicola* strain (KS22), harbouring the $bla_{OXA-181}$ carbapenemase gene (100 % identity after sequencing). Similarly, another study by (Zurfluh *et al*., 2021) showed that international trade is responsible for the spread of extended-spectrum β-lactamase producing *Enterobacteriaceae*, most of which were multidrug resistant.

1.6.3. Water

As a result of increased human population and their activities, lakes, rivers, and aquifers are running dry or are too contaminated to be used as a source of water (WWF, 2017). Gilbert (2015) argues that

the pollution brought on by agriculture and urbanisation is what has the greatest impact on water quality and has led to a significant loss of biodiversity in the majority of Europe's aquatic ecosystems.

Sewage discharges are the primary source of pollution. Sewage discharges may also contain biological pollutants from human waste and manure in addition to chemical pollutants from heavy metals, pesticides, and fertilisers (from agricultural activity) (Gilbert, 2015). These substances may enter the water cycle and travel up the food chain to reach us, endangering our environment and health.

Pharmaceuticals are a different source of pollution that is only now becoming detectable. Because of how long their chemical structure is active, they have long-lasting pollution-causing impacts. A portion of the molecule, either in its original form or as a metabolite, is expelled in the urine and faeces after it has been metabolised. They then enter wastewater and go there, where they should be destroyed once they reach wastewater treatment facilities (Iglesias *et al*., 2013). The issue is that sometimes it is impossible to get rid of them altogether. Such a large number of these substances make it past the purification filters and into the freshwater that will be used for irrigation or human consumption. Regarding veterinary medications, they are immediately disposed of in the environment, mostly through the usage of fertilisers made from animal faeces.

Beta-blockers, antidepressants, oestrogens, personal care items, and antibiotics are some of the medications that are most often used and, consequently, are also most frequently discovered in the environment. Between ng/L and g/L quantities of these compounds have been reported in water (Ternes *et al*., 2004).

It could also be related to the widespread use of prescription antibiotics in both humans and animals, as well as wastewater treatment facilities (WWTPs). Up to 95% of antibiotics can be excreted unmodified in both people and animals, which may eventually have an influence on human health. On the other hand, because wastewater treatment plants (WWTPs) are improperly made to remove micro-pollutants (pharmaceutical compounds), they are reintroduced into the environment where they can cause microorganisms to become more susceptible to selection pressure (Becerra-Castro *et al*., 2015).

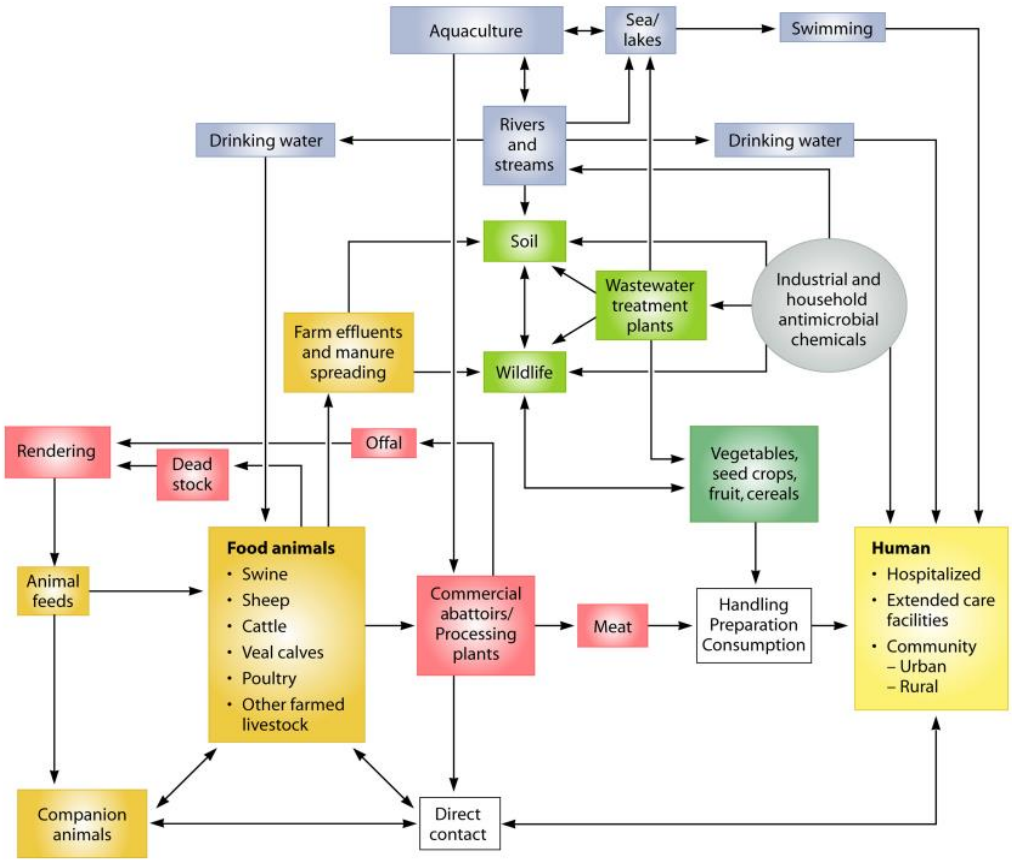

Figure 1.1. Dissemination of antibiotics and antibiotic resistance within agriculture, community, hospital, wastewater treatment and associated environment (Davies and Davies., 2010)

1.7. GENES OF RESISTANCE TO ANTIBIOTICS

According to [Li *et al*., 2007; Marti and Balcazar*.,* 2013], there are more than a hundred β-lactam resistance genes (*bla*) encode for about 400 different β-lactamases and are frequently present in sewage from sewage treatment plants and animal waste. Nevertheless, the number of β-lactamases stated by (Bush and Bradford *et al.,* 2019) is quite high. According to their studies, about 2,770 distinct naturally occurring β-lactamases have been identified till this moment. The genes for β-lactamase enzymes are probably the most international in distribution. Random mutations of the genes encoding the enzymes have given rise to modified catalysts with increasingly extended spectra of resistance (Davies and Davies., 2010). The first natural ESBLs were identified in the mid-1980s, but soon they became widely disseminated in Gram-negative populations worldwide. The major factor responsible for this accumulation is the strong selective pressure of oxyimino-β-lactam use (Gniadgowsky *et al*., 2007) Among the many substitutions observed in *TEM* and *SHV* ESBLs, several are more or less directly associated with ESBL activity. These include mutations at positions Glu104 (TEM), Ala146 (SHV), Gly156 (SHV), Arg164 (TEM), Leu169 (SHV), Asp179 (SHV and TEM), Arg205 (SHV), Ala237 (TEM), Gly238 (TEM and SHV), and Glu240 (TEM and SHV) (Gniadgowsky *et al*., 2007).

The most prevalent genes conferring resistance to quinolones are *qnrA, qnrB* and *qnrS* (Strahilevitz *et al*., 2009). A recently proposed consensus for qnr nomenclature defined qnr as a naturally occurring allele encoding a pentapeptide repeat protein that confers reduced susceptibility to nalidixic acid or a

fluoroquinolone. qnr families (such as *qnrA, qnrB*, or *qnrC*) are defined by a 30% or more difference in nucleotides or derived amino acids. Within each family, qnr alleles differ in one or more amino acids. qnr genes found on a bacterial chromosome are named after the host organism or assigned to a family if the gene is at least 70% identical to an established qnr family (e.g., SaqnrA3 from the chromosome of *Shewanella algae*). Resistance to fluoroquinolones is usually chromosomally mediated so the spread of resistant bacteria contributes to the high numbers of resistant strains reported by some institutions (Piddock, 1998). Fluoroquinolone-resistant *E. coli* could be found even in healthy broiler chickens which further aggravates the situation (Mahmud *et al*., 2018).

***bla*$_{TEM}$:** *bla*$_{TEM}$ refers to genes encoding TEM-type β-lactamases. TEM β-lactamases are class A enzymes that can hydrolyse penicillin antibiotics and some extended- spectrum cephalosporins. TEM-type β-lactamases are widespread in both Gram-negative and Gram-positive bacteria.
***bla*$_{SHV}$:** *bla*$_{SHV}$ denotes genes encoding SHV type β-lactamases. SHV β-lactamases are also class A enzymes that can confer resistance to β-lactam antibiotics, including extended-spectrum cephalosporins. They are commonly found in *Enterobacteriaceae* and other Gram-negative bacteria.
***bla*$_{CMY-2}$:** *bla*$_{CMY-2}$ refers to a type of β-lactamase gene that encodes the CMY-2 β-lactamase enzyme. CMY-2 is a class C β-lactamase commonly found in *Enterobacteriaceae,* and it confers resistance to cephalosporins, including extended-spectrum cephalosporins.
***bla*$_{OXA}$:** The OXA genes encode class D β-lactamase enzymes, which can hydrolyse β-lactam antibiotics. They are commonly found in Gram-negative bacteria and contribute to resistance against various β-lactam antibiotics.
***bla*$_{IMP}$:** IMP (imipenemase) refers to a class B β-lactamase gene that can confer resistance to carbapenem antibiotics, including imipenem. IMP-type β-lactamases are often found in metallo-β-lactamase producing bacteria which are particularly concerning due to their ability to hydrolyse carbapenems, a critically important class of antibiotics.
***bla*$_{KPC}$:** KPC (*Klebsiella pneumoniae* carbapenemase) is a class A β-lactamase gene that is commonly associated with carbapenem resistance in Gram-negative bacteria, particularly *Klebsiella pneumoniae*. KPC enzymes can hydrolyse carbapenem antibiotics and are often carried on mobile genetic elements such as plasmids.
***bla*$_{VIM}$:** VIM (Verona integron-encoded metallo-β-lactamase) is another type of class B β-lactamase gene that confers resistance to carbapenems. VIM-type enzymes are often found in various Gram-negative bacteria, including *Pseudomonas aeruginosa.*
***qnrA, qnrB, qnrS*:** These genes encode the qnr proteins, which confer resistance to quinolone antibiotics. Quinolones are a class of antibiotics commonly used to treat bacterial infections. The qnr proteins protect bacterial DNA gyrase and topoisomerase IV, the targets of quinolones, thereby reducing the effectiveness of these antibiotics.

1.8. ORGANIC FARMING

Organic farming, ecological farming or biological farming is a sustainable agricultural system that uses ecologically based pest controls and biological fertilisers derived largely from animal and plant wastes and nitrogen fixing cover crops. It is a kind of farming that puts a strong emphasis on using sustainable, all-natural methods to raise animals and crops. It strives to promote sustainable and regenerative agricultural practices while minimising the use of synthetic inputs such as pesticides, fertilisers, growth hormones, and genetically modified organisms (GMOs). This cutting-edge farming method was

created in reaction to the damage that traditional agriculture's use of chemical pesticides and synthetic fertilisers had on the environment (Dubey, 2023).

The EU Commission defines organic farming as a mode of production that attempts to produce food using natural ingredients and procedures. Spain, the fourth-largest country in the world, dedicates the greatest land area in the European Union to organic farming. With a rise in certified organic surface area of 81.2% between 2016 and 2020, and a current total of 146,767 ha, the Valencian Community (CV) is currently Spain's fourth-largest producer in the organic sector (Figure X) and exports 63% of its production despite a 7% rise in domestic consumption of organic goods in the CV between 2019 and 2020 (CAECV, 2020).

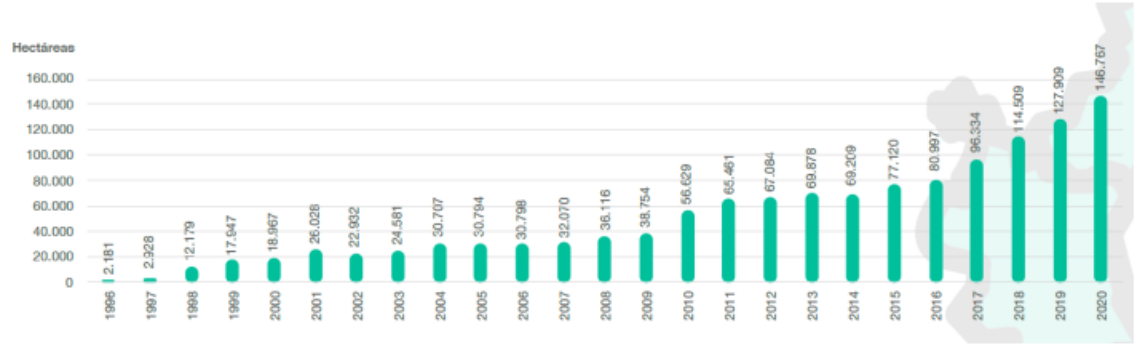

Figure 1.2. Evolution of the certified organic surface area in the Valencian community over the years (CAECV,2020)

The number of eco enterprises increased from 701 in 2019 to 748 in 2020, making the eco sector one of the industries that is experiencing rapid growth. In addition to its quick growth, it is also bringing about generational shifts and breaking stereotypes by giving women the chance to enter the workforce. Having said that, a study released by CAECV shows that there are presently 31% females and 69% males, a modest rise and drop in the percentages of females in 2019 (24.5%) and males (75.5%), respectively. In Valencia province, vegetables, fruit trees, citrus fruits, cereals, olive groves for oil, dried fruits, and vineyards are the main food products cultivated through ecological farming.

Overall, the CAECV 2020 research demonstrates a growing eco market and increased popularity of eco-friendly food (CAECV, 2020).

1.8.1. Distinguishing features of organic food

A study in Arizona in 2013 found out that there were no significant differences between organic and inorganic grown foods with the exceptions that Organic food had higher phosphorus and omega 3 fatty acid levels. Based on their investigations, organic food has 30% less pesticide contamination, however, they are not 100% pesticide free. Experts believe that whether we choose organic or non-organic foods, the best method to protect ourselves is to properly rinse all fruits and vegetables under running water (Misner and Florian., 2013).

While it is true that no significant differences between organic and inorganic food has been reported, Anna Dubey, in one of her articles “sustainable agriculture” published in 2023 states that organic farming presents some key advantages over conventional farming. Organic agriculture is productive and sustainable (Reganold *et al.*, 1993). Healthy soil ecosystems are the basic goal of organic farming.

It places a focus on techniques that improve soil fertility, structure, and biodiversity, such as composting, crop rotation, cover crops, and the use of natural soil additives. Crops that are nutrient-rich can flourish in healthy soil and this way reduces the need for artificial fertilisers. Preventive and multifaceted strategies are employed in organic farming to control diseases and pests. Pest and disease management techniques include crop rotation, use of natural predators, beneficial insects, trap crops, and physical barriers. As a last option, approved organic pest control treatments made from natural sources are employed. Organic farming uses a variety of methods, such as, Mulching, manual weeding, crop rotation, cover crops, and the use of power instruments like cultivators or flame weeders to manage weeds without significantly depending on artificial pesticides (RULNR, 2010).

Moreover, organic farming techniques aim to preserve and improve farmland biodiversity by protecting natural habitats, establishing buffer zones, providing places for beneficial insects and birds to breed, and refrain from using chemicals that might harm the species. In organic livestock farming, the welfare of animals is prioritised, and the animal must be provided with Pasteur and outdoor spaces, organic feed and forage must be encouraged, and the use of antibiotics and growth hormones must be limited. Livestock contributes directly to agriculture by producing manure and influencing the availability of organic carbon to soil. It contributes indirectly through its influence on income of the households. Integration of livestock and crop production, or mixed farming, allows the use of animal manure to increase soil fertility. Farmers recognize the benefits of using manure, and with the relatively high costs of mineral fertilisers, manuring could play a greater role in maintaining soil fertility (Powell and Williams, 1995). Better integrated crop and animal systems, crop residue recycling, and judicious use of other available nutrients can all increase agricultural output (Hilhorst and Muchena, 2000).

According to Swaminathan (1990), a farming system that attempts to maximise the revenue and employment potential of the small farm by concurrently focusing on crop and animal husbandry and post-harvest technology should be promoted more broadly. No large agricultural research initiative should begin without a thorough grasp of the existing farming systems (Diao *et al*., 2014).

1.8.2. Organic food certification EU/US

To ensure that the laws and regulations regarding organic farming are appropriately implemented, the EU maintains a stringent control and compliance assurance system. Imported organic food is also subject to control procedures to confirm that it has been produced and transported in compliance with organic farming principles.

Organic food is guided by certification standards and regulations that vary between countries and regions. Products that have been certified organic are labelled accordingly, giving customers peace of mind that they were made in accordance with organic standards. The standards for organic farming in the EU offer a clear framework for the production of organic goods within the EU. Each EU nation names its "control bodies or control authorities" to be in charge of examining participants in the supply chain for organic foods. Producers, distributors, and merchants must register with their regional inspection authority in order to promote their goods as organic. They receive a certificate indicating that their products meet organic requirements after inspection and control. At least once a year, all operators must successfully complete a check to confirm that they continue to comply with the standards (Agriculture and rural development EU, 2023)

In the United States (US) they can be classified as 100% organic, Organic, and Made with organic ingredients in which there are no synthetic ingredients, at least 95% of ingredients are organically produced, and at least 70% of ingredients are organic and 30% are from USDA approved ingredients respectively. Natural or All natural apply to meat or poultry which may not contain any artificial flavouring, colours, chemical preservatives, or synthetic ingredients. (Misner and Florian, 2013).

Overall, the goal is to meet consumer demand for reliable organic products. However, it also presents challenges, such as lower yields in certain crops and increased labour requirements (Wuerthner, 2020).

1.9. ONE HEALTH

One Health is an approach that recognizes the interdependence of human health, animal health, and the health of the environment. It emphasises the collaboration and integration of various disciplines, such as medicine, veterinary science, environmental science, and public health to address health issues that arise at the interface of humans, animals, and the environment. One Health is an integrated, unifying approach that aims to sustainably balance and optimise the health of people, animals and ecosystems. It recognizes the health of humans, domestic and wild animals, plants and the wider environment (including ecosystems) are closely linked and interdependent (OHHLEP, 2021).

Other international organisations, such as the Food and Agriculture Organisation (FAO)also advocate for the promotion of One Health. FAO promotes a One Health approach as part of the transformation of the agrifood system for the health of people, animals, plants, and the environment. Sustainable agriculture, animal, plant, forest, and aquaculture health, food safety, antimicrobial resistance (AMR), food security, nutrition, and livelihoods are among the issues addressed. Ensuring a One Health approach is critical for advances in anticipating, preventing, detecting, and controlling infections that transfer between animals and people, combating AMR, ensuring food safety, preventing environmental-related human and animal health concerns, and addressing a variety of other difficulties.

Bacterial resistance and One Health are two interconnected concepts that highlight the complex relationship between human health, animal health, and the environment in the context of combating antimicrobial resistance (AMR). Antimicrobial resistance is a natural phenomenon. But the overuse and misuse of antibiotics in both human and veterinary medicine is primarily associated with the dissipation and spread of resistance, to the point of endangering its therapeutic effectiveness. These ARBs and ARGs enter the environment after being excreted from the human or animal body and contaminate the environment.

Antibiotic resistance genes (ARGs) and antibiotic resistance bacteria (ARBs) are released through excreta. The main receptor compartments are soil, wastewater, and surface water. From the primary compartments, these AMs, ARBs, and ARG are released and go to other environmental compartments, or secondary receptors, as a result of climatic action and interactions between ecosystem constituents. The secondary receptors thus activate the dispersion agents and the AM, ARB, and ARG are spread between the effluent water and the atmosphere. The WWTPs contain as much as 90% of the undegraded antibiotic (BIO Intelligence Service, 2013). Sewage sludge, slurry, and waste from animal farms are applied to soils, hence contaminating the soil. The water absorbed by the soil, synanthropic fauna, aerosols and atmospheric dust serves as dispersion vector, carrying contaminants

to neighbouring surface bodies and groundwater. Since both humans and animals utilise soil for agricultural purposes, exposure to soil includes exposure to ARBs and ARGs both occupationally and through consumption of meals from plant and animal-based ingredients. Additionally, exposure to resistant microorganisms can also occur using irrigation water (particularly water for human use, including drinking and recreational water). The natural microbiota of an ecosystem may change due to an increased proportion of ARGs and ARBs, and this might have further consequences on the environment that are not yet recognized. Investigations are active in this field and promising results are anticipated (PRAN-MA).

A One Health approach is also necessary for accomplishing the SDGs (Sustainable Development Goals).The Sustainable Development Goals (SDGs) are a set of 17 global goals established by the United Nations (UN) in 2015 as part of the 2030 Agenda for Sustainable Development. The SDGs provide a framework for countries and stakeholders to work towards a more sustainable and equitable future.

Ecological farming supports the theory of Sustainable development goals. Keeping the Antimicrobial resistance in perspective, the trio, i.e., SDGs, organic farming and antimicrobial resistance are inter-related.

For instance, Goal 2 “Zero hunger” is directly linked to ecological farming practices. Ecological farming promotes sustainable agriculture focussing on soil health, biodiversity and natural resource conservation to ensure food security and nutrition. Goal 12 “Responsible Consumption and Production” aligns with ecological farming by promoting sustainable and environmentally friendly agricultural practices, reducing the use of synthetic inputs, minimising wastes, and promoting sustainable food systems. Goal 15 “Life on land” and ecological farming is interconnected as it emphasises the protection, restoration and sustainable use of terrestrial ecosystems. Ecological farming practices contribute to preserving biodiversity, soil conservation, and the promotion of agroecosystem resilience. Goal 3 “Good health and well-being” Goal 6 “Clean water and sanitation”. Goal 11 “Sustainable cities and communities” these goals are related to ecological farming in a way that by adopting ecological farming practices, the risk of environmental contamination with antimicrobial residues decreases, reducing the potential for AMR development in environmental bacteria. Ecological farming also promotes the use of alternative disease prevention methods, such as probiotics, herbal remedies, and vaccinations, which can reduce the reliance on antimicrobials.

2. OBJECTIVES

Human, animal, and environmental health are interconnected. One health is a concept in which human health depends both on the animal health and the environment in which they live, since they are inter-connected, making a circle or chain in which one thing influences the other.

Considering the concept of One Health and inter-relationship between food chain and antimicrobial resistance, this work was carried out to determine the distribution and emergence of resistance in a closed cycle of organic production and establish resistance load relationships.

Having said that, the following specific objectives were established:

1. To Isolate *E. coli (Escherichia coli), Acinetobacter* and specimens from *Enterobacteriaceae* family suspected of being resistant to β-lactams, carbapenems and fluoroquinolones.
2. To study the sensitivity isolates from 4 families (β-lactams, quinolones, tetracyclines and aminoglycosides) and detect the presence of multi-resistance.
3. To detect β-lactam resistance genes in both samples and isolates (bla_{TEM}, bla_{SHV}, bla_{CMY-2}).
4. To detect carbapenem resistance genes in both samples and isolates (bla_{OXA}, bla_{IMP}, bla_{KPC}, bla_{VIM})
5. To detect fluoroquinolones resistance genes in both samples and isolate (*qnrA, qnrB, qnrS*).
6. To compare the biochemical and molecular microbial identification methods.
7. To analyse the results and mention which source contributes to most of the resistance and the relationships between the appearance of resistance and the sources.

3. MATERIALS AND METHODS

3.1. SAMPLES: ORIGIN AND PROCESSING

A total of 29 samples of organically grown vegetables from a private farmland in Horta Nord in the city of Valencia were analysed, including, 10 organically grown vegetable/fruit samples, 10 soil samples, and 9 water samples. The vegetable/fruit sample included 3 cabbages (*Brassica oleracea var. capitata*), 3 red cabbages (*Brassica oleracea var. capitata f.rubra*), 2 strawberries (*Fragaria spp*.), 1 oak lettuce (*Lactuca scariola var. sativa L*.), and 1 lettuce four season marvel (*Lactuca sativa*). Soil samples included 3 cabbage soil, 3 soil samples from red cabbages, 2 soil samples from strawberries, 1 soil sample from oak lettuce and 1 soil sample from lettuce four season marvel. All of the 9 water samples obtained were from irrigation water supplied to these crops. The samples were named as C1-C29 and are given in the table.

Table 3.1. List of the samples collected from a field camp including samples from water, organic vegetables/fruit (cabbage, red cabbage, oak lettuce, lettuce marvel, strawberries) and organic soil samples (cabbage soil, red cabbage soil, oak lettuce soil, lettuce marvel soil, strawberries soil).

CAMP	ORGANIC	VEG/SOIL/WATER
C1	No	Irrigation water
C2	No	Irrigation water
C3	Yes	Cabbage (col repollo)
C4	Yes	Red cabbage (Lombarda)
C5	Yes	Strawberries (fresas)
C6	Yes	Oak lettuce (lechuga roble)
C7	Yes	Cabbage soil (Tierra repollo)
C8	Yes	Red cabbage soil (Tierra lombarda)
C9	Yes	Strawberries Soil (tierra fresa)
C10	Yes	Oak lettuce soil (tierra roble)
C11	No	Irrigation Water
C12	No	Irrigation Water
C13	No	Irrigation Water
C14	No	Irrigation Water
C15	No	Irrigation Water
C16	No	Irrigation Water
C17	No	Irrigation Water
C18	Yes	Cabbage
C19	Yes	Lettuce Marvel
C20	Yes	Red cabbage
C21	Yes	Cabbage
C22	Yes	Red cabbage
C23	Yes	Strawberries
C24	Yes	Cabbage soil
C25	Yes	Marvel lettuce soil
C26	Yes	Red cabbage soil
C27	Yes	Cabbage soil
C28	Yes	Red cabbage soil
C29	Yes	Strawberries soil

The samples were taken from a private grower on a block of land in Horta Nord that is watered by the Moncada irrigation canal, which receives water from the Turia River. The samples were collected near

the end of the growing season, between late May and June, when the vegetables and strawberries were ready to eat. The vegetable sample was obtained on three distinct dates over three weeks, while soil and water samples from the main irrigation channel were gathered from the same place where the vegetables were cultivated. The samples were collected aseptically in sterile bags and containers and transported to the laboratory for processing.

In the laboratory, 10 grams of each vegetable and strawberry were weighed and inoculated into each of the below mentioned enrichment broths supplemented with cefotaxime 2.5 mg/L and by meropenem1 mg/L and vancomycin 5 mg/L and then inoculated with the same broth.

Buffered peptone water (Scharlau, Barcelona, Spain) supplemented with Cefotaxime 2.5 mg/L.

Tryptic Soy broth (Scharlau, Barcelona, Spain) supplemented with Meropenem 1 mg/L

In addition, 10 ml of water was inoculated into 90 ml of each of the supplemented broths and into the soil. They were incubated for 24h at 37°C and then seeded in Supercarba (Chromagar®, France) and McConkey (Scharlau, Barcelona, Spain) supplemented with CTX and left to incubate for 24h at 37°C. From the supplemented selective media, 5 colonies were selected from each culture medium suspected to be *Enterobacteriaceae* or *Acinetobacter* as indicated by each manufacturer. They were frozen at -20°C in glycerine broth until use in this TFM.

3.2. ANTIBIOTICS EMPLOYED AND CULTURE MEDIUM

3.2.1. Antibiotics employed.

For this project, the antibiotics employed were Ampicillin, Amoxicillin, Ceftazidime, Cefotaxime, Imipenem, Meropenem, Ciprofloxacin, Levofloxacin, Nalidixic acid, Chloramphenicol, Gentamycin, Tetracyclines, and Ceftriaxone, all supplied by Sigma Aldrich.

Table 3.2. Antibiotics used, and concentrations added to the medium.

Antibiotic	Abbreviation	Concentration (µg/mL)
Ampicillin	AMP	30
Amoxicillin	AMC	30
Ceftazidime	CAZ	30
Cefotaxime	CTX	30
Imipenem	IMP	10
Meropenem	MEM	10
Ciprofloxacin	CIP	5
Levofloxacin	LEV	5
Nalidixic acid	NA	30
Chloramphenicol	C	30
Gentamicin	CN	10
Tetracyclines	TE	30
Ceftriaxone	CRO	30

These antibiotics have been chosen because they are commonly used to treat infections in both humans and animals, being the most responsible for the emergence of resistance and difficult-to-cure infections. The antibiotics for β-lactamases, carbapenemases and fluoroquinolones were used at concentrations of 30,10 and 5µg/mL respectively.

3.2.2. Culture media

Agar Plate count (PCA) (Scharlau, Barcelona, Spain): It is prepared by weighing 23.5 g of medium per litre of distilled water and sterilised in an autoclave, for 15 minutes at 121º C. Once sterile, it is tempered in a bath and distributed in Petri dishes.

Mueller Hinton (MH) (Scharlau, Barcelona, Spain): It is prepared by weighing 11.75 g of medium per litre of distilled water and sterilised in an autoclave, for 15 minutes at 121 ºC. Once sterile, it is tempered in a bath and distributed in petri dishes.

MacConkey agar (Scharlau, Barcelona, Spain), mSuperCARBA (Chromagar®, France), Buffered peptone water (Scharlau, Barcelona, Spain) and Tryptic soy broth (Scharlau, Barcelona, Spain) were all prepared according to the manufacturer's instructions.

3.3. ISOLATION OF ANTIBIOTIC RESISTANT GRAM-NEGATIVE MICROORGANISMS

3.3.1. Isolation of Gram-negative microorganisms resistant to β-lactams

In order to select micro-organisms resistant to 3rd generation cephalosporins, Buffered peptone water supplemented with cefotaxime (CTX) at a concentration of 2.5 mg/L, and vancomycin at 5 mg/L; the latter in order to inhibit Gram-positive bacteria (Liu *et al*., 2018).

Next, 10 g of each sample was taken, and 90 mL of Buffered peptone water was added to it, with antibiotics. After homogenisation in Stomacher (BAGPAGE, Interscience, Bag System) for about 5 minutes, the broths were incubated at 37 ± 1 °C for 24 ± 1 h. After this time, they were triple streak seeded onto MacConkey agar (MCA) agar (MacConkey AGAR, Scharlau, Barcelona, Spain) supplemented with CTX at a concentration of 2.5 mg/L and VAN at 5 mg/L, to isolate Gram-negative bacteria resistant to cefotaxime, mainly enterobacteria.

For each sample, 5 colonies grown on MacConkey agar were randomly selected from each sample and triple streaked in Plate Count Agar (37 ± 1°C, 24 h) and cultured as many times as necessary until pure cultures were obtained.

Also, from the incubated Buffered peptone water, 50 mL were taken in a sterile Falcon, which was stored at -20 °C for subsequent DNA extraction and direct identification of β-lactam resistance genes.

3.3.2. Isolation of Gram-negative microorganisms resistant to carbapenems

In order to isolate carbapenem-resistant microorganisms, containers with 90 mL of (Tryptic Soy Broth (TSB) Scharlau, Barcelona, Spain), supplemented with meropenem (MEM) 1 mg/L, and vancomycin (VAN) 8 mg/L (VAN in order to stop the growth of Gram-positive bacteria), were prepared in parallel and added to 10 g of sample. After homogenisation in Stomacher for approximately 5 minutes, the samples were incubated at 37 ± 1 °C for 24 ± 1 h. After this time, the samples were triple streaked on plates with mSuperCARBA (SC) agar (CHROMagar™mSuperCARBA™, Paris, France), to isolate Gram-negative bacteria resistant to carbapenem (carbapenem-resistant Gram-negative bacteria). According to the manufacturer's instructions, colonies grown on this medium are colour-coded, with *E. coli* can be identified by their pink colour, other *enterobacteria* (*Klebsiella spp*, *Citrobacter spp*, *Enterobacter spp*.) by their blue-green colour and *Acinetobacter spp.* by their light, opaque colour.

For each sample, 5 colonies grown on mSuperCARBA agar were randomly selected and triple streaked on PCA (37 ± 1°C, 24 h) and reseeded as many times as necessary until pure cultures were obtained.

In addition, 50 mL of the incubated TSB broth was stored at -20°C for subsequent DNA extraction and identification of resistance genes. DNA extraction and identification of resistance genes.

Subsequently, the bacterial strains already isolated from the samples were revived and triple streaked in order to get the pure culture. The process is carried out in a sterile cabin in the presence of a burner. STREAK-PLATE method is a pure culture technique which helps in isolating desired colonies from contaminants.

The procedure begins by sterilising a wire loop in flame. Cool the sterilised loop by allowing it to stand for a few seconds (Metal loop is recommended for this process). Collect a very small colony from the revived contaminated petri with the help of sterilised loop and make a streak in a new sterilised petri dish (with solidified plate-count agar). Sterilise the loop and repeat the process in the second direction (without picking more samples). The process is repeated for the third time with a streak in different directions in such a way that three different streaks are formed.

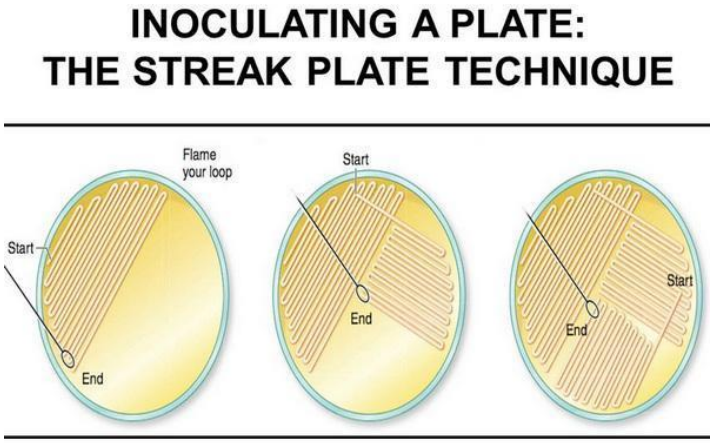

Figure 3.1. Representation of the streak plate technique (https://laboratoryinfo.com/streak-plate-method/).

3.4. IDENTIFICATION OF ISOLATES VIA BIOCHEMICAL TESTS

3.4.1. Preliminary tests

Once the pure culture was grown on Plate count Agar (PCA), different biochemical tests were performed in order to identify and select those suspicious micro-organisms according to the previously established criteria. The criteria include the micro-organism to be Gram-negative, oxidase-negative and catalase-positive.

Oxidase test: The first test to be performed was oxidase. The fundamental of this test is that it determines the presence of cytochrome (C). The cytochrome oxidises NNN´N´ tetramethyl, 1-4, phenylenediamine dihydrochloride (TMPD). NNN´N´ tetramethyl, 1-4, phenylenediamine dihydrochloride (TMPD) is a redox mediator used in the oxidase test for detecting bacterial cytochrome c oxidases.

The oxidation can be detected by the appearance of blue colour. For this purpose, (1) oxidase reagent strips (OXIDASE Reagent, Scharlau, Barcelona, Spain) were applied directly to the culture. (2) Scratch the petri on which you want to perform the test with the help of a cotton bud (baston) and add reagent (OXIDASE Reagent, Scharlau, Barcelona, Spain) to it. The positive reaction indicates intense blue/violet

colour within 10 seconds. In case it changes the colour, the result is positive, and the given sample will be positive for oxygenase. In case of oxygenase positive, the sample must be discarded since this project is based only on the selection of oxidase-negative microorganisms. Bacteria lacking cytochrome C don't change the colour.

Catalase test: Catalase is an enzyme possessed by most aerobic bacteria. It breaks down hydrogen peroxide into water and oxygen. The release of bubbles from the oxygen indicates that the test is positive. An isolated colony is deposited on a drop of H_2O_2. The occurrence of bubbles in less than a minute indicates the positivity of the test.

The analysis was followed by Gram staining. The aim was to be able to identify Gram-negative microorganisms, oxidase-negative and catalase-positive since this was the demand of the current project.

Gram staining: Gram staining includes extension of pure culture on water drop placed and subsequent drying and treating it with a special stain including Violeta, Lugol, alcohol and fuchsine for 1 min, 1min, 30 sec and 3 minutes respectively.

After drying for 15-20 minutes, bacteria can be seen under the microscope in an oil objective lens. Bacteria can be seen in round form (coccus) or enlarged stick form (bacillus). The bacteria can be Gram-positive with purplish colour or Gram-negative with a pinkish colour.

3.4.2. Identification by API strip 20E

All those strains found to be oxidase-negative, catalase-positive and Gram-negative were identified by API 20E miniaturised biochemical tests (API Strips, Biomèrieux, France). The Analytical Profile Index or API is a classification of bacteria based on experiments allowing fast identification. This system is developed for quick identification of clinically relevant bacteria. With the help of API only known bacteria can be identified.

For identification by the API 20E system, a 24- hour pure culture was used as the starting point from which an isolated colony was selected and resuspended in 5 mL of sterile water. The 20 microtubes of the system were filled with this inoculum, in such a way that both the tube and cupule of the tests CIT, VP, GEL were filled completely and only the tubes of the other tests were filled with the bacterial suspension. Then the cupula for the underlined tests, such as ADH, LDC, ODC, URE, H_2S were filled with Vaseline (using the same sterile pipette) in order to create an anaerobic condition.

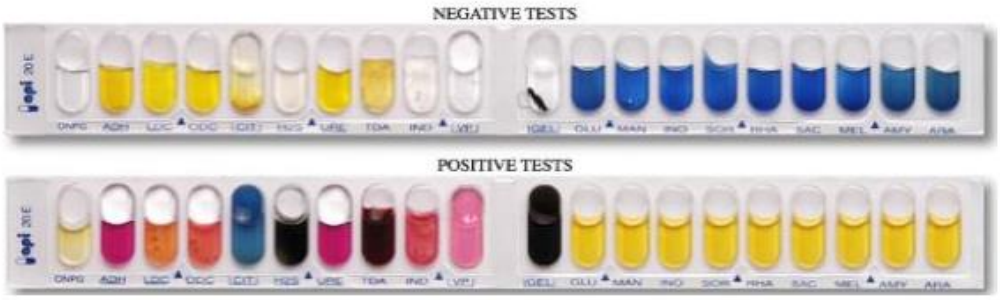

Figure 3.2. Colour coding corresponding to the negative and positive API 20E system tests

Bubble formation in the tube can lead to false positives or errors during analysis, therefore, special care was taken in this regard. Once inoculated, incubated at 37 ± 1°C for 24 hours.

The next day, the relevant reagents were added to the IND, VP and TDA tests. The results were entered into the APIWEB software tool (http://apiweb.biomerieux.com) and the identification of the isolate was obtained. All isolates identified were stored at -20 ºC in cryovials (Microbank, Prolab Diagnostics™, Thermo Fischer Scientific, UK), for further analysis.

3.5. ANTIMICROBIAL SUSCEPTIBILITY TESTING OF ISOLATES

Antibiograms: Antibiogram is a test to study the susceptibility of a microorganism to different antibiotics. Once the strains were identified, those who possessed pathogenic characteristics according to bibliography were selected for antibiograms. Antibiograms were performed using the CLSI (2014) disc-plate antibiogram method or Kirby-Bauer method, which is based on radial diffusion through Agar.

The antibiotics employed were from the β-lactam family, carbapenem, quinolones, phenicols, aminoglycosides, and tetracyclines (Table 3.3). They were selected as they are relevant in public health and/or epidemiology according to EFSA (2008) and were dispensed as Antibiotic discs (OXOID Antimicrobial Susceptibility test disc). The greater the inhibition ring is, the higher is the sensitivity of the strain studied to that antibiotic.

For this purpose, colonies were taken from a 24 h pure culture and inoculated in sterile 0.9% saline, adjusting their turbidity by comparison with the 0.5 Mc Farland scale. A swab was impregnated with this solution and two plates per strain of Mueller-Hinton (MH) agar (MUELLER-HUNTON AGAR, Scharlau, Barcelona, Spain) were inoculated onto the agar in all directions without leaving a free zone.

The *E. coli* strain *ATCC 25922* was used as quality control of the methodology employed, whose acceptable limits for inhibition zone diameters are established by the Clinical and Laboratory Standards Institute (CLSI, 2014).

Table 3.3. Selected antibiotics to study the resistance of the strains to them. Classifications by groups. Concentration of each antibiotic in each disk in µg. Values to classify each strain by resistant (R), intermediate sensitivity (I) or sensitive (S) depend on the diameter of the inhibition ring (Kirby-Bauer_Antibiotic_Sensitivity).

Group	Antibiotic	Abbreviation	Conc. (µg)	Diameter of the ring of inhibition		
				R	I	S
β-lactams	Amoxicillin	AMC	20/10	≤13	14-17	≥18
	Cefotaxime	CTX	30	≤22	23-25	≥26
	Ceftazidime	CAZ	30	≤17	18-20	≥21
	Ceftriaxone	CRO	30	≤19	20-22	≥23
	Ampicillin	AM	10	≤11	12-13	≥14
Quinolones	Levofloxacin	LEV	5	≤13	14-16	≥17
	Ciprofloxacin	CIP-5	5	≤15	16-20	≥21
	Nalidixic acid	NA	30	≤13	14-18	≥19
Carbapenems	Imipenem	IMP	10	≤19	20-22	≥23
	Meropenem	MEM	10	≤19	20-22	≥23
Aminoglycosides	Gentamycin	GM	10	≤12	13-14	≥15
Tetracyclines	Tetracycline	TE-30	30	≤14	15-18	≥19
Phenicols	Chloramphenicol	C	30	≤12	13-17	≥18

A disc dispenser (OXOID 16 Antimicrobial Susceptibility Testing Disc Dispenser) and a maximum of 6 antibiotics were placed per plate and distributed in such a way that the inhibition halos did not overlap, as shown in Figure 3.3.

Plates were incubated for 16 to 18 h at 37 ± 1 °C and in groups of 5 plates maximum. After this time, the diameters of the different inhibition halos were measured in millimetres and their resistance was categorised as sensitive (S), intermediate (I) and resistant (R). millimetres, categorised as sensitive (S), intermediate (I) and resistant (R), according to CLSI standards. (CLSI, 2014) for the different bacteria as shown in Table 3.3.

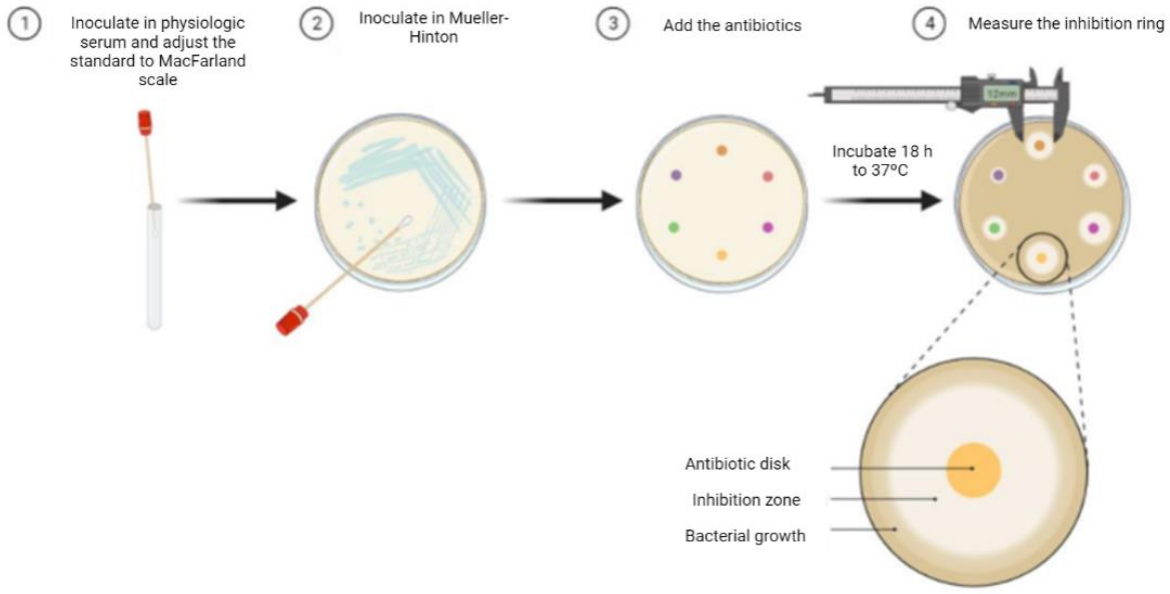

Figure 3.3. Representation of the antibiogram method plate-disk to study the sensitivity to antibiotics.

3.6. IDENTIFICATION OF *Acinetobacter baumannii* BY MOLECULAR METHODS

Identification using the miniaturised API 20E tests (API Strips, Biomèrieux, France), optimal for the identification of *Enterobacteriaceae*, finding results with an acceptable identification percentage. In the case of *Acinetobacter baumannii*, as the strip used was not specific and the results were very disparate, we proceeded to an identification by amplification and sequencing of 16S rRNA for all strains suspected of being this species. Of the 17 isolated bacteria, 5 potential pathogenic strains (C1M1, C1M2, C2SC2, C25M1, C29SC1) were chosen for molecular identification purposes after classification by phenotypic and biochemical identification. The DNA extractions of strains were obtained by the Bacterial Genomic DNA Kit (Sigma-Aldrich).

For this, DNA was extracted using the protocol explained in 3.7.1 and PCR was performed with a final volume of 25 µL to obtain sufficient amplification product required for sequencing, of which 2.5 µL corresponded to the extracted DNA and the remaining 22.5 µL to the reaction mix. This reaction mix consisted of 1X PCR buffer 1X, 2 mmol of $MgCl_2$ 200 µmol of each dNTP, 0.4 µmol of each primer used and 1.25 U of BIOTAQTM DNA Polymerase (Bioline), and sterile milliQ water to complete the 25 µL mix.

Purified DNA samples were used as a template to amplify the 16s rRNA gene (1,500 bp) using universal bacterial primers *27F* and *1525R (5'-AGAAAGGAGGTGATCCAGCC-3')* for the *Acinetobacter baumannii* strains (Khosravi, Sadeghi, Shahraki, Heidarieh, & Sheikhi, 2015). The polymerase chain reaction (PCR) conditions of the equipment (MJ Research PTC-100 Thermal Cycler, USA) were set up for the *A. baumannii* strains as follows: (denaturation) one cycle at 95 °C for 2 min, (extension and annealing) 35 cycles at 94 °C for 30 s, 65 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. To determine fragment size, PCR products were electrophoresed on 2% agarose gel (Pronadisa, Madrid, Spain) with 100 mL of Tris-AcetateEDTA buffer and 5 µL of Red Safe™ solution (Intron Biotechnology, Washington, USA). PCR products were scanned by the gel monitoring system (TransilluminatorVilber, Lourmat, France).

Subsequently, the PCR products were purified to obtain only the amplified DNA fragments, thus eliminating the components of the reaction mix so that sequencing could be carried out correctly. The kit for purifying PCR products (GenElute PCR Clean-Up Kit, Sigma-Aldrich, Missouri, USA) was used, following the protocol described by the manufacturer. The products obtained were stored at -20 °C until sequencing.

The amplified 16S rRNA gene fragments were sequenced by extension cycles on an ABI 373 DNA sequencer (Applied Biosystems). The sequences were verified by using the Chromas 2.6.6 software (http://technelysium.com.au/wp/chromas/) and compared with those stored in GenBank using the software BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) (Orhan-Yanıkan *et al*., 2019).

3.7. STUDY OF THE PRESENCE OF ANTIBIOTIC RESISTANCE GENES

After a series of biochemical test and antibiograms, TE solution was prepared for the subsequent DNA extraction of the bacterial strains and later multiplex PCR was performed on the strains turned out to be oxidase-negative, catalase-positive and Gram-negative that belonged to a specific group of pathogenic bacteria and broths (in which the specificity of bacteria was not known).

With the help of a loop, small amounts from the strains that were oxidase-negative, catalase-positive and Gram-negative were taken and put into Eppendorf of 1 ml containing TE solution. The solution was labelled according to the name of the sample, mixed on a vortex and placed in a refrigerator for later DNA extraction.

For PCR multiplex, DNA was extracted through the following protocol.

3.7.1 DNA extraction

To extract DNA from the isolated strains, a 24-hour pure culture in PCA was started and half a culture loop was resuspended in 1 mL of TE 1X buffer. half a culture loop was resuspended in 1 mL of 1X TE buffer. In the case of the samples from the broth samples in PCA and pre-enrichment broths, the 50 mL collected were centrifuged at 4000 rpm for 10 minutes, the supernatant was removed, and the precipitate was resuspended in 1 mL of 1X TE buffer.

DNA extraction was performed using the commercial GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, Missouri, USA). Genomic DNA kit (Sigma-Aldrich,Missouri, USA), according to the manufacturer's indications for Gram-negative bacteria

3.7.2. Multiplex PCR for the detection of β-lactam resistance genes (*bla_{TEM}, bla_{SHV}, bla_{CMY-2}, bla_{OXA}, bla_{IMP}, bla_{KPC}, bla_{VIM}, qnrA, qnrB, qnrS*).

The detection of resistance genes was performed on DNA extracted from all the 17 isolates as well as from the 29 samples already placed in enrichment broths, i.e, BPW (Buffered peptone water) broth supplemented with cefotaxime 2.5 mg/L and vancomycin 5 mg/L. And Tripto Soya broth, previously enriched with meropenem 1 mg/L and vancomycin 5 mg/L.

A multiplex PCR was performed to detect resistance to β-lactam antibiotics: specifically, the *bla_{TEM}*, *bla_{SHV}* , *bla_{CMY-2}*, *bla_{OXA}*, *bla_{IMP}*, *bla_{KPC}*, *bla_{VIM}*, *qnrA, qnrB, qnrS* genes, whose primers, sequences and expected amplicon sizes are shown in Table 3.4.

To perform the multiplex PCR, 2.5 µL of DNA were used out of a final volume of 25µL, the remaining 22.5 µL corresponding to the reagent mix. In addition, three positive controls and sterile MiliQ water as a negative control were used as in the previous work.

For β-lactam PCR, the *bla*$_{TEM}$ and *bla*$_{SHV}$ genes were selected as they encode two of the three most important extended-spectrum beta-lactamases, and *bla*$_{CMY-2}$, which encodes the plasmid of AmpC- β-lactamase CMY2 conferring resistance to extended-spectrum cephalosporins (Kader *et al*., 2022).

The reaction mix used consisted of 1X PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM for each dNTP, 0.4 µM of each primer for the *bla*$_{SHV}$ gene, 0.2 µM of each primer for the *bla*$_{TEM}$ and *bla*$_{CMY-2}$ genes, 5U of BIOTAQTM DNA Polymerase (Bioline), and sterile milliQ water to complete the 22.5 µL mix. The 25 µL were supplemented with 2.5 µL of DNA.

Table 3.4. Primer sequences of different types of genes to resistance to β-lactams, carbapenems and quinolones.

Gene	Size (bp)	Primer sequences	Reference
bla$_{TEM}$	247	F: 5'-TTAACTGGCGAACTACTTAC-3' R: 5'-GTCTATTTCGTTCATCCATA-3'	Kozak *et al*., (2009)
bla$_{SHV}$	393	F: 5'-AGGATTGACTGCCTTTTTG-3' R: 5'ATTTGCTGATTTCGCTCG-3'	
bla$_{CMY-2}$	1000	F: 5'GACAGCCTCTTTCTCCACA-3' R: 5'-TGGACACGAAGGCTACGTA-3'	
bla$_{OXA}$	438	F: 5'-GCGTGGTTAAGGATGAACAC-3' R: 5'-CATCAAGTTCAACCCAACCG-3'	van der Zee *et al*., (2014)
bla$_{IMP}$	232	F: 5'-GGCGGAATAGAGTGGCTTAATTCTC-3' R: 5'-GAATTTTTAGCTTGTACTTTACCGTCTTT-3'	
bla$_{KPC}$	798	F: 5'-TGCAGAGCCCAGTGTCAGTTT-3' R: 5'-CGCTCTATCGGCGATACCA-3'	
bla$_{VIM}$	390	F: 5'-GAGATTCCCACGCA[C/T]TCTCTAGA-3' R: 5'-AATGCGCAGCACCAGGATAG-3'	
qnrA	580	F: 5'-AGAGGATTTCTCACGCCAGG-3' R: 5'-TGCCAGGCACAGATCTTGAC-3'	Cattoir *et al*., (2007)
qnrB	264	F: 5'-GGMATHGAAATTCGCCACTG-3' R: 5'-TTTGCYGYYCGCCAGTCGAA-3'	
qnrS	428	F: 5'-GCAAGTTCATTGAACAGGGT-3' R: 5'-TCTAAACCGTCGAGTTCGGCG-3'	

F: forward primer; R: reverse primer; M: A or C; H: A or C or T; Y: C or T.

The thermocycler conditions for this PCR were as described by (Kozak et al., 2009): 15 minutes at 94 °C, 30 amplification cycles consisting of 1 minute at 94 °C (denaturing), 1 minute at 55 °C, 1 minute at 72 °C (annealing and extension) and a final extension of 10 minutes at 72 °C, and 1 minute at 72 °C and a final extension of 10 minutes at 72 °C.

As a positive control, we used our own strains from previous work. 700603, 6021b V2, M1D Mec 8, and 35218 were used for the detection of bla_{TEM}, bla_{SHV}, bla_{CMY-2} and bla_{TEM} respectively.

Similarly, the positive controls used for carbapenem resistance genes bla_{OXA}, bla_{IMP}, bla_{KPC}, bla_{VIM} were 13442, 13476,13438,13440 respectively. The thermocycler conditions for this PCR were 94 ºC for 10 min as initial activation, denaturation at 94ºC for 30 sec, annealing 52ºC for 40 sec/72ºC for 50 sec and the final extension at 72ºC for 5 minutes as described by (Van Der Zee *et al*., 2014).

Finally, the positive control used for fluoroquinolones resistance genes *qnrA, qnrB, qnrS* were ET1(IV) and ET2(IV) and the thermocycler conditions were 95ºC for 10 min as initial activation, denaturation at 95ºC for 1 min, annealing at 54ºC for 1min/ 72ºC for 1 min and final extension at 72ºC for 10 minutes as described by (Cattoir *et al*., 2007).

3.7.3. Agarose gel electrophoresis

To visualise the results obtained from the PCRs performed, the amplified DNA fragments were analysed using 1.5% agarose E gel (Condalab) in 1X TAE buffer (40 mM TrisHCl [pH 8.3], 2 mM acetate, 1 mM EDTA), to which 5 µL of RedSafe™ (iNtRON Biotechnology, Washington, USA) per 100 mL was added and gel was prepared, in order to reveal the bands posteriorly. The electrophoresis conditions were 90V for 60 minutes, after which the results were observed with a transilluminator (TransilluminatorVilber, Lourmat, France) under UV light.

4. RESULTS

4.1. BIOCHEMICAL IDENTIFICATION OF ANTIBIOTIC RESISTANT ISOLATES

From 29 samples analysed, 76 isolates were obtained, in which we identified 17 strains that were oxidase-negative, catalase-positive and Gram-negative bacteria. These 17 strains were suspected to be antibiotic-resistant. 33 isolates were from ditch water, 7 from cabbage, 2 from strawberries, 3 from wonder lettuce, 8 from red cabbage (in total 20 from vegetables), 3 from strawberries soil, 5 from wonder lettuce soil, 5 from red cabbage soil, 9 from cabbage soil and 1 from oak lettuce soil (in total were 23 samples from soil). 36 isolates were identified from MacConkey agar (resistant to cephalosporins, CTX) and the rest of the 40 were cultured in agar mSuperCARBA (resistant to carbapenems).

The results from identification of the strains after doing API 20E strips is illustrated in Table 4.1; 6 strains were identified as *E. coli*, 4 as *Stenotrophomonas maltophilia*, 2 as *Acinetobacter baumannii* and 1 for each strain of the following: *Citrobacter freundii, Pseudomonas oryzhabitans, Pseudomonas aeruginosa, Enterobacter cloacae* and *Pasteurella pneumotropica.*

Table 4.1. Identification of the isolated strains through API 20E stripes.

Samples	Number of strains	Identification API 20E
C11M1, C12M1, C13M2, C14M1, C16M1, C16M2,	6	*E. coli*
C1SC2, C17M3, C25SC1, C26SC2	4	*Stenotrophomonas maltophilia*
C1M1, C1M2	2	*Acinetobacter baumannii*
C17M1B1	1	*Citrobacter freundii*
C25M1	1	*Pseudomonas oryzhabitans*
C2SC2	1	*Pseudomonas aeruginosa*
C27M1	1	*Enterobacter cloacae*
C29SC1	1	*Pasteurella pneumotropica*

M: isolates from MacConkey+CTX; SC: isolates from mSuperCARBA

4.2. SENSITIVITY OF ISOLATES TO ANTIBIOTICS

The Table 4.2 shows the results of antibiograms of the 17 strains detected, grouped according to resistance patterns.

Table 4.2. Strains' resistance patterns to antibiotics.

Resistance patterns	Number of strains	Strain code
AMP-AMC	1	C1M2
AMP-AMC-CAZ-C	1	C1M1
AMP-AMC-CTX-C	1	C25M1
AMP-AMC-CTX-CRO	2	C11M1, C16M2
AMP-AMC-MEM-CN	1	C29SC1
AMP-AMC-CTX-CRO-CAZ	4	C27M1, C17M1B1, C16M1, C13M2
AMP-AMC-CTX-CRO-IMP-MEM	1	C11SC2
AMP-AMC-CTX-CRO-CAZ-MEM-C	1	C2SC2
AMP-AMC-CTX-CRO-IMP-MEM-CN	2	C25SC1, C26SC2
AMP-AMC-CTX-CRO-NA-LEV-CIP-TE	1	C14M1
AMP-AMC-CTX-CRO-CAZ-IMP-MEM-CN-TE	1	C17M3
AMP-AMC-CTX-CRO-CAZ-NA-LEV-CIP-CN-TE-C	1	C12M1

Strain codes in blue were from irrigation water; in brown were from soil

Antibiotics can be classified as: tetracyclines, quinolones, aminoglycosides, penicillins, third generation cephalosporins, carbapenems, and phenicols, as stated by WHO (WHO, 2019).

12 resistance patterns for 17 strains were detected (Table 4.2). All strains studied were resistant to ampicillin and amoxicillin (both ampicillin and amoxicillin are penicillins).

Penicillins (AMP and AMC, among others), cephalosporins (such as CTX, CAZ, CRO) and carbapenems (such as IMP and MEM), are all included in the β-lactams group of antibiotics, so that, there were 8 strains resistant to β-lactams exclusively. Interestingly, the most common resistant pattern (found in 4 strains) was AMP-AMC-CTX-CRO-CAZ, and the second one was AMP-AMC-CTX-CRO (found in 2 strains).

9 strains had resistance to some β-lactams and other types of antibiotics (aminoglycosides such as gentamicin (CN), quinolones (CIP, LEV, NA), tetracyclines or phenicols). Of them, two strains had resistance to AMP-AMC-CTX-CRO-IMP-MEM-CN.

One strain (C12M1) was resistant to 11 antibiotics of 13 studied, specifically for AMP-AMC-CTX-CRO-CAZ-NA-LEV-CIP-CN-TE-C (this strain, however, didn´t show any resistance to carbapenems- IMP and MEM).

It was considered a multiresistant strain when it contained 3 or more resistances to antibiotics' classes (Magiorakos *et al*., 2012). It was found that 9 resistance patterns met the criteria, so that 10 strains of the total 17 were multiresistant (a 58.8%). The multiresistant strains were resistant at 11.8% to penicillins, cephalosporins and phenicols; 5.9% to penicillins, carbapenems and aminoglycosides; 5.9% to penicillins, cephalosporins and carbapenems; 5.9% to penicillins, cephalosporins, carbapenems, chloramphenicol; 11.8% to penicillins, cephalosporins, carbapenems and aminoglycoside; 5.9% to penicillins, cephalosporins, quinolones and tetracycline; 5.9% to penicillins, cephalosporins, carbapenems, aminoglycoside and tetracycline; and 5.9% to penicillins, cephalosporins, quinolones, aminoglycoside, tetracycline and chloramphenicol.

Figure 4.1 shows the susceptibility of the 13 antibiotics tested in the 17 strains, grouped by antibiotics family (β-lactams (penicillins, cephalosporins and carbapenems), quinolones, aminoglycoside, tetracycline and phenicol).

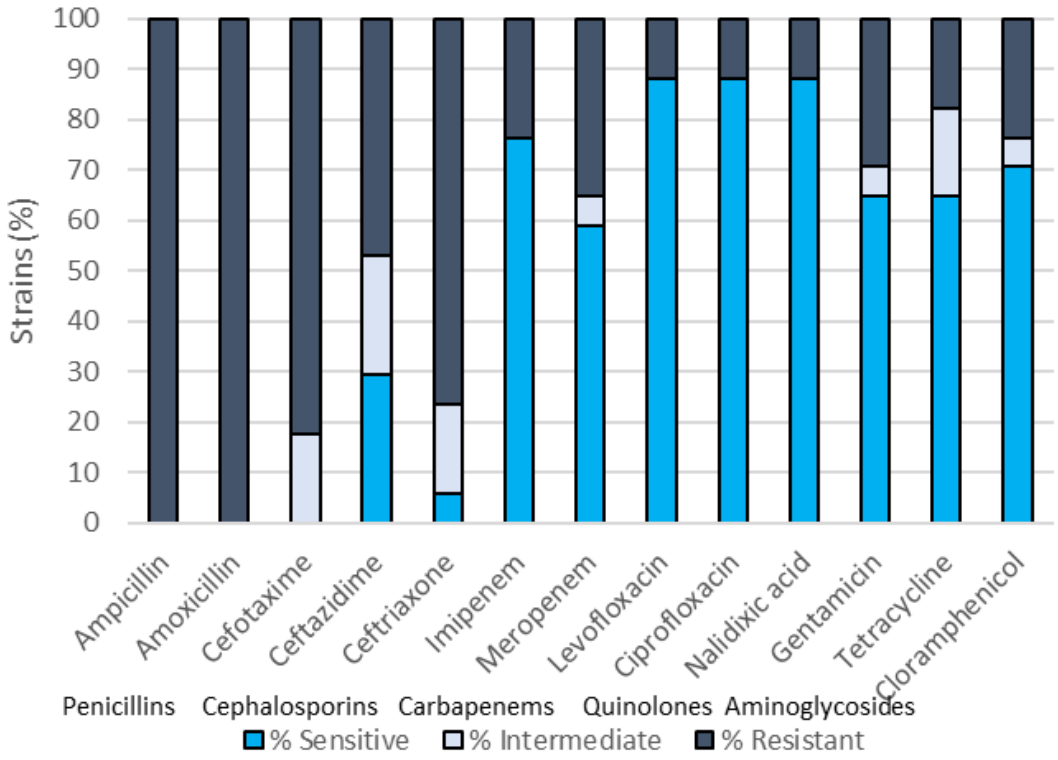

Figure 4.1. Sensitivity to antimicrobials of the different strains studied.

There was great variability between the sensibility of the strains analysed to different antimicrobial families in the antibiogram; nonetheless, there could be seen a clear predominance of the resistance to β-lactams, especially AMP and AMC (both with 100% of resistance) (as mentioned before, Figure 4.1). The second antibiotic with more percentage of resistant strains was the cephalosporin cefotaxime-CTX (82.4%), followed by ceftriaxone-CRO (76.5%), ceftazidime-CAZ (47.1%), meropenem-MEM (35.3%), gentamicin-CN (29.4%), imipenem-IMP (23.5%), chloramphenicol-C (23.5%), and tetracycline-TE (17.7%). The lowest resistance was observed for quinolones (levofloxacin-LEV, ciprofloxacin-CIP, nalidixic acid-NA) (11.8% for each type of quinolone).

Thereafter, it is shown the results from the sensitivity to antimicrobials of the strains clustered by the type of samples, which were either from irrigation water or soil from different vegetables and fruits (Figures 4.2 and 4.3).

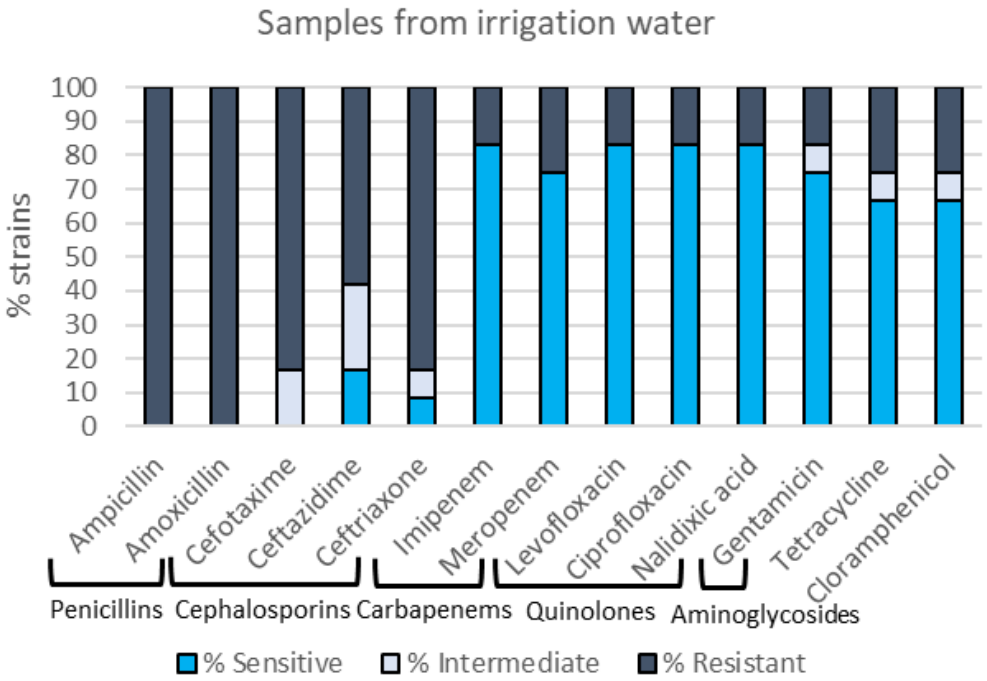

Figure 4.2. Sensitivity to antimicrobials from irrigation water's samples.

Of the total of 12 microorganisms from irrigation water (Figure 4.2), 100% were resistant to penicillins, followed by cephalosporins: cefotaxime-CTX (83.3%), ceftriaxone-CRO (83.3%) and ceftazidime-CAZ (58.3%). Then, the resistance was for carbapenem meropenem-MEM (25%), for tetracycline-TE (25%) and chloramphenicol-C (25%). For carbapenem imipenem-IMP, quinolones (LEV, CIP and NA) and gentamicin the resistance was 16.7%.

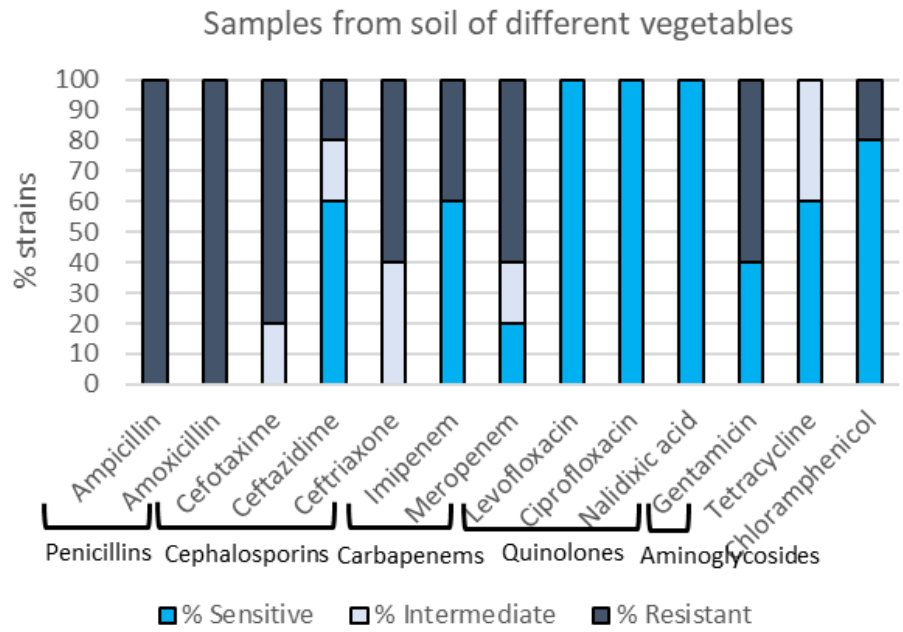

Figure 4.3. Sensitivity to antimicrobials from soil's samples.

The rest of the microorganisms (5 in total) were from soil. They were resistant to penicillins (100%), 80% to cefotaxime-CTX, 60% to ceftriaxone-CRO, meropenem-MEM and gentamicin, 40% to imipenem-IMP and 20% to ceftazidime-CAZ and chloramphenicol-C. For quinolones and tetracycline there weren't resistant microorganisms from this type of sample (Figure 4.3).

4.3. MOLECULAR IDENTIFICATION OF SOME STRAINS

Through biochemical identification with API 20E strips of 17 strains, 5 strains were identified as *Acinetobacter baumannii* or suspicious to be that strain, so the amplification by PCR of the 16S rRNA and Sanger sequencing was done. Some strains of *A. baumannii* are multi-resistant to several antibiotics and can cause nosocomial infections, such as septicaemia, pneumonia, urinary tract infections, meningitis and even endocarditis (López and López-Brea, 2000), due to these effects this strain is studied in more detail. The results obtained by sequencing were then analysed by BLAST and compared with the one achieved by the API 20E strip (Table 4.3).

Only 1 strain was confirmed by molecular method to be *A. baumannii* (20% of the total) (Table 4.3). A strain previously identified as *A. baumannii* was reidentified as the *Acinetobacter sp.* genus without the achievement of species identification. In another sample, the micro-organism was found to be *Pseudomonas aeruginosa* by API strip. However, it was revealed as *A. pitti* by molecular identification. In another sample (C25M1), the molecular and biochemical methods had similar findings (*Pseudomonas sp*. and *P. oryzhabitans*, respectively). Nevertheless, *Pseudomonas* genus' microorganisms are oxidase-positive different from *Acinetobacter* genus. Ultimately, *Pasteurella pneumotropica* identified by API strip (which is oxidase-positive) was designated as the genus *Sphingobacterium sp.* (also oxidase-positive) by the sequencing method (Table 4.3).

Table 4.3. Molecular identification by 16S rRNA sequencing compared with biochemical identification by API 20E strip of 5 strains.

Strain	Accession number	Identity %	Identification by BLAST	Identification by API 20E
C1M1	MK014194.1	99.9	*Acinetobacter baumannii*	*Acinetobacter baumannii*
C1M2	MK583584.1	99.45	*Acinetobacter sp.*	*Acinetobacter baumannii*
C2SC2	KY941122.1	99.81	*Acinetobacter pittii*	*Pseudomonas aeruginosa*
C25M1	MG674323.1	99.43	*Pseudomonas sp.*	*Pseudomonas oryzhabitans*
C29SC1	MT238678.1	99.34	*Sphingobacterium sp.*	*Pasteurella pneumotropica*

4.4. GENES OF RESISTANCE TO β-LACTAMS: bla_{TEM}, bla_{SHV}, bla_{CMY-2}; CARBAPENEMS: bla_{IMP}, bla_{OXA}, bla_{VIM}, bla_{KPC} and QUINOLONES: *qnrA*, *qnrB*, *qnrS*

A multiplex PCR was done to detect resistance genes to β-lactams, carbapenems or quinolones of 17 isolated strains (Table 4.4).

Table 4.4. Resistance genes detected in the 17 isolated strains, classified by type of sample (irrigation water or soil of vegetables), code and original strain.

Sample and code	Strain (API strip)	*bla*$_{TEM}$	*bla*$_{SHV}$	*bla*$_{CMY2}$	*bla*$_{IMP}$	*bla*$_{OXA}$	*bla*$_{VIM}$	*bla*$_{KPC}$	*qnrA*	*qnrB*	*qnrS*
Ditch water, C1M1	*Acinetobacter baumannii*	-	-	-	-	+	+	-	-	-	-
Ditch water, C1M2	*Acinetobacter baumannii*	-	-	-	-	+	+	-	-	-	-
Ditch water, C2SC2	*Pseudomonas aeruginosa*	-	-	-	-	-	-	-	-	-	-
Ditch water, C11M1	*Escherichia coli*	-	-	-	-	-	-	+	-	+	+
Ditch water, C11SC2	*Stenotrophomonas maltophilia*	+	-	-	-	-	-	-	-	-	-
Ditch water, C12M1	*Escherichia coli*	+	+	-	-	+	-	+	-	-	-
Ditch water, C13M2	*Escherichia coli*	+	-	-	-	+	-	+	+	-	-
Ditch water, C14M1	*Escherichia coli*	+	-	-	-	+	-	+	-	-	+
Ditch water, C16M1	*Escherichia coli*	+	-	-	-	-	-	-	-	-	+
Ditch water, C16M2	*Escherichia coli*	-	-	-	-	-	-	-	-	-	+
Ditch water, C17M3	*Stenotrophomonas maltophilia*	+	-	-	-	-	-	+	-	-	-
Ditch water, C17M1B1	*Citrobacter freundii*	-	-	+	-	+	-	-	-	-	-
Wonder lettuce soil, C25M1	*Pseudomonas oryzhabitans*	+	+	-	-	+	-	+	-	-	-
Wonder lettuce soil, C25SC1	*Stenotrophomonas maltophilia*	+	-	-	-	-	-	-	-	-	-
Red cabbage soil, C26SC2	*Stenotrophomonas maltophilia*	-	+	-	-	-	-	-	-	-	-
Cabbage soil, C27M1	*Enterobacter cloacae*	-	-	-	+	+	-	+	-	-	-
Strawbewrries soil, C29SC1	*Pasteurella pneumotropica*	-	-	-	-	+	-	-	-	-	-

Genes for resistance to β-lactams (*bla*$_{TEM}$, *bla*$_{SHV}$, *bla*$_{CMY-2}$) were detected in 3 different samples, which were ditch water, wonder lettuce soil, and red cabbage soil. Also, these resistance genes to β-lactams were found in 4 different *Enterobacteriaceae* strains (*S .maltophilia*, *E. coli*, *C. freundii* and *P. oryzhabitans*) (Table 4.4). The genes that indicate resistance to carbapenems (*bla*$_{IMP}$, *bla*$_{OXA}$, *bla*$_{VIM}$, *bla*$_{KPC}$) were detected in 4 different samples (ditch water, wonder lettuce soil, cabbage soil and strawberries soil) and 7 *Enterobacteriaceae* strains: *A. baumanni*, *E. coli*, *S. maltophilia*, *C. freundii*, *P. oryzhabitans*, *E. cloacae*, *P. pneumotropica*. Lastly, genes for resistance to quinolones (*qnrA*, *qnrB*, *qnrS*) were found only in samples from ditch water and only detected in *E. coli* strains (Table 4.4).

One sample from ditch water where it was isolated, a strain from *P. aeruginosa*, hadn't any of these resistance genes studied (Table 4.4).

Additionally, there are 2 strains of *E .coli* isolated from ditch water (C13M2 and C14M1) which had resistance genes to β-lactams, carbapenems, and quinolones (Table 4.4). Specifically, both contained 4 resistances; C13M2 had *bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{KPC}$, and *qnrA*; whereas C14M1 had *bla*$_{TEM}$, *bla*$_{OXA}$, *bla*$_{KPC}$ and *qnrS*. In the antibiograms C13M2 presented this pattern of resistance: AMP-AMC-CTX-CRO-CAZ while

C14M1 had this pattern: AMP-AMC-CTX-CRO-NA-LEV-CIP-TE. To conclude, in C13M2 the results agreed in the resistance to β-lactams and not for carbapenems and quinolones; on the other hand in C14M1 agreed in the resistance to β-lactams and quinolones but not for carbapenems. This may be due to these 2 samples being from MacConkey agar and were selected to be resistant to a cephalosporin CTX and not for carbapenems.

Table 4.5. Frequency of detection of the different resistance genes studied by type of sample.

	Resistance to β-lactams			Resistance to carbapenems				Resistance to quinolones		
Sample	***bla*$_{TEM}$**	***bla*$_{SHV}$**	***bla*$_{CMY2}$**	***bla*$_{IMP}$**	***bla*$_{OXA}$**	***bla*$_{VIM}$**	***bla*$_{KPC}$**	***qnrA***	***qnrB***	***qnrS***
Ditch water	50%	8.3%	8.3%	0%	50%	16.7%	41.7%	8.3%	8.3%	33.3%
Wonder lettuce soil	100%	50%	0%	0%	50%	0%	50%	0%	0%	0%
Red cabbage soil	0%	100%	0%	0%	0%	0%	0%	0%	0%	0%
Cabbage soil	0%	0%	0%	100%	100%	0%	100%	0%	0%	0%
Strawberries soil	0%	0%	0%	0%	100%	0%	0%	0%	0%	0%

Table 4.5 shows the frequency of resistance genes in the 17 strains isolated in different types of samples from ditch water or soil from vegetables and fruits. In ditch water were detected genes to all types of resistance genes between a percentage of 8.3% and 50%, except for *bla*$_{IMP}$. For different types of samples from soil it was detected some resistance genes for β-lactams, carbapenems or both**,** the percentage was situated between 50% or 100%. However, it should be noted that the number of soil samples of vegetables and fruits is lower.

Besides that, 10 samples from soil, 9 from irrigation water and 10 from vegetables (a total of 29 samples) were analysed and cultured in buffered peptone water supplemented with cefotaxime-CTX or tryptic soy broth supplemented with meropenem-MEM (Table 4.6).

Table 4.6. Study of resistance genes to antimicrobials of 29 samples sow in buffered peptone water (A) or in tryptic soy broth (T).

Sample	***bla*$_{TEM}$**	***bla*$_{SHV}$**	***bla*$_{CMY2}$**	***bla*$_{IMP}$**	***bla*$_{OXA}$**	***bla*$_{VIM}$**	***bla*$_{KPC}$**	***qnrA***	***qnrB***	***qnrS***
C1 A	+	-	-	-	+	-	-	-	-	-
C1 T	-	-	-	-	+	-	-	-	-	-
C2 A	-	-	-	-	-	-	-	-	-	-
C2 T	-	-	-	-	-	-	-	-	-	-
C3 A	-	-	-	-	-	+	-	-	-	-
C3 T	-	-	-	-	-	-	-	-	-	-
C4 A	-	-	-	-	-	-	-	-	-	-
C4 T	-	-	-	-	-	-	-	-	-	-
C5 A	+	+	-	-	-	-	-	-	-	-
C5 T	-	-	-	-	-	-	-	-	-	-
C6 A	-	-	-	-	-	-	-	-	-	-
C6 T	-	-	-	-	-	-	-	-	-	-
C7 A	-	-	-	-	-	-	-	-	-	-
C7 T	-	-	-	-	-	-	-	-	-	-
C8 A	-	-	-	-	-	-	-	-	+	-
C8 T	-	-	-	-	-	-	-	-	-	-
C9 A	-	-	-	-	-	-	-	-	-	-
C9 T	-	-	-	-	-	-	-	-	-	-
C10 A	-	-	-	-	-	-	-	-	-	-
C10 T	-	-	-	-	-	-	-	-	-	-
C11 A	-	-	-	-	-	-	-	-	-	-
C11 T	-	-	-	-	-	-	-	-	-	-
C12 A	-	-	-	-	-	-	-	-	-	-
C12 T	-	-	-	-	-	-	-	-	-	+
C13 A	-	-	-	-	-	-	-	-	-	-

Sample	bla_{TEM}	bla_{SHV}	bla_{CMY2}	bla_{IMP}	bla_{OXA}	bla_{VIM}	bla_{KPC}	*qnrA*	*qnrB*	*qnrS*
C13 T	-	-	-	-	-	-	-	-	-	-
C14 A	-	-	-	-	-	-	-	-	-	-
C14 T	-	-	-	+	-	+	-	-	-	-
C15 A	-	-	-	-	-	-	-	-	-	-
C15 T	-	-	+	-	-	-	-	-	+	-
C16 A	+	+	-	-	+	+	-	-	-	+
C16 T	-	-	-	-	-	-	+	-	-	-
C17 A	+	+	-	-	+	+	-	-	-	+
C17 T	+	+	-	-	+	-	+	-	-	-
C18 A	-	-	-	-	-	-	-	-	-	-
C18 T	-	-	-	-	-	-	+	-	-	-
C19 A	-	-	-	-	+	-	-	-	-	-
C19 T	-	-	-	-	-	-	-	-	-	-
C20 A	-	-	-	-	-	-	-	-	-	-
C20 T	-	-	+	-	-	-	-	-	-	-
C21 A	-	-	-	-	-	-	+	-	-	-
C21 T	-	-	-	-	-	-	+	-	-	-
C22 A	-	-	-	-	-	-	-	-	-	-
C22 T	+	-	-	+	-	-	+	-	-	-
C23 A	-	-	-	-	-	-	-	-	-	-
C23 T	-	-	-	-	-	-	-	-	-	-
C24 A	+	-	-	-	-	-	-	-	-	-
C24 T	-	-	-	-	-	-	-	-	-	-
C25 A	-	-	-	-	-	-	-	-	-	-
C25 T	-	-	-	-	-	-	-	-	-	-
C26 A	-	-	-	-	-	-	-	-	-	-
C26 T	-	-	-	+	-	-	-	-	-	-
C27 A	-	-	-	-	-	-	-	-	-	-
C27 T	-	-	-	-	-	-	-	-	-	-
C28 A	-	-	-	-	-	-	-	-	-	-
C28 T	-	-	-	+	-	-	-	-	-	-
C29 A	-	-	-	-	-	-	-	-	-	-
C29 T	-	-	+	-	-	-	-	-	-	-

Samples remarked in blue: from irrigation water; in green: from vegetables; in brown: from soil.

Ten samples had microorganisms with genes for resistance to β-lactams, of these, 5 samples were from ditch water, 3 from vegetables, and 2 from soil. Additionally, 5 samples were detected in the medium of peptone water with cefotaxime-CTX and the other 5 in the medium of tryptic soy broth with meropenem-MEM (Table 4.6). 15 samples had microorganisms with resistance to carbapenems, in which 7 were from irrigation water, 6 from vegetables, and 2 from soil; furthermore, 6 of the samples were identified in peptone water broth-CTX and 9 in tryptic soy broth-MEM (Table 4.6). Eventually, 5 samples had resistance genes to quinolones, 4 of them were detected in irrigation water and 1 in soil; moreover, 3 were detected in peptone water-CTX and 2 in tryptic soy broth-MEM (Table 4.6).

There were 36 samples out of a total of 58 which hadn't any gene of resistance; on the contrary, 22 samples had one or more resistance genes. 8 samples had 2 or more resistance genes, and of these 8 samples, 2 had origin from vegetables and 6 from irrigation water (Table 4.6).

Finally, the β-lactamase gene bla_{TEM} was the resistance gene more common (6 out of 29 samples presented it) with 20,7%. Then, carbapenemases bla_{IMP} *and* bla_{KPC} followed with percentages of 17,2% (both were found in 5 samples) and carbapenemases bla_{OXA} *and* bla_{VIM} were in 4 samples each one (13,8%).

5. DISCUSSION

5.1. BIOCHEMICAL AND MOLECULAR IDENTIFICATION OF THE ISOLATES RESISTANT TO ANTIMICROBIALS

A total of 17 strains (22.4%) were oxidase-negative, catalase-positive, and Gram-negative, in addition to being suspicious of being resistant to antibiotics, since they were isolated from enrichment broths and culture media supplemented with antibiotics. All 17 strains were identified using API 20E strips. However, the results were compared with molecular identification by sequencing of the 16S rRNA gene, and it was found that the degree of similarity was different as expected. Some of the strains (40%) were identified through molecular methods since the results obtained from API 20E were not convincing. Their percentages upon the API 20E testing were quite low. Upon molecular identification, only 20% of the strains analysed by sequencing agreed with the results obtained with API strip.

According to Reller *et al*. (2007), identification by gene sequencing is more objective, does not require optimal growth or even a viable microorganism, and has the added capability of defining taxonomic relationships among bacteria. Bosshard *et al*. (2006), in a study, identified by 16S rRNA gene sequence analyses the isolates of nonfermenting Gram-negative bacilli (non-*P. aeruginosa*) at the level of specie in a 92% of the cases and 8% at genus level; however, using API 20 NE strip, 54% of isolates were assigned to species, and 7% to genus level, and 39% of the isolates could not be discriminated at any taxonomic level. In the case of this work, the results are presumably against what was expected because only 40% of strains analysed by sequencing of 16S rRNA achieved specie level and 60% achieved genus level. This could be due to the low number of isolates analysed by this molecular method (5 in total).

Two of the strains identified by API 20 E strip as *A. baumannii* were confirmed by sequencing of 16S rRNA in one case and in the other one, only reached genus level with the molecular method. Antimicrobial resistance is one of the major public health problems, ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.) are responsible for over 80% of deaths (Chapartegui-González *et al.*, 2022; Osman *et al.*, 2020), so that the strain *A. baumanni* identified in a sample of ditch water has to be kept in mind and take preventive measures to avoid harmful effects. One strain identified previously as *P. aeruginosa* with API 20E strip was reidentified as *A. pitti* with 16S rRNA sequencing method. This strain is not included in the group of ESKAPE, but one study with strains isolated in a hospital reveals the ability of them to survive under stressful conditions (e.g., starvation, desiccation), also acting as a reservoir for antimicrobial resistance genes in clinical facilities that could be easily disseminated to other pathogens (Chapartegui-González *et al.*, 2022). Nevertheless, it should be noted that these strains have been identified in hospitals and not in food and ecological environments.

Finally, one strain was identified as the *Sphingobacterium sp.* by sequencing of 16S rRNA. *Sphingobacterium* are usually found in soil, stagnant water, and vegetation. They are described as opportunistic pathogens. However, they may cause infections in immunocompetent hosts (Gupta *et al.*, 2016). Also, bacteria of this genera are oxidase-positive.

Ahmed *et al*. (2015) emphasised the importance of using molecular biology techniques as diagnostic tools in microbiology laboratories, and the combination of microbiology and molecular biology

resulted in high sensitivity and specificity. Because of their low contamination levels and relatively fast outcomes, molecular approaches are intriguing ways for diagnosing many infectious disorders. So that these molecular methods could be implemented in order to study antibiotic resistance and know the potential impact on the environment and on human health. However, because molecular approaches need expensive equipment and knowledge, they may not be available in many Low-Middle Income Countries (LMICs) due to lack of available finances.

5.2. SENSITIVITY OF THE STRAINS TO ANTIMICROBIALS

The antibiograms conducted on the 17 isolated strains revealed a notable pattern of antibiotic resistance. It was observed that all of these strains displayed resistance to at least two antibiotics, specifically ampicillin (AMP) and amoxicillin (AMC).

Furthermore, the most significant resistance pattern observed was related to β-lactam antibiotics. This class of antibiotics displayed the highest resistance rates, with a complete resistance (100%) to penicillins such as AMP and AMC. Following this, resistance percentages were notably high for third-generation cephalosporins, including 82.4% for cefotaxime (CTX), 76.5% for ceftriaxone (CRO), and 47.1% for ceftazidime (CAZ). Carbapenems, another subgroup of β-lactam antibiotics, exhibited resistance rates of 35.3% for meropenem (MEM)and 23.5% for imipenem (IMP).

It is remarkable that nearly half of the strains (47.1%) displayed resistance exclusively to β-lactam antibiotics.

These strains originating from ditch water used for ecological food irrigation and soils within ecological food production displayed a consistent resistance trend, primarily characterised by resistance to β-lactams, followed by cephalosporins and carbapenems.

Comparing these findings to a study conducted in the Yangtze River Basin in China, which investigated β-lactam antibiotic resistance, a similar trend was observed. The study identified 319 species, mainly *E.coli* and *Klebsiella pneumoniae*, with high resistance rates, including 97.5% resistance to CTX, 78.1% to CRO, 34.5% to CAZ, and 5% to IMP. However, it is noteworthy that only 50.2% of the strains in that study were resistant to penicillin (Ana *et al*., 2021).

The presence of such antibiotic resistance in aquatic systems, which often serve as sources for irrigation of crops and domestic use, poses potential risks to public health, as highlighted by these authors.

In alignment with these results, Chelaghma *et al*., (2021) found that fresh vegetables and fruit, frequently consumed raw, served as reservoirs of resistance genes to β-lactam antibiotics.

Furthermore, it's important to note that samples obtained from the MacConkey medium (M) were cultured with cefotaxime (CTX), indicating that strains from this medium should exhibit resistance to this cephalosporin. Similarly, for samples from the mSuperCARBA medium, resistance to carbapenems like meropenem (MEM) and imipenem (IMP) would be expected. This antibiotic selection approach in the media contributed to the enrichment of strains resistant to cephalosporins and carbapenems.

However, it's worth acknowledging that while this selection process effectively imposed resistance to these specific antibiotics, it did not prevent the co-selection of resistance to other antibiotics. Notably,

resistance to aminoglycosides (gentamicin-CN) was observed in 29.4% of the strains, followed by resistance to chloramphenicol-C at 23.5% and tetracycline-TE at 17.7%.

On the other hand, resistance to quinolones (levofloxacin-LEV, ciprofloxacin-CIP, and nalidixic acid-NA) was less common, with a percentage of 11.8% for each of these antibiotics.

These results highlight the potential for co-selection of resistance to non-β-lactam antibiotics alongside cephalosporin and carbapenem resistance due to the antibiotic selection used in the mediums. It's worth noting that resistance levels to non-β-lactam antibiotics observed in this study are somewhat higher than those reported in the study conducted by Jiménez-Belenguer *et al.*, (2023). Their study found resistance levels ranging from 2% for levofloxacin-LEV to 16% for nalidixic acid-NA in organic vegetable samples. This variation underscores the importance of monitoring antibiotic resistance patterns and the potential impact of selection pressures in different environments and sample sources.

In this study, the concept of multi-resistance, defined as resistance to three or more classes of antibiotics according to Magiorakos *et al*., (2012), was examined. It was found that 10 out of the 17 strains (58.8%) exhibited multiresistance, showing resistance to 2 or 3 classes of β-lactam antibiotics, along with resistance to an antibiotic from a different antibiotic family.

This observation aligns with findings from a study conducted by Asfaw *et al*., (2023), which investigated bacterial isolates from various vegetables purchased at a local market in Debre Berhan town, Ethiopia. In their study, 64.8% of bacterial isolates displayed multi-resistance, a value that is notably similar to the results obtained in this study.

Despite the relatively small number of samples used in this study, it appears that isolates from ditch water may exhibit more antibiotic resistance compared to those from soil. However, it is important to consider the numbers of sample discrepancy, with 12 samples from water and only 5 from soil.

Notably, 50% of the water samples (6 out of 12) and 80% (4 out of 5) soil samples showed multiresistance. Examples of strains from water with diverse resistance patterns, such as *E.coli* strain C12M1 (resistant to AMP-AMC-CTX-CRO-CAZ-NA-LEV-CIP-CN-TE-C), and *E.coli* strain C14M1 (resistant to AMP-AMC-CTX-CRO-NA-LEV-CIP-TE) and *S. maltophilia* strain C17M3 (resistant to AMP-AMC-CTX-CRO-CAZ-IMP-MEM-CN-TE), illustrate the complexity of resistance profiles in water isolates. Similarly, in soil samples, strains like *Stenotrophomonas maltophilia* C25SC1 and C26SC2 with the same pattern of resistance: AMP-AMC-CTX-CRO-IMP-MEM-CN exhibited multiresistant patterns.

Given these findings, it's difficult to establish which source contributes more to the antibiotic resistance. It's evident that both water and soil reservoirs contain diverse strains with various resistance profiles. Expanding the sample size for soil isolates may provide a more comprehensive understanding of resistance dynamics within this environment.

In a study conducted in the Turia River in Valencia and its associated irrigation ditches network, it was discovered that a significant proportion of *E.coli* isolates exhibited multiresistance. Specifically, 70.4% of these isolates displayed resistance to multiple classes of antibiotics. In this context, the most common pattern of multiresistance involved resistance against 4 and 5 different antibiotic classes (Amato *et al.*, 2021).

In contrast, in this Master´s thesis, isolates obtained from ditch water exhibited the highest levels of multiresistance, with resistance observed against 4,5, or even 6 different antibiotic classes. Meanwhile, isolates from the soil of ecological food production primarily exhibited multiresistance against 4 classes of antibiotics. This discrepancy underscores the diverse resistance patterns observed in different environmental niches.

Soil ecosystems are highlighted as particularly favourable environments for the acquisition and selection of antimicrobial resistance due to the presence of antibiotic-producing microorganisms. Furthermore, in the context of organic farming, where manure is used as a fertiliser, there is a potential pathway for antimicrobial-resistant bacteria from the animal gut microbiota to disseminate into soil ecosystems. When humans consume foods grown in these soils, it can facilitate the exchange of resistance genes with their own gut microbiota, potentially leading to further dissemination of resistance in the environment. The study by Armalytė *et al*., (2019) exemplifies this, showing that bacteria belonging to genera such as *Pseudomonas*, *Acinetobacter*, and *Enterobacteriaceae* from rapeseed field soil in organic farming were more antibiotic-resistant compared to those recovered from conventional farming sites.

While humans consume raw vegetables and fruits, which can serve as reservoirs of antibiotic-resistant bacteria, it is crucial not to overlook other potential sources of such bacteria, such as irrigation water or the soil from which these foods derive their nutrients.

5.3. DETECTION OF GENES OF RESISTANCE TOWARDS β-LACTAMS, CARBAPENEMS AND QUINOLONES

In the PCR multiplex analysis, of the 17 strains, several important findings were revealed regarding antibiotic resistance genes and their relationship to antibiogram results.

Among the strains analysed, 11 out of 17(64.7%) exhibited resistance genes to **carbapenems**. These strains belonged to diverse species, including *A. baumannii*, *E. coli*, *S. maltophilia*, *C. freundii*, *P. oryzhabitans*, *E. cloacae* and *P. pneumotropica*. Notably, one of these strains, *A. baumannii* from ditch water (C1M1) which is also included in the ESKAPE group, showed the presence of both bla_{OXA} and bla_{VIM} carbapenemase genes, despite not showing resistance to carbapenems in the antibiogram. This discrepancy highlights the potential presence of resistance genes even in strain that may not exhibit resistance phenotypically.

Similarly, 10 out of 17 strains (58.8%), resistance genes to β-lactam antibiotics were detected. These genes were found in strains of *S. maltophilia*, *E. coli*, *C. freundii,* and *P. oryzihabitans*. The presence of these genes aligns with the antibiogram results, reflecting the phenotypic resistance to β-lactams observed in these strains.

Resistance genes to quinolones were identified in 5 strains (29.4%) of *E.coli* isolated from ditch water. This finding corresponds to the lower prevalence of quinolone resistance in both the PCR analysis and the antibiogram results. For instance, the strain *E.coli* (C14M1) exhibited resistance to various antibiotics in the quinolone class, which matched the presence of the quinolone resistance gene *qnrS*.

These results highlight the intricate relationship between resistance genes and phenotypic resistance as determined by antibiograms. It's essential to consider that the presence of resistance genes, even

when not phenotypically expressed, may have potential implications for antibiotic resistance dynamics in the environment and human health.

In certain instances, it's evident that there can be agreement between the findings obtained from antibiograms and the detection of resistance genes using PCR. However, in other cases, such agreement may not be present, and instead, the results tend to complement each other.

In our study, which involved 17 strains isolated from samples of irrigation water and soil samples from ecological vegetables/fruit, the presence of genes of resistance was observed. Specifically, resistance genes to carbapenems were detected in 64.7% of the strains, β-lactam resistance genes were found in 58.8% of the strains, and resistance genes to quinolones were identified in 29.4% of the strains. These results exhibit slight differences compared to the antibiogram results. In the case of resistance genes through PCR, a higher percentage was associated with carbapenem resistance, followed by b-lactam and quinolones. In contrast, the antibiogram results indicated higher resistance to penicillins, followed by third-generation cephalosporins and carbapenems.

Furthermore, in our study, we cultured 29 samples obtained from irrigation water, ecological food, and soil. These samples were cultured in a medium containing peptone water with a third generation cephalosporins (CTX) or tryptic soy broth with a carbapenem (MEM). Our intention was to selectively culture strains that exhibited resistance to cephalosporins or carbapenems.

However, it is noteworthy that despite our efforts to select for resistance to these specific antibiotics, some samples surprisingly exhibited resistance genes to quinolones or to other antibiotics for which they were not expected to be resistant initially.

Our choice to focus on these two types of antibiotics was deliberate. Firstly, ceftriaxone (CTX), being a third-generation cephalosporin, possesses outstanding efficacy against a wide range of Gram-negative and most Gram-positive microorganisms, as previously documented (McLeod *et al*., 1985). Secondly, carbapenems are renowned for their exceptionally broad spectrum of activity and high potency against both Gram-positive and Gram-negative bacteria. Consequently, carbapenems are frequently regarded as “last-line agents” or “antibiotics of last resort”, as highlighted by Papp-Wallace *et al*., (2011).

These antibiotic selections were made due to the clinical significance and relevance of these antibiotic classes in combating bacterial infections, underscoring their importance in both research and clinical practice.

In this particular case, there were a total of 29 samples, out of which 9 samples were from irrigation water, 10 samples from ecological food, and an additional 10 samples from the soil of ecological food. This equalisation of sample numbers could provide a balanced and fair assessment, in contrast to the analysis of the 17 strains mentioned in 5.2.

The results from this approach revealed interesting insights. Among the samples, it was observed that in ditch water, 6 samples (66.7%) contained one or more resistance genes. Among the ecological food samples, 7 samples (70%) displayed one or more resistance genes. In soils from ecological food, 5 samples (50%) exhibited the presence of at least one resistance gene.

Remarkably, the highest level of resistance was found in ecological vegetables/fruit, followed by ditch water and soil. This observation reinforces previous findings and aligns with existing studies, such as those conducted by Araújo *et al.*, (2017) and Hölzel *et al.*, (2018), which have highlighted a correlation between the presence of resistance genes in vegetables, fruit, soil and irrigation water.

In this study, the most prevalent resistance gene identified was bla_{TEM} which was found in 20.7% of the samples. Interestingly, a study conducted by Su *et al*., (2020) reported a similar observation. In their research, the absolute abundance of $bla_{TEM,}$ a β-lactam resistance gene, was also notably high in various aquatic environments, including a river, lake and sea in China.

These findings not only highlight the prevalence of bla_{TEM} but also emphasise the global nature of antibiotic concerns. These results underscore the importance of considering all potential sources surrounding raw vegetables and fruit consumed by the population when addressing antibiotic resistance concerns. The presence of antibiotic resistance genes in these sources emphasises the need for a comprehensive approach to understanding and managing antibiotic resistance, taking into account various environmental reservoirs.

5.4. PRESENCE OF ANTIBIOTIC-RESISTANT BACTERIA IN ORGANICALLY GROWN FOOD

Only 4 out of the 17 selected isolated strains (oxidase-negative, catalase-positive and Gram-negative) and 20 out of 29 samples analysed were organic in nature. The 4 organic isolated strains were all isolated from soil, i.e; soil from wonder lettuce MacConkey agar (C25M1), soil from wonder lettuce SuperCarba (C25SC1), red cabbage soil (C26SC2), cabbage soil (C27M1) and soil from strawberry (C29SC1). While the other 20 samples, which were placed in non- selective pre-enrichment broths, including 10 organic soil samples and 10 organic vegetable/fruit samples.

The antibiogram results for these strains showed that all of them are multi-resistant (80% of the strains from soils) except for C27M1, showing resistance to 3 or more classes of antibiotics. In Jimenez Belenguer *et al.* (2023) 33.5% of isolates from organic vegetable samples (from a total of 161 isolates) had multidrug resistance, these differences could be due to the low number of isolates employed in this work. C25M1 (*Pseudomonas oryzihabitans*) showing resistance to CTX-C-AMP-AMC. The PCR of this strain has also identified the presence of β-lactam and carbapenem resistance genes, such as, bla_{TEM}, bla_{SHV}, bla_{OXA}, bla_{KPC}. C25SC1 and C26SC2 both identified as (*Stenotrophomonas maltophilia)* through biochemical testing and showed resistance to CTX-CRO-IMP-MEM-CN-AMP-AMC. This shows a little contrasting results since its PCR showed only the presence of β-lactam resistance genes. Another strain, C27M1 (*Enterobacter cloacae*) was resistant to CTX-CRO-CAZ-AMP-AMC and its PCR showed only the presence of carbapenem-resistant genes ($bla_{IMP,}$ $bla_{OXA,}$ bla_{KPC}). Finally, C29SC1 showed resistance to MEM-CN-AMP-AMC, while its PCR has shown only the presence of carbapenem resistant genes (bla_{OXA}).

Similarly, the PCR for the soil and vegetable samples in broth (A/T) showed the presence of resistance genes. i.e; C3A/C18T/C21A/C21T-cabbage, showing the presence of resistance genes bla_{VIM}, bla_{KPC} while C24A-cabbage soil sample showing positivity for bla_{TEM}. C5A-strawberry samples were positive for bla_{TEM} and bla_{SHV} while C29T-strawberry soil samples were positive for bla_{CMY-2}. C8A/C20T/C22T-red cabbage showed the presence of *qnrB*, $bla_{CMY-2,}$ and bla_{TEM}/bla_{IMP}/bla_{KPC} respectively, while

C26T/C28T-red cabbage soil is positive for bla_{IMP}. Finally, the C19A-lettuce marvel sample turned out to be positive for bla_{OXA}. Goudarzi *et al.*, (2019) found in clinical isolates of *A. baumannii* the carbapenemases genes bla_{IMP} in 3.9% and bla_{VIM} in 7% of the isolates, so that exists the possibility of spreading of these resistance genes to the human environment, too.

While it is true that organic food is produced according to specific standards, with lesser use of pesticides and with the aim to yield healthy and nutritious food (Dangour *et al*., 2009), nevertheless there is no evidence which shows that organically grown food does not contribute to antibiotic resistance as it could be seen with the results of this work.

6. CONCLUSIONS

Successful isolation of antibiotic-resistant strains of *E.coli* and *Acinetobacter baumannii* from water and organic soil samples were observed which poses a significant danger to human health. Moreover, ARGs can transfer from environmental bacteria to pathogenic bacteria and can render antibiotics ineffective against diseases caused by these pathogens. Besides, if ARGs are present in these sources, they can enter the food chain, leading to antibiotic-resistant bacteria in the food supply. Consuming such contaminated food or water can introduce antibiotic resistance into the human gut microbiome.

All of the 17 isolated strains (oxidase-negative, catalase-positive and Gram-negative) were resistant to AMP and AMC upon sensitivity testing, which suggests a widespread resistance to this class of antibiotics among these strains. Following penicillins, the strains showed resistance to third-generation cephalosporins and carbapenems (β-lactams). While β-lactams showed the highest resistance rates, there were also varying degrees of resistance to other antibiotic classes, including gentamicin (CN), chloramphenicol (C), tetracycline (TE), and quinolones (levofloxacin-LEV, ciprofloxacin-CIP, nalidixic acid-NA). These resistance patterns suggest a complex antibiotic resistance profile in these strains. The resistance to multiple β-lactam antibiotics is a cause for concern as these are commonly used for treating a wide range of infections.

Analysis of resistance genes in these strains revealed similar findings. A significant proportion of resistance genes were associated with carbapenems and β-lactams, suggesting a genetic basis for these resistances. Additionally, resistance genes to quinolones were also identified, though to a lesser extent.

A notable finding was the high prevalence of multi-resistance, defined as resistance to 3 or more antibiotic classes, which was observed in 58.8% of the 17 strains. Multi-resistance is a significant concern as it limits treatment options.

It was observed that molecular identification methods were more accurate and specific, while biochemical methods were quicker and required less specialised equipment.

It was challenging to definitively attribute the source of antibiotic resistance among the 17 strains to either irrigation water or the soil of ecological vegetables/fruit. Apparently, the water source showed higher resistance. The analysis of the 29 samples in broths revealed that the highest content of resistance genes was found in organic vegetables/fruit, followed by ditch water and soil. Despite the challenges in pinpointing the primary source, there is a correlation between the presence of resistance genes in organic vegetables/fruit, soil and irrigation water. This suggests a complex interplay between organic vegetables/fruit, soil and irrigation water in terms of antibiotic resistance. While the specific source of resistance remains challenging to determine, the observed correlation underscores the importance of further investigation into how antibiotic resistance is disseminated within ecological systems and its potential impact on human health and agriculture.

While it is true that antibiotic resistance is on surge, nevertheless, it could be controlled by taking appropriate measures, such as responsible use of antibiotics in healthcare, agriculture, and aquaculture, as well as to improve wastewater treatment practices. Additionally, practising good hygiene, vaccination to prevent infections, investment in research, development of new antibiotics and alternative treatments, establishing robust surveillance systems, raising awareness and educating

the public are essential to understanding the extent of the problem and taking measures to address it.

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