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Identification and Characterization of a Novel Bacterial Species Found in the Lebanese Soil

By

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A thesis

Submitted in partial fulfillment of the requirements for the degree of Master of Science in Pharmaceutical Development and Management

School of Pharmacy

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DEDICATION

To my parents, Georges and May, for your endless love and unwavering support and to my brother, Majid, for making me laugh, no matter how stressed I am. I love you, forever

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Identification and Characterization of a Novel Bacterial Species Found on a Mushroom Compost in the Lebanese Soil

Shirine Kyprianos

ABSTRACT

The disruption of the soil's ecosystem causes a disturbance in microbial communities and leads to the emergence of dangerous resistant pathogens and most antibiotic resistance genes. An unknown bacterial strain was collected from a mushroom compost in the Lebanese soil as part of another project aiming to identify bacteria capable of being used in bio-cementation. Our study aims to identify and characterize the unknown strain, its features, behavior, and infectious potential. Standardized techniques were used to determine the morphology and staining properties of the bacteria, biochemical and physiological reactions, and susceptibility and resistance to different antimicrobial agents. Since the strain originated from a mushroom compost in the soil, potassium hydroxide ruled out fungal entities. The bacteria are Gram-negative, rod-shaped, obligate aerobes, non-spore forming and non-encapsulated, urease, coagulase, and catalase-positive, oxidase-negative, possessing β-hemolytic activity, and resistant to UV light. The bacterial strain did not form biofilms at the air liquid interface and produced calcium carbonate precipitates. Antimicrobial susceptibility tests were carried out using antibiotics of major classes and *Cannabidiol* (CBD) oil. The strain conferred resistance to azithromycin, doxycycline, and trimethoprim-sulfamethoxazole. CBD demonstrated bactericidal activity at low doses but did not have an effect at higher concentrations. Based on the results obtained, we determined that the strain belonged to the genus *Gluconacetobacter.* All in all, these findings provide preliminary results related to the potential virulence of the bacterial species and its resistance to different antimicrobials. Future *in vivo* studies are required to determine the infectivity spectrum of the bacteria. Whole genome sequencing is also essential to detect and analyze resistance genes, virulence factors, and clusters for the production of bio-cement.

Keywords: Bacteria, Antimicrobial Resistance, Soil, Identification Tests, Antimicrobial Susceptibility Tests.

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LIST OF ABBREVIATIONS

AFB ………………………………………………………………….. Acid-fast bacilli
A-L ………………………………………………………………………….. Air-liquid
AMR ………………………………………………………… Antimicrobial resistance
ARB……………………………………………………….Antibiotic-resistant bacteria
ARGs …………………………………………………...Antimicrobial resistance genes
AST…………………………………………………….Antibiotic susceptibility testing
$CaCO_3$ ………………………………………………………………Calcium carbonate
CBD ……………………………………………………………………….Cannabidiol
CLSI…………………………………………Clinical and Laboratory Standards Institute
dH_2O……………………………………………………………………Distilled water
ESBL …………………………………………………...Extended-spectrum β-lactamases
FTM…………………………………………………………Fluid thioglycollate medium
HCl……………………………………………………………………...Hydrochloric acid
H_2O_2 ……………………………………..…………………Hydrogen peroxide
KOH………………………………………………………………Potassium hydroxide
LBA………………………………………………………………….Luria Bertani agar
LBB…………………………………………………………………...Luria Bertani broth
MICP……………………………Microbially induced calcium carbonate precipitation
MHA………………………………………………………………Mueller-Hinton agar
MRSA…………………………………Methicillin-resistant *Staphylococcus aureus*
RBC…………………………………………………………………….Red blood cells
PG……………………………………………………………………..Peptidoglycan
PBP…………………………………………………….Penicillin-binding proteins
SASPs…………………………………………………….Small acid-soluble proteins
SMC………………………………………………………Spent mushroom compost
TGB…………………………………………………………………..Thioglycollate broth
TMP-SMX…………………………………………Trimethoprim-sulfamethoxazole
TSB…………………………………………………………………..Trypticase soy broth
ZOI……………………………………………………………………..Zone of inhibition

PREAMBLE

The soil is known to be a great host for a large number of microorganisms. Some of these are beneficial to human life while others are considered harmful human pathogens. Soil pathogens originate from a disturbed ecosystem caused by the presence of fecal matter, wastes, manure, sewage, heavy human interference, climate change, and the thawing of permafrost and burial sites (Heuer et al., 2011). The soil is also considered a major contributor to AMR as it comes into direct contact with different antibiotics used in livestock rearing and plant agriculture. Moreover, it is the habitat for *Streptomyces*, a bacterial genus known for its ability to yield the majority of naturally produced antibiotics such as chloramphenicol, clavulanic acid, tetracycline, vancomycin, and others (Sethi et al., 2013). The prevalence of antimicrobial resistance genes (ARGs) in agricultural soils has risen since antibiotics were employed for growth promotion in animal farming and spread to agricultural areas through manure application. Compared to non-manured soils, manure application can transmit antibiotic-resistant bacteria (ARB) and ARGs to soils, increasing the growth of antibiotic-resistance reservoirs (McMillan et al., 2019).

Bio-cementation, known as "microbially induced calcium carbonate precipitation" or MICP, is a green technique that utilizes ureolytic bacteria to produce calcium carbonate, ultimately improving the mechanical properties of cement. MICP has been studied using various bacterial species. Typically, *Bacillus* bacteria are applied to fix the structural cracks of cement-based products (Leeprasert et al., 2022). However, only pure and safe bacterial cultures should be utilized to protect those who come in contact with the biocement produced. Another criterion for selecting microorganisms for MICP is their susceptibility or resistance to antimicrobials.

The bacterial strain was isolated from mushroom compost in the Lebanese soil, in the frame of utilizing it in bio-cement applications. Upon 16S rRNA gene sequencing, the strain displayed scores below the threshold of defining the bacterial genus and species. Therefore, this study aims to identify and characterize the novel bacterial species using standardized microbial and biochemical techniques.

CHAPTER ONE

BACKGROUND AND LITERATURE REVIEW

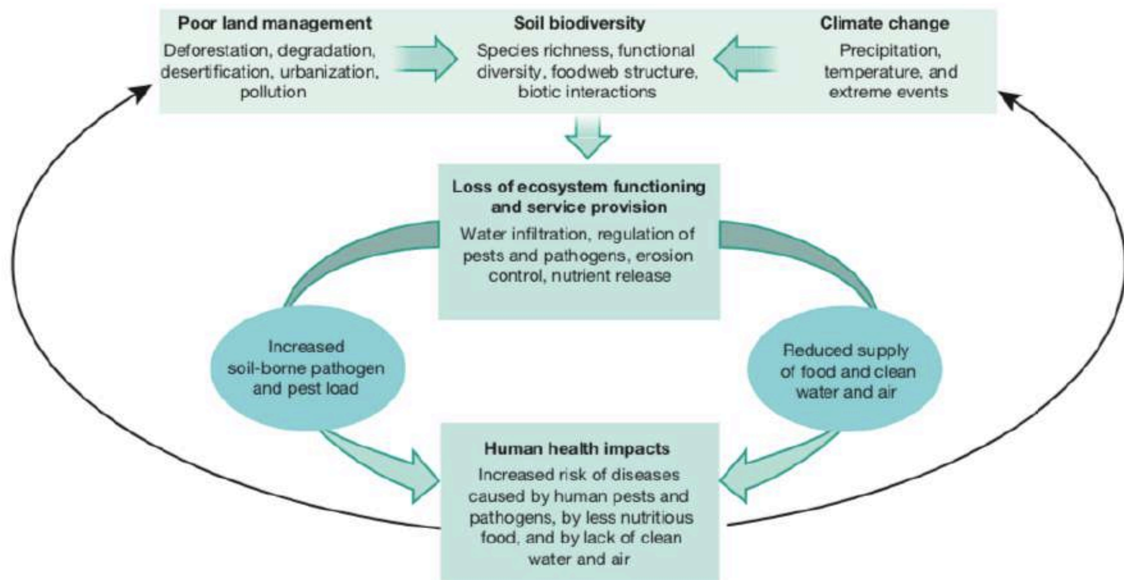
1.1. Soil as a habitat for a large bacterial community

1.1.1. The biological environment of soil and its link to human health

Soil is one of the most diversified, complex, and heterogeneous habitats on Earth. It houses one of the largest and richest microbial communities (Aleklett *et al.,* 2018; Thakur *et al.*, 2020). Soil is regarded as a valuable resource for the environment, agriculture, and human health. A key source of fiber, food, fuel, and biodiversity, it possesses the nutrients required for human and plant life to function properly (Steffan *et al.,* 2017). Soil microorganisms drive many biological cycles necessitating essential elements such as nitrogen, carbon, iron, and phosphorous. This largely contributes to a variety of functions, including healthy plant production and proper nutrient cycling, which are tightly linked to a healthy ecosystem. As such, these organisms have beneficial effects on water and air quality, pollution reduction, and suppress the emergence of harmful pathogens (Tecon and Or, 2017).

Ultimately, soil biodiversity is linked to human health, typically affected by different types of soils. Natural soils usually yield very little anthropogenic contamination and disturbance. Their soil-borne pathogens do not threaten human health as they are kept in check by a proper ecosystem (Jeffery and van der Putten, 2011). On the other hand, soils in mines, urban areas, oil and gas extraction zones, landfill and permafrost sites, and agricultural sites using pesticides and other chemicals share a higher anthropogenic disruption of their microbial communities (Steffan *et al.,* 2017). Such disturbed soils caused by human interferences, changes in the climate, and poor land management, have negative effects on the microbiota, leading to harmful effects on human health, as shown in figure 1.1 (Wall *et al.,* 2015).

Figure 1.1 - Flow diagram adapted from Wall et al. (2015) representing the link between human health and soil biodiversity.



Humans can be exposed to soil pathogens (such as fungi, actinomycetes, and bacteria) through three main routes: ingestion, inhalation, and skin absorption and penetration (Brevik *et al.,* 2020). Infection by ingestion is more common in children. It can occur accidentally when raw vegetables or fruits are consumed, or through hand-to-mouth contact (von Lindern *et al.,* 2016). Although ingestion of soil materials can potentially deliver vital nutrients, nonetheless, it can expose the individual to harmful pathogens, heavy metals, and other chemicals (Lupolt *et al.,* 2020). Inhalation of soils may lead to serious problems affecting the lungs, such as coccidioidomycosis, commonly known as "valley fever" (Dobos *et al.,* 2021), chronic bronchitis, inflammation of the bronchioles, and emphysema (Zosky *et al.,* 2013). Soil pathogens and chemicals may also penetrate the skin. The absorption of harmful elements can lead to both infectious diseases including tetanus and helminthiasis (Moynan *et al.,* 2018; Mascarini-Serra, 2011), and non-infectious diseases, such as podoconiosis, a type of non-filarial elephantiasis affecting individuals who walk barefoot on volcanically-derived clay for a long period (Deribe *et al.,* 2013).

As humans continue to disrupt the environment and the soil ecosystem, both novel and ancient pathogens have a high chance of emerging from the soil (Steffan *et al.,* 2020). *Bacillus anthracis* are Gram-positive bacteria responsible for anthrax disease. They have been found in the soil all over the world as dormant endospores (Dragon and Rennie, 1995). Climate change, heavy rains, and soil degradation can transport the spores to the surface, increasing the exposure to a serious infection that can be acquired through touch, inhalation, or ingestion (Steffan *et al.,* 2020). Other soil bacteria having the potential to infect people include *Campylobacter* spp., *Escherichia coli*, *Legionella* spp., *Mycobacteria leprae*, *Salmonella enterica*, and *Shigella* spp. Moreover, the melting of the permafrost (a frozen layer under the surface of the Earth) and northern areas, unthaws microorganisms that have long been eradicated. One example would be the re-emergence of an ancient bacterial species, *Yersinia pestis*, responsible for the deadly Black Plague. *Y. pestis* had re-emerged in specific geographical locations after staying dormant for a long time in the soil (Ayyadurai *et al.,* 2008). Madagascar for instance is one of the most affected countries in the world by plague outbreaks (Ditchburn and Hodgkins, 2019).

1.1.2. Bacterial diversity in the soil

Soil bacteria have yet to be fully identified (Fierer and Jackson, 2008). This resonates with the fact that soil bacteria are the most abundant and distinct organisms on Earth (Young and Crawford, 2004). Moreover, more than 99% of the bacterial species found in the soil are difficult to culture, and less than 1% of these are representative of the soil's biodiversity (Pham and Kim, 2012). They can adapt to a changing soil matrix which can alter the structure, diversity, and activity of the microbial community (Tang *et al.*, 2017).

Bacterial distribution in the soil is neither random nor uniform. Bacterial activity reflects the soil's conditions, transforming the soil-microbe complex into a self-organizing entity (Pulleman *et al.*, 2012). With constantly changing environmental circumstances, soil-bacteria interactions are very dynamic. For example, the distribution of bacteria and their supply of substrates and oxygen is affected by water content (Li *et al.*, 2017). Other parameters influencing the diversity of the soil microbiota include soil pH, particle size,

and enzyme activity (König *et al.*, 2020). Moreover, it has been shown that healthy soils possess a more abundant diversity in terms of beneficial bacteria than infected soils. Such beneficial bacteria include *Agromyces, Bacillus, Bradyrhizobium, Lysobacter, Mesorhizobium, Micromonospora, Microvirga,* and *Pseudonocardia*, which increase the soil's quality while decreasing disease incidence. Infected soils, however, share higher urease and invertase activities, a higher soil pH, and a larger potassium and phosphorous content (Wang *et al.*, 2017).

As such, not all bacterial species are equally ample in the soil. *Bradyrhizobium* spp. for example, is highly abundant in North American forest soils (Chalasani *et al.*, 2020), whereas *Spartobacteria* spp. is dominant in grassland soils (Bergmann *et al.*, 2011). 16S rRNA gene sequencing has allowed a more direct classification of soil bacteria without the restrictions of cultivation-based studies (Boomer *et al.*, 2002). In 1968, Vagn Jensen determined that bacterial colonies formed through cultivation-based approaches were not representative of the overall soil bacterial community (Janssen, 2006). 16S rRNA gene sequencing has also allowed the global analysis of soil samples from 237 locations spread across six continents and eighteen countries. Different soil samples were tested and included soils with high and low pH, low productivity, and soils from drylands and dry forests. It was determined that *Proteobacteria* spp., *Actinobacteria* spp., *Acidobacteria* spp., *Planctomycetes* spp., *Chloroflexi* spp., *Verrucomicrobia* spp., *Bacteroidetes* spp., *Gemmatimonadetes* spp., and *Firmicutes* spp. were the most abundant species globally (Delgado-Baquerizo *et al.*, 2018).

Nonetheless, 16S rRNA genes and PCR-amplified 16S rRNA libraries may not be a comprehensive or accurate representation of the bacterial population in soils. Combining all of the reported sequences would appear to be an insufficient count of all 16S rRNA genes on the planet. 79% to 89% of the 16S rRNA gene sequences come from bacteria that don't belong to any known genera (Schloss and Handelsman, 2004). As such, the use of conventional biochemical and staining tests may distinguish, characterize, and identify bacteria that are not found in the current 16S rRNA sequencing databases (Poretsky *et al.*, 2014). In Lebanon, the most common oleaginous microorganisms were identified, using

standard identification techniques. The samples originated from different soil sites (wetland, sand, lawn, and farmland) across the country. The most common oleaginous bacteria detected were *Escherichia coli*, *Arthrobacter* spp., *Pontoea* spp., *Agrobacterium* spp., and *Chryseobacterium* spp. (El-Haj *et al.*, 2015).

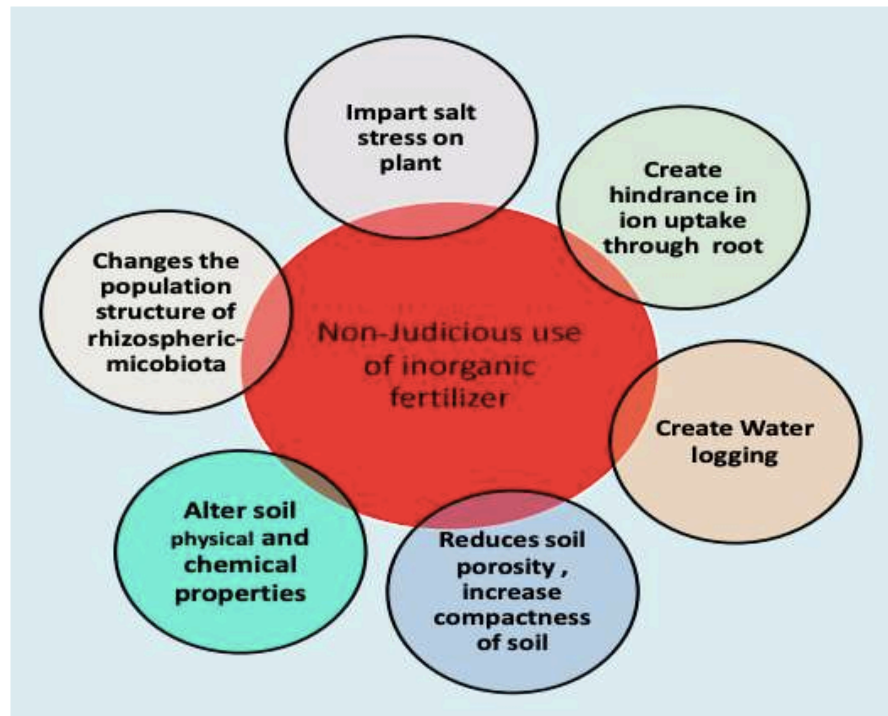
1.2. Mushroom composts and the spectrum of microbial diversity

1.2.1. Characteristics and benefits of mushroom composts

Mushroom composts, commonly called “spent mushroom compost” (SMC), are organic plant fertilizers. They are produced using organic materials such as corn cobs, poultry or horse manure, gypsum, and water. They enrich the soil with nutrients, increase its water-holding capacity, and allow the organic growth of plants, fruits, herbs, and vegetables (Uzun, 2004). From a microbiological perspective, mushroom composts represent an intriguing example of an ecosystem, rich in different microbial species including thermophilic and mesophilic bacteria, actinomycetes, and fungi (Agrawal *et al.*, 2015).

SMC contains a high amount of salt, organic elements, enzymes, and nutrients, making it a great habitat for a range of microorganisms (Singh *et al.*, 2020). These microorganisms, including bacteria and fungi, are synergistically beneficial in plant growth and disease suppression (Patil *et al.*, 2018). Pesticides are commonly used in agriculture to reduce pests and illnesses from crops that endanger the Earth’s food supply. However, inorganic fertilizers and specifically pesticides have a lot of negative and toxic effects on human health, mainly by affecting the soil and water quality (Masiá *et al*, 2015), as demonstrated in figure 1.2. In contrast, due to its high organic matter and low toxic materials, SMC serves as an attractive alternative to fertilizers and pesticides (Marín-Benito *et al.*, 2016). Moreover, using SMC as plant fertilizer improves the soil’s physicochemical properties (Pathak *et al.*, 2021).

Figure 1.2 - Diagram adapted from Pathak et al. (2021) representing the relationship between the use of inorganic fertilizers and soil properties.



1.2.2. Microbial diversity in mushroom composts

Microorganisms in mushroom composts play an essential role in breaking down organic material for plant consumption. The biological, physical, and chemical environments of SMC differ greatly based on the cultivation techniques, composting processes, mushroom species used, and weather conditions (Catal and Peksen, 2020). The dominant bacteria found in different compost ecosystems are *Bacilli* (Ferreira Silva *et al.*, 2009). The pioneer bacteria, however, are *Pseudomonas* and *Arthrobacter*, two fast-growing bacteria that rapidly degrade a high amount of organic material (Hayes *et al.*, 1967). Kleyn and Wetzler (1981) isolated and identified the microorganisms found in different mushroom compost samples. They determined that the most common bacteria found in all types of samples was *Bacillus licheniformis*, a Gram-positive, endospore-forming, saprophytic organism. Zhang *et al.* (2021) conducted a 16S rRNA gene sequencing study to detect the common microorganisms found in SMC samples. The results showed that the predominant fungi were *Ascomycota*, while the predominant

bacteria were *Firmicutes* and *Actinobacteria* (Gram-positive), *Bacteroidetes*, and *Proteobacteria* (Gram-negative). However, the dominant microorganism detected in all samples was *Planifilum fulgidum*, a Gram-positive, thermophilic bacterium. Of eight different bacterial genera and species identified by 16S rDNA sequencing of SMC samples and in phase II composting samples, four were found to be associated with human pathogens: *Bacillus licheniformis*, *Bacillus subtilis*, *Klebsiella/Enterobacter* spp., and *Sphingobacterium multivorum* (Watabe *et al.*, 2003). Table 1.1. reviews the pathogenicity of the above-mentioned pathogens.

Table 1.1 - Potential infectious role of pathogens found in spent mushroom compost and phase II composting samples.

Bacteria detected in the samples	**Type of infection caused and description**	**References**
Bacillus licheniformis	Sepsis in immunocompromised patients	Haydushka *et al.* (2012)
	Peritonitis and food poisoning	Park *et al.* (2006)
Bacillus subtilis	Gastrointestinal infections post-ingestion of bacteria and spores	Richard *et al.* (1988)
Klebsiella/Enterobacter spp.	Septicemia in nosocomial infections	Kim *et al.* (2002)
Sphingobacterium multivorum	Meningitis and fatal case of bacteremia in a 28-year-old immunocompromised male	Abro *et al.* (2016)

1.3. The alarming rise of antimicrobial resistance

1.3.1. A probable return to the pre-antibiotic era

Antimicrobial resistance (AMR) refers to the resistance acquired by microorganisms to an antimicrobial agent against which they were initially sensitive (Jindal *et al.,* 2014). It arises when antibiotics predominantly fail to completely eradicate

infections. This allows bacteria to become resistant to the antibiotic used (Rechel *et al.,* 2018). Although AMR is a natural evolutionary phenomenon, it is much encouraged by the suboptimal use and misuse of antimicrobials and their widespread use in agriculture and farming (Llor and Bjerrum, 2014). Antibiotic-resistant bacteria (ARB) cause severe infections and illnesses and increase the risk of complications, length of hospital stays, and mortality rates (Kollef, 2008). Two important factors have contributed to the tremendous bacterial resistance witnessed around the globe:

1. The improper stewardship and prescription of antimicrobial agents: prescribing unnecessary broad-spectrum or ineffective antibiotics increases the resistance of the bacteria causing the infection (L Yu, 2011).
2. The overuse and excessive consumption of antibiotics (Griffith *et al.,* 2012): prior use of antibiotics increases the chance of acquiring a drug-resistant organism. Patients who were highly exposed to antibiotics are more likely to be infected later on by resistant bacteria (Tacconelli, 2009).

A pandemic-like spread of antimicrobial-resistant genes (ARG) is slowly developing and may grow into a big threat if no immediate and global corrective action is taken.

Figure 1.3 - Timeline adapted from Davies and Davies (2010) displaying the evolution of discovery and the development of resistance to the major antibiotic classes.

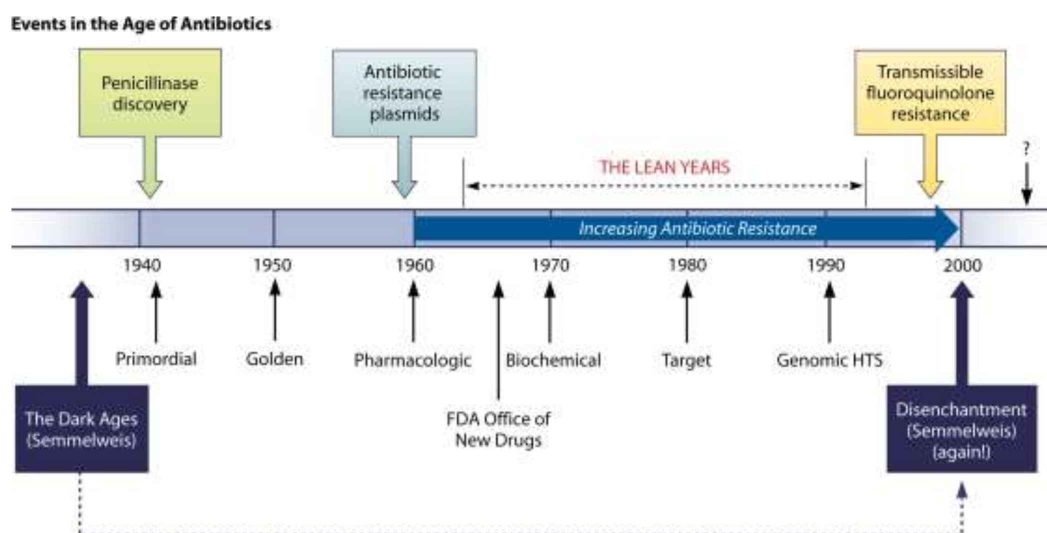


Figure 1.3. details the evolution of the discovery of antibiotics and the resistance of bacteria. The dark ages represent the pre-antibiotic era of the 1900s, where antibiotics were not available to treat common germs, causing enormous human suffering and loss. Prior to the discovery of antibiotics, Ignaz Semmelweis, a Hungarian physician, emphasized the importance of hand washing to prevent infections. The 1950s (primordial era) are highlighted by the introduction of chemotherapy. The halcyon years of the golden era represent the time when most antibiotics were discovered. Soon after, antibiotic-resistant plasmids and resistance mechanisms became evident. The lean years describe the decreased discovery of novel antibiotics and the increasing antibiotic resistance. Different strategies were studied to limit resistance related to pharmacology, biochemistry, and genetics (high throughput sequencing (HTS) and genome sequencing) (Davies and Davies, 2010). Today, the healthcare system faces the disenchantment era, where pharmaceutical companies discontinue their search for novel drugs after the failure of genome-based strategies (Plackett *et al.,* 2020). As such, frequent hand washing (as initially advocated by Semmelweis) is necessary to prevent transmission.

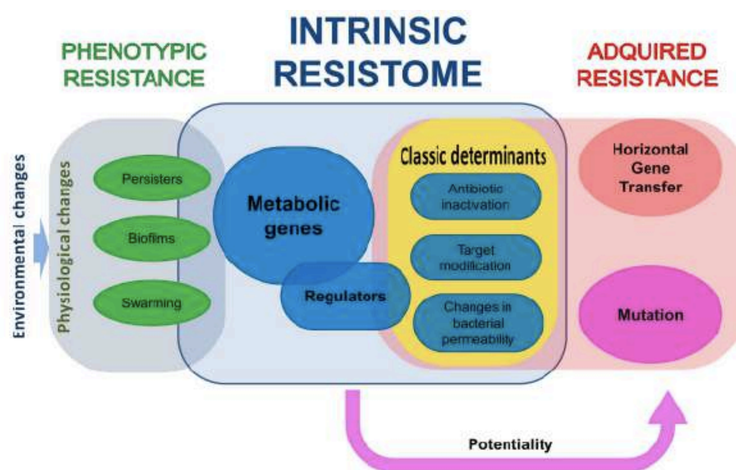
1.3.2. Mechanism of bacterial resistance

Bacterial resistance was first reported in the late 1930s to sulfonamides, soon after their introduction in 1937. Today, it has affected other antibiotic agents and almost all infectious diseases face the menace of AMR (Davies and Davies, 2010). In 1928, Alexander Fleming discovered penicillin, but it was not introduced as a therapeutic drug until the mid-late 1940s. However, in 1940, years before its use by the general public, a bacterial penicillinase was detected, able to degrade and destroy penicillin (Abraham and Chain, 1940). Soon after bacteria became widely resistant to penicillin, β-lactamase inhibitors were synthesized to salvage the drug.

Interestingly, the discovery of an enzyme capable of inactivating a drug before its introduction to the general public begs the question of what came first. The antibiotic or the resistance?

Bacteria may become resistant to antibiotics through natural or acquired resistance (Hughes and Andersson, 2017). A resistome is a collection of all the ARGs of both pathogenic and non-pathogenic bacteria. The intrinsic resistome represents the repertoire of elements that contribute to the susceptibility and resistance of bacteria to antibiotics (Kim and Cha, 2021). As displayed in figure 1.4, these elements may be classical determinants of resistance (i.e., antibiotic-inactivating enzymes, target modification, and changes in cell membrane permeability). Mutations in these enzymes might make some bacteria more susceptible to antibiotics, while others may acquire an increased resistance. Phenotypic resistance is achieved by the development of persistence, growth of bacterial biofilms, swarming adaptation, and others (Olivarez *et al.,* 2013).

Figure 1.4 - Figure adapted from Olivarez et al. (2013) displaying the various elements in bacterial resistance.



Natural resistance

Natural resistance occurs when resistance genes are present in the bacteria but are only expressed when exposed to an antibiotic. Intrinsic antimicrobial resistance (IAR) is another form of natural resistance, where it is always expressed (Reygaert, 2018). IAR is a commonly shared trait within a bacterial species, unrelated to horizontal gene transfer, and independent of prior antibiotic exposure (Martinez, 2014). IAR occurs through efflux pumps or decreased permeability of the outer cell membrane, most importantly that of the lipopolysaccharide layer of Gram-negative bacteria (Cox, 2013). Table 1.2. displays some common intrinsically resistant bacteria (Reygaert, 2018).

Table 1.2 - Antibiotic groups and drugs based on their mechanism of action.

Bacteria	**Intrinsic resistance to antibiotics**
All Gram-positive bacteria	Aztreonam
All Gram-negative bacteria	Glycopeptides and lipopeptides
Bacteroides (anaerobes)	- Aminoglycosides - Fluoroquinolones - Many β-lactams
Enterococci	- Aminoglycosides - Cephalosporins - Lincosamides
Pseudomonas aeruginosa	- Ampicillin - First and second generation cephalosporins - Chloramphenicol - Sulfonamides - Tetracyclines
Escherichia coli	Macrolides

Acquired resistance

Bacteria acquire resistance through mutations of a chromosomal gene or the acquisition of mobile resistance genes, known as "horizontal gene transfer" (Cloeckaert *et al.*, 2017). The acquired genes are not mobile themselves but are rather carried by different structures allowing their "horizontal" transfer. Such mobile genetic elements include plasmids and genomic islands (Juhas *et al.*, 2008). Acquired resistance genes code for three fundamental mechanisms of resistance (Schwarz *et al.*, 2016):

1) Decreased intracellular accumulation of antibiotics.
2) Alteration of antibiotic target sites.
3) Enzymatic inactivation of antibiotics.

Decreased intracellular accumulation of antibiotics:

As previously discussed, bacteria may become resistant to antibiotics by preventing their accumulation inside the cells either by decreased uptake or increased efflux. To exert their antimicrobial effects, antibiotics act on intracellular targets. In the case of Gram-negative bacteria, these targets are located on the inner (cytoplasmic) membrane. Thus, the drug has to penetrate the outer membrane through water-filled channels called "porins" (Munita and Arias, 2016). Over time, bacteria have developed mechanisms to block the entry of antibiotics inside their cells. Hydrophilic drugs (i.e., β-lactams, fluoroquinolones, and tetracyclines) penetrate the cell through the porins (Pagès *et al.,* 2008). Vancomycin serves as a great model for this resistance phenomenon. It is a glycopeptide antibiotic, inactive against Gram-negative bacteria, as it cannot penetrate the outer membrane. Acinetobacter baumanii and Pseudomonas aeruginosa are not susceptible to β-lactams partly due to a reduced number and/or expression of porins (Nikaido, 2003; Hancock and Brinkman, 2002).

Bacteria also produce complex types of machinery called "efflux pumps", capable of extruding antibiotics and other toxic agents out of their cells. Mutations in a repressor of an efflux pump make the bacteria more resistant to antibiotics (Alonso and Martínez, 2000). As soon as the antimicrobial agent enters the cell, the efflux systems pump them out before reaching their target (Webber and Piddock, 2003). Unlike porins present on the outer membrane, efflux pumps are located on the cytoplasmic membrane of the bacterial cell. Except for polymyxin, all antibiotics are susceptible to the effects of efflux pumps (Henrichfreise *et al.,* 2007). These pumps are also multidrug transporters, pumping a variety of antibiotics (e.g., fluoroquinolones, macrolides, and tetracyclines) (Džidić *et al.,* 2007).

Alteration of antibiotic target sites:

Bacteria often employ a strategy of altering antibiotic target sites through spontaneous gene mutations. Antibiotics generally interact with their targets quite specifically, to the point that even a slightly modified target site generates important outcomes on antibiotic

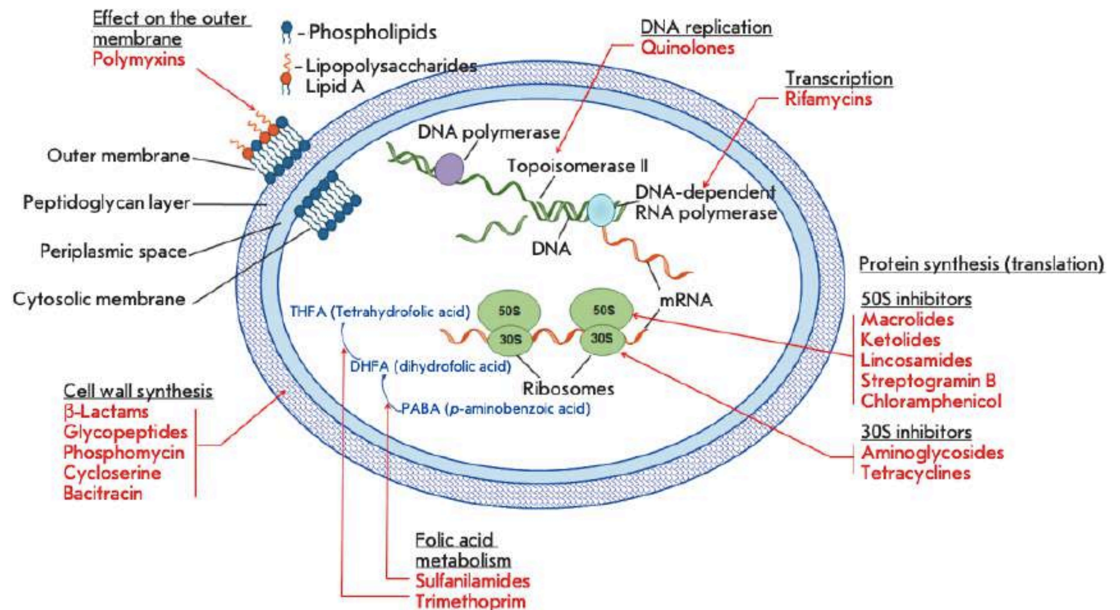
binding (Lambert, 2005). Based on their mechanisms of action (MOA), antibiotics are grouped into five categories (Kapoor *et al.*, 2017):

1. Cell wall synthesis inhibitors
2. Cell membrane depolarizers
3. Protein synthesis inhibitors
4. Nucleic acid synthesis inhibitors
5. Metabolic pathways inhibitors

Table 1.3 - Antibiotic groups and drugs based on their mechanism of action.

Mechanism of action	Antibiotic groups
Cell wall synthesis inhibitors	1) β-lactams - Carbapenems (e.g., imipenem, meropenem) - Cephalosporins (e.g., ceftriaxone, cephalexin) - Monobactams (e.g., aztreonam) - Penicillins (e.g., amoxicillin, ampicillin) 2) Glycopeptides (e.g., vancomycin)
Cell membrane depolarizers	Lipopeptides (e.g., daptomycin)
Protein synthesis inhibitors	1) Binding to 30S ribosomal subunit - Aminoglycosides (e.g., gentamicin, amikacin) - Tetracyclines (e.g., doxycycline) 2) Binding to 50S ribosomal subunit - Macrolides (e.g., azithromycin, erythromycin) - Lincosamides (e.g., clindamycin) - Oxazolidinones (e.g., linezolid) - Chloramphenicol - Streptogramins (e.g., quinupristin)
Nucleic acid synthesis inhibitors	Fluoroquinolones (e.g., ciprofloxacin, levofloxacin)
Metabolic pathways inhibitors	1) Sulfonamides (e.g., sulfamethoxazole) 2) Trimethoprim

Figure 1.5 - Figure adapted from Egorov et al. (2018) illustrating the mechanism of action of different antibiotics.



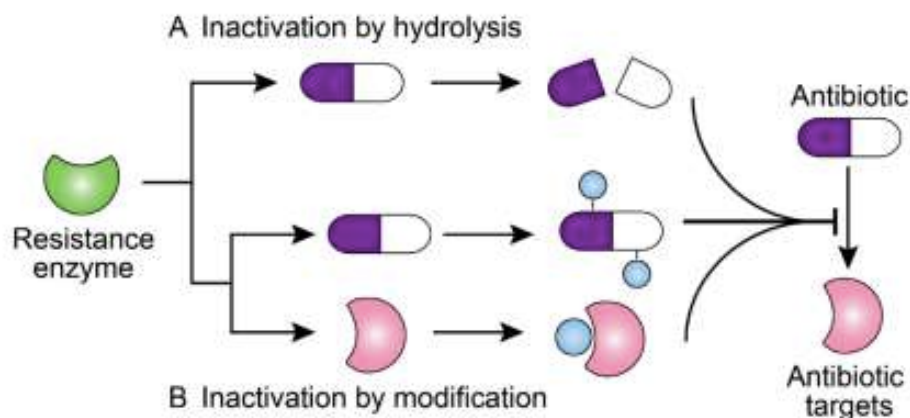
As illustrated in figure 1.5, alterations in the different target sites trigger resistance to the concerning antibiotics. For example, modifications of the 30S or 50S ribosomal subunits create resistance to aminoglycosides, tetracyclines, macrolides, clindamycin, linezolid, chloramphenicol, and streptogramins. Penicillin-binding proteins (PBP) are a group of essential proteins involved in the synthesis of the bacterial cell wall. Penicillins and other β-lactam antibiotics bind to PBP to produce their antimicrobial effect (Sauvage *et al.,* 2008). Mutations in PBP are the preferred mechanism of resistance of Gram-positive bacteria. Such mutations reduce the affinity of β-lactams to PBP, thereby decreasing their therapeutic efficacies. Moreover, fluoroquinolone-resistant bacteria produce mutations in the genes encoding for DNA gyrase and topoisomerase IV.

Enzymatic inactivation of antibiotics:

Just as Gram-positive bacteria favor the modification of PBP to overcome the effects of β-lactams, Gram-negative bacteria favor the production of β-lactamases to inactive these antibiotics. Antibiotic-inactivating enzymes, specifically β-lactamases, are the most threatening and widespread mechanism of resistance (Zeng and Lin, 2013). The modification and/or destruction of antibiotics is one of the most common modes of

resistance. Depending on the type of reaction, the resistance enzymes can be classified as either hydrolytic or modifying (Liu *et al.*, 2018).

Figure 1.6 - Scheme adapted from Liu et al. (2018) illustrating of the main antibiotic resistance mechanisms mediated by bacterial inactivating enzymes.



(A) Bacterial resistance enzymes hydrolyze and inactivate the antibiotic, conferring resistance.
(B) Bacterial resistance enzymes modify the structure of both the antibiotic and its target, conferring resistance.

β-lactamases are a superfamily of hydrolyzing enzymes, that inactivate a large group of antibiotics: penicillins, cephalosporins, carbapenems, and monobactams (Jacoby and Munoz-Price, 2005). The production of β-lactams with improved properties and a wider coverage has triggered the development of bacterial β-lactamases with bigger spectra of activity. For instance, penicillinases (which are early β-lactamases) are only active against the first generation of β-lactams (i.e., penicillins) (Pollock, 1967). On the other hand, extended-spectrum β-lactamases (ESBL), produced primarily by *E. coli* and *Klebsiella pneumoniae*, hydrolyze and destroy penicillins, third generation cephalosporins, aztreonam, cefoperazone, and cefamandole (Paterson and Bonomo, 2005). However, ESBL-producing microorganisms are resistant to carbapenems and can be killed by β-lactamase inhibitors (i.e., clavulanic acid, tazobactam, and sulbactam). Table 1.4 summarizes the resistance mechanisms of antibiotics.

Table 1.4 - Resistance mechanisms of common antibiotics.

Antibiotic class	Mechanism of resistance	Example
β-lactams	Altered PBP	*S. aureus*, *S. pneumoniae*
	Enzymatic hydrolysis (e.g., Penicillinase)	Gram-negative bacteria
Aminoglycosides	Decreased uptake	*P. aeruginosa*
	Enzymatic modification of antibiotic	Gram-negative bacteria
Macrolides	Altered target site	Vancomycin-resistant *E. faecalis* and *E. faecium*
Fluoroquinolones	Altered target site	Enteric Gram-negative bacteria
	Increased efflux	*S. pneumoniae* and *S. aureus*
Tetracyclines	Increased efflux Altered target site	Gram-positive and negative bacteria

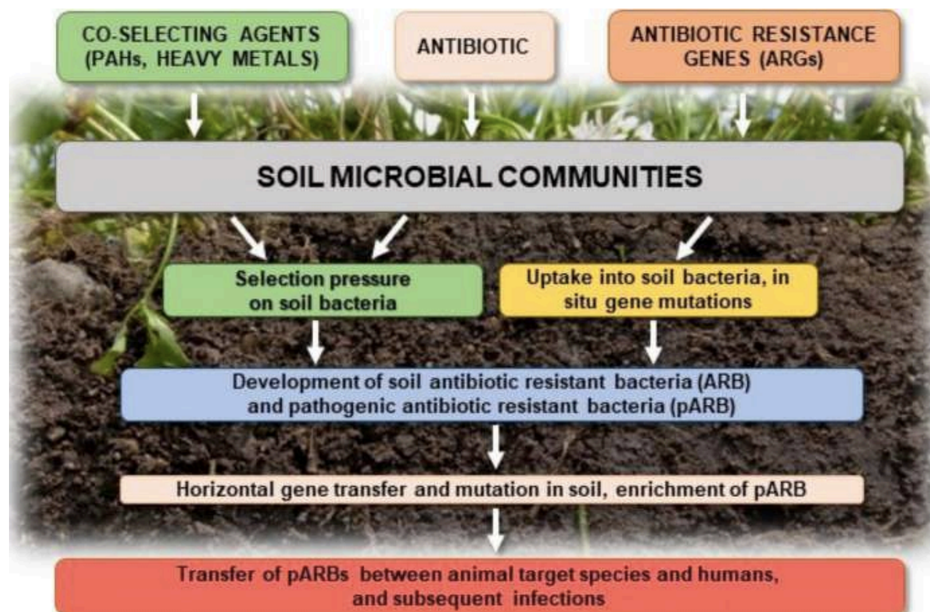
1.3.3. The soil: a reservoir of antimicrobial-resistant genes

The soil is a major environmental source of AMR and a reservoir of ARG (Cytryn, 2013). A large amount of ARG is found in soil, where they can spread across various ecosystems and be acquired by diseases that pose a threat to both human and animal health (Forsberg et al., 2012). The proliferation of ARG globally is largely caused by anthropogenic practices that disperse antimicrobial agents into the environment (Zhu et al., 2017). As previously discussed, bacteria can acquire resistance after being exposed to antibiotics at sub-therapeutic concentrations (natural selection) and/or through horizontal gene transfer between species. Such transfers between species can occur through mobile genetic elements such as transposons, integrons, or plasmids (Allen et al., 2010). ARG can remain in the soil matrix for a long period by cation binding, easing the exchange between members of the soil microbial communities (Levy-Booth et al., 2007).

Of note, soils with minimum anthropogenic contamination of synthetic and semi-synthetic antibiotics and human interference reflect natural antibiotics and their respective resistance mechanisms (D'Costa et al., 2011). For instance, soils obtained from a secluded area in Alaska displayed high amounts of β-lactamases (Allen et al., 2009). On the other hand, ancient permafrost showed genes encoding for resistance to glycopeptides and tetracyclines, and soils obtained from cave samples included multiple ARG encoding for macrolide resistance (Bhullar *et al.,* 2012). Moreover, a novel resistance mechanism has been detected in remote forest soil sites which had no previous exposure to synthetic antibiotics. Genes encoding for a non-mobile dihydropteroate synthase conferred resistance to sulfonamide antibiotics (Willms *et al.,* 2019). This suggests that sulfonamide resistance occurs naturally in bacterial communities of forest soils. Shotgun metagenomics was performed to determine ARG distribution in 17 remote and unspoiled surface soils in the Mackay Glacier region of Antarctica (Van Goethem *et al.,* 2018). The majority of the 177 naturally occurring ARGs are encoded for efflux pumps (single or multi-drug). Aminoglycosides, β-lactams, and chloramphenicol resistance were also noted. 71% of the ARGs detected were confined within Gram-negative bacteria, and 9% in the Gram-positive Bacilli and Actinobacteria. Mobile genetic elements were absent and there is a significant negative association ($p<0.05$) between the number of ARG and species diversity and richness. This suggests that although the samples were obtained from a pristine region, there is a possibility that horizontal gene transfer occurred a long time ago.

Soils harboring high concentrations of antibiotics favor the production of resistant bacteria, altering the antibiotic sensitivity of all microbial communities (Ashbolt et al., 2013). Figure 1.7 illustrates the transfer of antibiotic resistance in soils and the elevated risk of resistant infections in humans. Soils used for livestock rearing or that have been treated with manure usually contain the highest concentrations of antibiotics (DeVries and Zhang, 2016). In manure applications, tetracyclines are the most widely used antibiotics, followed by fluoroquinolones and sulfonamides (Massé et al., 2014).

Figure 1.7 - Scheme adapted from Cycoń et al. (2018) illustrating sources and fate of antibiotics in the soil.



Once in the soil environment, antibiotics become subject to different degradation and transformation mechanisms (Duan *et al.,* 2017). Antibiotic degradation depends on several factors, including the catabolic activity of soil microbes, soil pH, moisture, organic matter content, oxygen status, temperature, and texture (Cycoń *et al.,* 2018). The spreading of antibiotics and their products affects the abundance and diversity of ARG (Kyselková *et al.*, 2015). Moreover, such misuse and overuse of antibiotics in the soil affect soil bacteria by modifying their enzyme activities and altering the bacterial biomass and abundance of diverse species (Ma *et al.,* 2016).

1.4. Cannabidiol as a potential candidate to overcome resistant pathogens

1.4.1. The vital search for alternatives

As the world is entering the post-antibiotic era, the search for alternatives to antibiotics is essential. As discussed in earlier sections, bacteria are becoming highly resistant to available antibiotics, putting people's lives at risk. For instance, patients undergoing surgeries, organ transplants, dialysis, cancer care, or those with low immunity (i.e., diabetic patients), will inevitably face the wrath of resistant bacteria. The major

players in the pharmaceutical field have abandoned antibiotic research due to unfavorable World economics (Roope *et al.*, 2019). This left smaller biotech companies to pick up the slack and endure the precarious financials of antimicrobial development (Butler and Paterson, 2019). In contrast to nearly 4,000 new immuno-oncology drugs under development, only 30 to 40 new antimicrobial agents are in clinical trial development (Beyer and Paulin, 2020). Less than 25% of those represent novel classes and/or mechanisms of action, none being active against Gram-negative bacteria, which cause substantial mortality (World Health Organization, 2019). Thus, new therapeutic options are urgently needed to combat AMR.

1.4.2. The antimicrobial activity of Cannabidiol oil

Cannabidiol, commonly known as CBD, is one of more than 100 cannabinoids extracted from the *Cannabis sativa L*. plant and is its primary non-psychoactive compound. In contrast, delta9-tetrahydrocannabinol (THC) is the principal psychoactive compound of the plant. CBD is a small, phytocannabinoid with a molecular weight of 314 Da. It possesses remarkable polypharmacological properties and has been examined extensively for a wide array of diseases. Clinical studies have heavily tested the effect of CBD on cancers, appetite stimulation in HIV/AIDs patients, chronic pain management, reduction of spasticity in multiple sclerosis patients, psychological problems (i.e., anxiety, depression, sleep disorders), and more (Whiting *et al.*, 2015).

In addition to its anti-inflammatory and neuroprotective effects (Atalay *et al.*, 2019; Fernández-Ruiz *et al.*, 2012), CBD has been shown to have bactericidal activities, largely against Gram-positive bacteria (Karas *et al.*, 2020). Cannabinoids, including CBD, bind to the widely spread cannabinoid receptors in the human body, CB1, and CB2. CB1 and CB2 are present in immune cells, suggesting that CBD activates these receptors during an infection (Hernández-Cervantes *et al.*, 2017).

The research on the antibacterial activity of CBD started in the 1950s, with an interesting study published in 1976 by Klingeren and Ham. They measured the minimum inhibitory concentration (MIC) of CBD and THC for Gram-positive *Staphylococci* and

Streptococci. MIC ranged from 1-5 μg/mL. Both compounds appeared to be bactericidal in this range. On the other hand, Gram-negative *E. coli*, Salmonella typhi, and *Proteus vulgaris* were resistant to both THC and CBD (MIC>100 μm/mL) (Klingeren and Ham, 1976). CBD has also shown high antimicrobial activity against *S. aureus* and *S. epidermidis*; however, no activity was observed on Gram-negative *Pseudomonas aeruginosa* and *E. coli* (Martinenghi *et al.*, 2020). The lower efficacy of CBD on Gram-negative bacteria is present in many pieces of literature. Sarmadyan *et al.* (2013) studied the antibacterial properties of Hashish against hospital-acquired bacteria, notably *S. aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), *E. coli*, and *Klebsiella pneumoniae*. The disc diffusion experiments determined that the greatest effects of Hashish were seen on *S. aureus* with a zone of inhibition (ZOI) of 14 mm followed by MRSA (ZOI = 12 mm). Lower effects were seen on Gram-negative *E. coli* (ZOI = 10 mm) and *K. pneumoniae* (ZOI = 7 mm). No effects were observed on *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Lelario *et al.* (2018) determined that the major constituents of *C. sativa* extracts (i.e., cannabinoids, glucosinolates, and glycoalkaloids) only displayed modest activity and only against Gram-positive bacteria.

A new study conducted in 2021 demonstrated the activity of CBD oil on highly resistant strains of *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *Clostridium difficile*. It was however inactive against *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *Acinetobacter baumannii*. Interestingly, CBD did demonstrate bactericidal activity against some Gram-negative bacteria including the "urgent threat" bacteria *Neisseria gonorrhea*, *Neisseria meningitides*, *Moraxella catarrhalis*, and *Legionella pneumophila* (Blaskovich *et al.*, 2021). This report is noteworthy as previous studies suggest that CBD oil is highly effective against Gram-positive pathogens, and to a much lower extent against Gram-negative ones (Klahn, 2020). Recently, in 2022, Gildea *et al.* examined the antimicrobial effect of CBD oil on Gram-negative *Salmonella newington* and *Salmonella typhimurium* using the Kirby-Bauer disc diffusion assay. They demonstrated that CBD oil inhibits the growth of the two *Salmonella* strains. The results also suggest that as the concentration of CBD decreases, so does the ZOI (Gildea *et al.*, 2022).

1.5. Identification and characterization of unknown bacteria

1.5.1. Culturing bacteria

Bacteria don't generally require a lot of nutrients to grow. They essentially need water, carbon and nitrogen sources, and mineral salts (most importantly phosphate, sulfate, magnesium, and calcium) (Giuliano et al., 2019). Culture media are a simple combination of water and nutrients, enhancing growth. There are two main types of media: solid and liquid. Liquid media are composed of nutrients dissolved in water. They are utilized for profuse growth, which can be noticed by the formation of turbidity. Such media facilitate the access of nutrients by bacteria as they become more accessible. This is because liquid media are incubated under agitation, which allows the regeneration of nutrients. One downfall, however, is the difficulty of separating mixed organisms or colonies. To grow and isolate bacterial colonies, solid media are used, formed of liquid broths and agar, a bacteriologically-inert gelling agent (Bonnet et al., 2020). They facilitate identification by studying the colony's appearance (size, shape, color). Moreover, mixed organisms can be separated. However, the access of bacteria to nutrients in this type of medium is limited. The uses of liquid and solid media are presented in table 1.5.

Table 1.5 - Uses of liquid and solid culture media.

Liquid Media	**Solid Media**
Grow bacteria for inoculum production for various tests (e.g., motility test, antibiograms, staining, etc.)	Isolate bacteria from specimen.
Revive bacteria from stock or lyophilized cultures.	Determine colony characteristics (i.e., morphology, pigmentation, hemolysis).
Study bacterial metabolism, enzyme and toxin production.	Perform antimicrobial susceptibility test (Kirby-Bauer disc diffusion).

Solid culture media can be classified into three main types: nutritive, differential, and selective (Lagier *et al.*, 2015). They facilitate the enumeration, isolation, and identification of diverse bacteria. Nutritive media are used to support the growth of microorganisms without differentiating between species. Such media are ideal for bacteria that don't require an enriched vehicle to grow. Luria Bertani agar (LBA) is the most commonly used nutritive media used for the general routine growth of many bacteria, as it is not targeted towards a particular bacterial species (Mitsuhashi, 2001). Differential media are enriched with different compounds, allowing specific genera and species to be visually differentiated. They promote the growth of different bacterial species, each with a distinct pattern (van Netten *et al.*, 1989). In contrast, selective media contain compounds that will inhibit the growth of organisms, allowing only a specific type to grow. Commonly, antibiotics are added to such media to inhibit the growth of certain bacteria and allow the proliferation of specific ones (Subramanyam *et al.*, 2012).

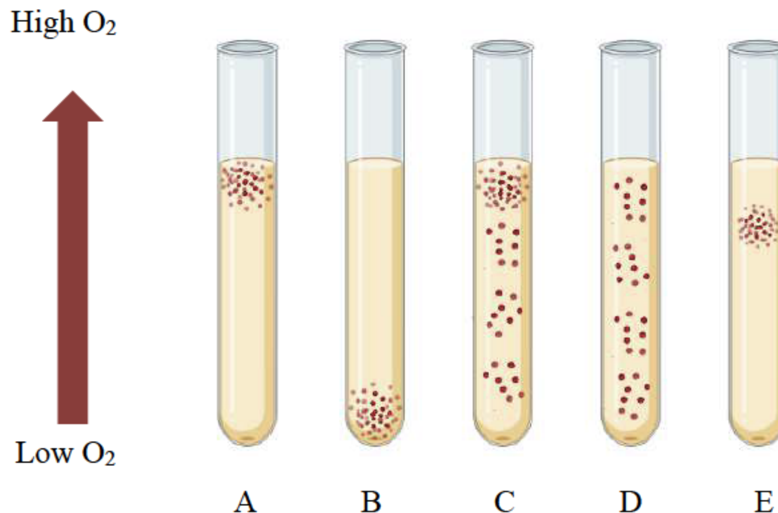
1.5.2. Morphological and physiological identification

Bacterial morphology extends beyond the most common shapes of cocci, such as *Staphylococcus aureus*; bacilli, such as *Clostridium* species; and spirals, such as *Vibrio cholera*. Some bacteria possess an exotic form, such as serpentines, stars, mustaches, or branches, which are often undefined (Young, 2006). Bacterial cell shape is determined by the cell wall structure. The peptidoglycan (PG) layer influences the cell wall's strength. This layer is composed of repeating disaccharide molecules and a peptide stem. Sugar strands are linked together followed by the transpeptidation of the peptide stems, thus forming a firm meshwork. In return, this rigid product wraps around the bacterial cell, creating turgor pressure, therefore preventing its lysis (Yang *et al.,* 2016). However, not all bacteria have the same cell wall strength. Some bacteria have a thin PG layer (Gram-negative). Their cell wall lies between the inner and outer membranes of the cell. The outer membrane protects the PG layer from lytic enzymes in the surrounding environment (Young, 2010). Other bacteria have a thicker cell wall (Gram-positive). They lack an outer membrane, and their thick PG layer, exposed to the environment, is reinforced by additional anionic polymers such as teichoic acids and mycolic acids (Randich and Brun, 2015). Research conducted on *Bacillus subtilis* cell walls proves that those lacking

teichoic acids have dramatic shape defects such as bigger cell diameters, bulges, uneven wall thickening, and septation anomalies (Bhavsar *et al.,* 2004). However, the notion that the peptidoglycan layer is the ultimate source of morphology cannot be generalized. Some bacteria lack a cell wall and have complex cell morphologies. For example, a wall-less bacterium detected in the Red Sea actively modifies its shape from a long straight filament to one with curves, bumps, curls, and spirals, in a matter of 8 to 10 seconds (Antunes *et al.,* 2008).

In 1974, Thomas D. Brock classified bacteria into five groups based on their oxygen requirements (Kikuchi and Suzuki, 1986), as depicted in figure 1.8. Fluid thioglycollate medium (FTM) is the medium of choice to determine the aerotolerance of bacteria. In addition to the essential nutrients required for bacterial growth, FTM contains L-cystine, methylene blue, sodium thioglycollate, thioglycolic acid, and 0.05% agar. Each possesses a specific function. L-cystine, sodium thioglycollate, and thioglycolic acid reduce oxygen to water. Methylene blue is a colorless indicator but turns greenish-blue in the presence of oxygen. Agar delays oxygen diffusion (Jensen and Ussery, 2013).

Figure 1.8 - Oxygen requirements for bacterial growth.



The red dots represent the bacterial colonies growing on the thioglycollate medium. (A) Obligate aerobes; (B) Obligate anaerobes; (C) Facultative anaerobes; (D) Aerotolerant anaerobes; (E) Microaerophiles. O_2: oxygen.

The high temperature and pressure of autoclaving drive oxygen out of the tubes. Once the broth returns to room temperature, oxygen diffuses back, displaying a small blueish layer at the top of the tube. Obligate aerobes (tube A) are microorganisms requiring oxygen to survive and grow. The optimal atmospheric oxygen concentrations supporting their growth is around 20%. Such organisms only grow at the top of the broth. On the other hand, oxygen is detrimental to obligate anaerobes (tube B), which grow at the bottom of the tube, where the oxygen concentration is at its lowest (Morris and Schmidt, 2013). Facultative anaerobes (tube C) respire in the presence of oxygen but can also grow anaerobically via fermentation. They grow primarily at the top of the broth, and throughout to a lower extent. Aerotolerant anaerobes (tube D) grow optimally in anaerobic conditions and tolerate oxygen but don't gain any energy from aerobic respiration. Finally, microaerophilic organisms (tube E) grow below the highly oxygenated layer and the normal atmospheric oxygen concentrations (Riedel *et al.,* 2013).

As part of their survival mechanisms, some bacteria develop a protective surface appendage, known as the biofilm. It is composed of DNA, proteins (fibrin), and polysaccharides (alginate) which provides a refuge for bacteria employing different survival strategies (Alav *et al.*, 2020). Within the biofilm matrix, bacteria remain dormant and evade the host's immune system, causing damage to the surrounding tissues. They adapt to their environment and remain in a state of anoxia and nutrient limitation, decreasing their metabolic rate and cell division (Hassan Muhammad *et al.*, 2020). Some bacteria are capable of colonizing the surface layer of liquids, known as the air-liquid (A-L) interface. Pathogenic and saprophytic strains of *Escherichia* and *Salmonella* present robust biofilms at the A-L interface of static liquid media (Wu *et al.*, 2012; Medrano-Félix *et al.*, 2018). A commonly used medium to grow biofilms is trypticase soy broth (TSB). In this niche, bacteria have access to abundant oxygen from the air above and nutrients from the solution below. The biofilm produced from such colonization attaches to the meniscus of the liquid (Koza *et al*., 2009).

1.5.3. Differential staining tests

1.5.3.1. Gram stain

In 1884, Hans Christian Gram, a Danish bacteriologist, created the Gram staining technique to better visualize bacteria under a microscope. This technique was invented for the sole purpose of increasing the visibility of bacteria in the lung tissues of patients who died of pneumonia (Bartholomew and Mittwer, 1952). Today, the Gram stain is used to classify bacteria into two groups based on their cell wall structures: Gram-positive and Gram-negative. Gram-positive bacteria possess a thick and highly cross-linked peptidoglycan layer (20 to 80 nm). They trap the primary stain-mordant complex and stain purple. On the other hand, Gram-negative bacteria have a thin and loosely cross-linked peptidoglycan layer (1 to 3 nm), followed by a thin outer membrane. They don't retain the primary-mordant complex after adding alcohol, and stain pink when the counterstain safranin is added (O'Toole, 2016). The four basic steps of a successful Gram stain are the application of a primary stain (crystal violet) to a heat-fixed smear, followed by the addition of a mordant (Gram's Iodine), quick decolorization with alcohol, acetone, or a combination of alcohol and acetone, and finally counterstaining with safranin O (Beveridge and Davies, 1983).

Gram-positive cocci include *Staphylococcus* and *Streptococcus genera* and Gram-positive bacilli include *Clostridia* and *Bacillus* genera. Gram-negative bacteria represent one of the most important public health problems as they are highly and more resistant to antibiotics than Gram-positive. Common Gram-negative bacteria include *Enterobacteriaceae* species, *Pseudomonas* species, *Klebsiella* species, *Acinetobacter* species, *Proteus* species, and *Escherichia coli* (Silhavy *et al.*, 2015).

1.5.3.2. String test

Another method for the classification of bacteria is the 3% potassium hydroxide (KOH) string test. As with the Gram stain, the string test is based on cell wall differences as the cell wall of Gram-negative bacteria is easily disrupted by alkali solutions (in this case, KOH solution) (Leong *et al.*, 2003). While KOH dissolves the thin PG layer of Gram-negative bacteria, it does not affect the thick wall of Gram-positive cells. The

disintegration of the cell wall lyses the bacterial cell, triggering the release of its contents and its DNA. The DNA makes the bacterial suspension viscous, thick, and stringy. The suspension sticks to the inoculating loop when touched. Since Gram-positive cell walls are not destroyed, their DNA is not released, therefore such bacteria don't form a viscous solution (Imperiale *et al.*, 2018). This technique represents a fast way of identifying Gram-negative bacteria, especially in clinical settings.

1.5.3.3. Acid-fast stain

In 1882, Robert Koch identified the bacteria *Tubercule bacillus* known today as *Mycobacterium tuberculosis,* and described its physical characteristics using a complex staining procedure: the acid-fast stain (Bishop and Neumann, 1970). Acid-fast bacteria, also called acid-fast bacilli (AFB), stain poorly with Gram stain and appear weakly Gram-positive. Bacteria that do not stain well with Gram stain are from the *Mycobacteria* and *Nocardia* genera. The high lipid content of the cell's outer membrane, especially mycolic acids in the *Mycobacterium* genus, makes the Gram stain dyes hard to penetrate and stain the cell wall (Riello *et al.*, 2016). AFBs share the same physical characteristics of "acid-fastness". Acid-fastness gives a bacterium the ability to resist decolorization by acids during the staining process. Therefore, this method gives the ability to further classify bacilli bacteria as acid-fast and non-acid-fast. Because their cell wall is highly resistant to various compounds, a special staining technique is used (Dvorská *et al.*, 2001). The Ziehl-Neelsen method is the most commonly used acid-fast staining technique. Carbolfuchsin stain is the primary staining agent used in the procedure. It solubilizes the waxy lipoidal material, mycolic acid, found on the bacterial cell wall. Heat is then applied to further allow the dye to penetrate the lipid layer and enter the cytoplasm by softening it. At this step, all cells appear red. A decolorizing agent (3% hydrochloric acid in 95% ethanol) is applied. The acid-fast cells are resistant to it due to the presence of a large number of lipids in their cell walls. This prevents the decolorizing agent to penetrate and decolorize the cells. On the other hand, non-AFB are easily decolorized as they lack lipoidal material. They lose their red color and become colorless. Methylene blue, the counterstain, is then used. The colorless cells, or non-AFB, stain blue, whereas the AFB retain their red color (Hussey and Zayaitz, 2008).

1.5.3.4. 20% Potassium hydroxide preparation

The 20% KOH test is another differential test that is typically done to identify fungi. When identifying an unknown microorganism obtained from the soil, and when suspecting the presence of bacteria, 20% KOH preparation is used to rule out fungi (Bunyaratavej *et al.*, 2016). KOH is a strong alkali. It digests and clears the surrounding environment of the specimens, only allowing the fungal hyphae and spores to be seen under a microscope (Ponka and Baddar, 2014).

1.5.3.5. Endospore stain

Bacteria sense changes in their environment and adapt accordingly. One difficult change for them is the lack of nutrients. Some bacteria become motile to search for nutrients, others produce enzymes to exploit other resources. Others form endospores, a complex process that allows the bacterium to form a dormant and highly resistant cell that preserves the cell's DNA (Beskrovnaya *et al.*, 2021). These endospores can generally survive any environmental factor that would normally kill the bacterium, such as high UV irradiation, high temperature, desiccation, and enzymatic destruction. While protecting bacteria, these endospores pose a great challenge for healthcare workers as they are resistant to or not easily killed by antibiotics (Flores and Popham, 2020).

Endospores are tough because of their outer proteinaceous coat surrounding a very thick peptidoglycan layer called the cortex. The cortex causes dehydration of the spore core which makes it resistant to high temperatures. The spore core houses the DNA. Underneath the cortex lies the gem cell wall, which becomes the bacterial cell wall after the spore germinates. The inner membrane serves as a major permeability barrier against damaging chemicals (Leggett *et al.*, 2012). Finally, small acid-soluble proteins (SASPs) tightly bind and condense the DNA. They are the ones responsible for resistance against UV light and DNA-damaging reagents and chemicals (Raju *et al.*, 2006).

Gram staining and other staining techniques do not usually reveal the presence of endospores. Relatively, only a few bacterial species produce endospores, therefore a

positive result is important in the identification process. Some endospores appear within vegetative bacterial cells whereas others appear outside of the cell. These are called free spores. The most common endospore staining technique is the Schaeffer-Fulton method (Oktari *et al.*, 2017). Malachite green is the primary stain used. It is forced into the cells by the action of steaming since the endospore coat is very tough. This is a water-soluble chemical that does not adhere to the cell. Thus, since vegetative cells have been disrupted by the heat, they will be decolorized after adding water. Spores will retain the malachite green. The counterstain used after decolorization is Safranin. It will counterstain any cell that has been decolorized. In the end, vegetative cells stain pink whereas endospores stain green. The most common bacteria that produce spores are *Bacillus cereus, Bacillus anthracis, Clostridium tetani,* and *Clostridium botulinum* (Hussey and Zayaitz, 2007).

1.5.3.6. Capsule stain

Some bacteria develop a capsule, a specific surface layer, which represents the outermost layer of the cell. The glycocalyx is a polysaccharide layer that becomes a capsule when it tightly binds and attaches to the bacterial cell. It is a thick layer outside of the cell wall (Cooper, 1925). Capsules are important determinants of pathogenic virulence as they confer resistance to host phagocytosis but are not essential for viability. They increase the tolerance to desiccation, mediate the adherence to host surfaces and play an important role in the access of certain molecules to the cell membrane. They also decrease the action of complement-mediated killing (Boyce and Adler, 2000).

The bacterial capsule is detected by various techniques, the most common of which is Anthony's method. The primary stain used is crystal violet. A mordant, copper sulfate, is required to precipitate the capsule. By counterstaining with crystal violet, the cell wall retains the dye. Both the bacterial cell and the background stain purple (crystal violet) and the capsule appears white or colorless. Since the capsule is a thick polysaccharide layer and is non-ionic, the crystal violet dye does not bind to it. It does however bind to the bacterial cell. This is why the capsule appears white/colorless (Hughes and Smith, 2007).

1.5.4. Biochemical and enzymatic reactions

1.5.4.1. Urease test

Urease, found in various pathogenic bacteria, is an important determinant of virulence. It is an essential enzyme for the colonization of bacteria in the host and its conservation in tissues (Konieczna *et al.*, 2012). Urease is responsible for hydrolyzing urea, a widely spread compound in the environment (soil and water) and the human body. It is associated with protein degradation and is a factor in proper kidney function (Weiner *et al.*, 2015). During urea hydrolysis, ammonia is produced. This compound, along with urease, is toxic to human tissues and has a role in chronic illnesses, such as rheumatoid arthritis and atherosclerosis (Mora and Arioli, 2014). Moreover, bacterial ureases negatively affect the immune system. *H. pylori* is a known ureolytic bacteria that activates neutrophils and monocytes. Such activation triggers the release of inflammatory cytokines, ultimately damaging epithelial cells. During the infection, phosphorylation of myosin regulatory light chains is increased, disrupting the tight junctions, and linking *H. pylori* urease to gastric cancer (Wroblewski *et al.*, 2010).

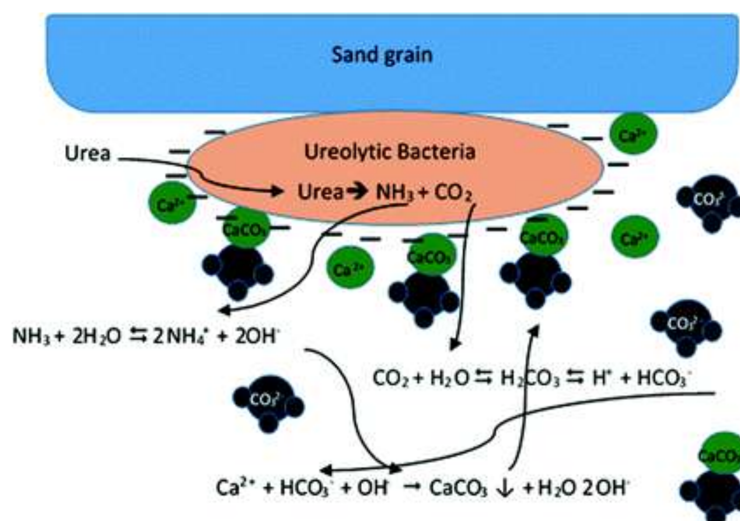
Aerobic bacteria such as *Proteus, Pseudomonas, Clostridium, Streptococcus*, and *Enterobacter*, produce urease in the soil and hydrolyze urea into ammonium and carbonate ions. The release of ammonium in the soil increases the pH and launches the precipitation of calcium carbonate, an essential component of bio-cementation (Mekonnen *et al.*, 2021). To determine the urease activity of bacteria, a urease test medium is used, containing 2% urea and a pH indicator, phenol red. The production of positively charged ammonia ions increases the pH and changes the media color from yellow (pH = 6.8) to bright pink/fuchsia (pH = 8.2). Bacteria can further be classified as rapid urease-positive, turning the whole media pink within 24 hours; weakly urease-positive, taking several days to change the color; or urease-negative, producing no color change (Brink, 2010).

1.5.4.2. Precipitation of calcium carbonate by ureolytic bacteria

Microorganisms, in particular bacteria, contribute to the soil's landscape by producing mineral deposits through a process known as microbially induced calcite precipitation (MICP). These deposits occur as by-products of bacterial metabolism, creating a "microenvironment", that supports the precipitation of calcium carbonate ($CaCO_3$) (Hoffmann *et al.,* 2021). One of the most important components for facilitating bio-mineralization and attaining bio-cementation is urease. The fastest route of calcite precipitation is ureolysis, whereby bacteria break down soil urea into ammonia and carbon dioxide, increasing the pH (Phillips *et al.,* 2014). Urea hydrolysis is the least complex metabolic process involved in bio-cementation. It also provides the highest calcite precipitation, ranging from 20 to 80%, when compared to other metabolic pathways such as amino acid ammonification, photosynthesis, and sulfate reduction (Achal *et al.,* 2011). Urea is found extensively in the soil as it represents the final product of the nitrogen metabolism of mammals (Sun *et al.,* 2019). Bicarbonate ions (HCO_3^-) are produced from the hydrolysis of urea, which in the presence of calcium ions (heavily present in the soil), yields $CaCO_3$ (figure 1.9).

Bio-cementation refers to the ecological process that results in the deposition of $CaCO_3$ to enhance the properties of cement. Ureolytic bacteria can either be introduced into the cement or concrete, stopping the cracks and sealing them by the action of CaCO3; or applied as a bacterial solution from the outside to seal the cracks (Bibi *et al.*, 2018). The bacteria are employed to produce organic-inorganic minerals as binding agents (Graddy *et al.*, 2021). Typically, *Bacillus* spp. are used in bio-cement applications and the most studied species is *Sporosarcina pasteurii*, a gram- and urease-positive bacterium that is capable of precipitating calcite. It can induce sufficient calcium carbonate precipitates to fix concrete fractures (Bundur *et al.*, 2017).

Figure 1.9 - Scheme adapted from Cheng and Shahin (2018) demonstrating the production of CaCO3 by soil bacteria.



1.5.4.3. Catalase test

Bacteria, like other organisms, rely on different defense mechanisms to survive and protect themselves against toxic agents. One toxic agent is hydrogen peroxide (H_2O_2), causing oxidative damage to the cells. Some bacterial species produce an enzyme to aid in cellular detoxification. This enzyme is the catalase enzyme, which neutralizes the bactericidal effects of H_2O_2 by breaking it down into water and oxygen (Juven and Pierson, 1996). Catalase-positive bacteria are correlated with virulence and pathogenicity as it produces toxic by-products (i.e., superoxide radicals O_2^-). Such products are toxic to the human body and result in cell lysis (Messina *et al.*, 2002). Catalase-positive bacteria can be identified by the addition of H_2O_2 to a bacterial inoculum, resulting in the production of oxygen bubbles (Reiner, 2010).

1.5.4.4. Oxidase test

Bacterial respiration typically ends with the electron transport chain. Cytochrome c oxidase (CcO) is the final electron acceptor in the chain of aerobic bacteria. CcO functions as an oxygen binding component, catalyzing the reduction of oxygen to water and oxidation of cytochrome c. Generally, only aerobic microorganisms contain the cytochrome system. (Noodleman *et al.*, 2020). The oxidase test often uses an electron donor to cytochrome c called Kovács reagent (tetramethyl-p-phenylenediamine

dihydrochloride solution), a colorless reduction agent. In the presence of oxidase-positive organisms, the reagent becomes oxidized by cytochrome c and produces a dark blue/purple color (Steel, 1961). Bacteria can be classified as:

- Oxidase-positive, when the color changes within 5-10 seconds.
- Delayed oxidase-positive, when the color changes within 60-90 seconds.
- Oxidase-negative, when there is no color change, or when the color change takes more than 2 minutes (Shields and Cathcart, 2010).

1.5.4.5. Coagulase test

Coagulase, an enzyme-like protein, is a key virulence factor in *S. aureus*, contributing to the production of bacterial pseudo-capsules. Evidently, it stimulates the formation of abscesses and causes the infection to persist (Velázquez-Guadarrama *et al.*, 2016). Coagulase converts fibrinogen to fibrin, causing the plasma to clot (Sperber and Tatini, 1975). The ability of bacteria to cause blood clots has significant implications on human health, whereby it plays an important role in thrombosis and fibrinous exudate formation (Loeb, 1903; Loof *et al.*, 2015). Coagulase also serves as a defensive shield. The formation of abscesses allows the bacteria to remain and proliferate in the body without being attacked by the immune cells, whereas the pseudo-capsules act as a mechanical barrier (Cheng *et al.*, 2010; Guggenberger *et al.*, 2012). Coagulase is detected by the formation of clumps when the bacterial inoculum is mixed with plasma, typically rabbit plasma. Coagulase-positive bacteria cause the rabbit plasma to clot, forming a clump. The speed of clumping, however, is not a factor of virulency (Katz, 2010).

1.5.4.6. Hemolysis test

Hemolysins are important virulent enzymes produced by various bacteria. They cause membrane damage, cell lysis, and the destruction of adjacent cells and tissues. The main role of hemolysins is to provide iron to the hemolytic bacteria through the destruction of red blood cells (RBC) (Bullen *et al.*, 2005). The expression of hemolysins has been associated with pathogenic bacteria (Nizet, 2002; Ruch *et al.*, 2019). Blood agar plates were first introduced in 1902 to differentiate bacteria based on their hemolytic activities. In 1919, J. Howard Brown classified bacteria into 3 groups based on the bacterial colonies

observed: alpha (α), beta (β), and gamma (γ) hemolytic (Brown *et al.*, 1926). Blood agar is a differential medium enriched with defibrinated mammalian blood containing RBC and hemoglobin.

Bacteria are grown on the medium and incubated overnight.

- α-hemolytic bacteria reduce hemoglobin to methemoglobin and display a green or dark discoloration around the bacterial colony. Such bacteria don't fully lyse the RBC. α-hemolytic bacteria include *S. pneumoniae* and *S. mitis*.
- β-hemolytic bacteria completely destruct the RBC and produce a clear and transparent color around the colony. Some β-hemolytic bacteria include *S. pyogenes*, *S. agalactiae*, and *L. monocytogenes*.
- γ-hemolytic bacteria, such as *Enterococcus* spp. don't produce hemolysins. No reaction is observed in the surrounding medium (Buxton, 2005).

1.5.5. Antimicrobial susceptibility testing using the Kirby-Bauer method

Antibiotic susceptibility testing (AST) of bacterial isolates is a crucial responsibility of the clinical microbiology laboratory. Bacteria are tested for potential drug resistance as well as for susceptibility to the drug of choice as depicted by clinical guidelines (Jorgensen and Ferraro, 2009). The increased bacterial resistance limits the choice of susceptible antibiotics (Arslan *et al.*, 2017). The Kirby-Bauer disk diffusion is the most widely used method for routine AST. It is simple, practical, and well-standardized (Nassar *et al.*, 2019). The results are qualitative in nature, determining the susceptibility and resistance of the isolate to different antibiotics. It also offers reliable and valid results while predicting the accurate clinical efficacy of the antimicrobials tested (King and Brown, 2001).

A bacterial inoculum is smeared onto the surface of a Mueller-Hinton agar (MHA) plate, as it allows for a better diffusion of the drug. Antibiotic discs with fixed concentrations are placed on the inoculated surface and the plates are incubated for 16-24 hours at 37°C. Zones of inhibition are measured around each disc. The diameter reflects the susceptibility of the bacterial strain to the drug and is interpreted based on the Clinical

and Laboratory Standards Institute (CLSI) guides. However, resistance and susceptibility cannot be fully confirmed until *in vivo* studies are conducted on blood and urine cultures (Hudzicki, 2009).

1.6. Study rationale, aim, and objectives

1.6.1. Study rationale

Many pathogens with high urease activity have been identified, such as *Helicobacter pylori* (*H. pylori*), *Proteus*, *Klebsiella*, and *Cryptococcus* spp. (Kappaun *et al*., 2018). Yet, only pure and non-infectious bacteria can be used in the context of MICP, to protect all individuals who come into contact with the biocement produced. Unknown microorganisms cannot be allowed to propagate in the environment without first identifying them and characterizing their features and behaviors. Moreover, the heavy use and misuse of antibiotics in both humans and animals perpetuated the appearance of superbugs (Lu *et al*., 2018). Thus, a clear identification and characterization analysis should be obtained before the application and release of unknown and novel ureolytic bacteria. This relates notably to their potential virulence and their susceptibility to various antimicrobials.

1.6.2. Aim of the present study

As a preliminary study, our study aims to identify and characterize an unknown bacterial strain isolated from mushroom compost in the Lebanese soil. We will also offer our collaborative team the data needed to use the bacteria safely in their bio-cement experiments.

1.6.3. Specific objectives

The objectives of our study are as follows:

1. Identify the unknown bacterial strain.
2. Characterize its features, behavior, and susceptibility to various antimicrobial agents.
3. Characterize its virulence and infectious potential.
4. Determine whether or not it can be employed in MICP.

CHAPTER TWO

MATERIALS AND METHODS

2.1. Chemicals and reagents

2.1.1. Culture media

Luria Bertani broth (LBB) (with 5 g/L sodium chloride, 5 g/L yeast extract, and 10 g/L tryptone) was obtained from Condalab (Madrid, Spain). Agar powder and fluid thioglycollate media (FTM) (with 15 g/L tryptone, 5 g/L yeast extract, 5.5 g/L dextrose as glucose, 2.5 g/L sodium chloride, 0.5 g/L L-cystine, 0.5 g/L sodium thioglycollate, 0.001 g/L resazurin sodium, and 0.75 g/L agar) were purchased from HiMedia Laboratories Pvt Ltd (Mumbai, India). Dehydrated TSB (with 17 g/L tryptone (pancreatic digest of casein), 3 g/L of soytone (peptic digest of soybean), 2.5 g/L glucose as dextrose, 5 g/L of sodium chloride, and 2.5 g/L of dipotassium phosphate) was obtained from VWR Chemicals (Pennsylvania, USA). Ready-to-use blood agar plates (with Schaedler agar and 5% sheep blood) were purchased from Bioteckno SAL (Beirut, Lebanon). Mueller-Hinton agar (MHA) (with 2 g/L beef extract, 17.5 g/L acid hydrolysate of casein, 1.5 g/L starch, and 17 g/L agar) was acquired from Bio-Rad Laboratories (California, USA). LB agar plates were prepared in the lab at a concentration of 1.5%. 1.5 g of agar was dispersed in 100 ml of distilled water and sterilized by autoclaving at 121°C for 15 minutes. After sterilization, the solution was allowed to cool to 55°C and then poured into Petri dishes. The media was allowed to solidify before taping them with parafilm and storing them in the fridge at 4°C for later use. MHA plates were also prepared in the lab by dissolving 10.5 g of MHA powder in 300 mL of distilled water. The solution was sterilized by autoclaving at 121°C for 15 minutes. After cooling, the media was poured into Petri dishes and stored in the fridge and 4°C.

2.1.2. Differential staining

Safranin O solution, crystal violet solution, methylene blue hydrate, malachite green powder, and iodine were obtained from Sigma-Aldrich Merck (Burlington, MA,

United States). Carbolfuchsin powder was purchased from Ibra Hadad & Fils (Beirut, Lebanon). Potassium hydroxide was acquired from Thermo Fisher Scientific (New Hampshire, USA). Acid-alcohol solution (with 97 mL 95% ethanol and 3 mL hydrochloric acid) and 20% copper sulfate solution were prepared in the lab by dissolving 20 g of copper sulfate in 100 mL of distilled water. Gram's iodine solution was also prepared in the lab by dissolving 1 g iodine and 2 g potassium iodide in 300 mL of water.

2.1.3. Biochemical and enzymatic reactions

Urea agar base (with 1 g/L dextrose, 1.5 g/L peptic digest of animal tissue, 5 g/L sodium chloride, 2 g/L monopotassium phosphate, 0.012 g/L phenol red, and 15 g/L agar) was obtained from HiMedia Laboratories (Mumbai, India). Urea powder was purchased from Sigma-Aldrich Merck (Burlington, MA, United States). 1% Kovács reagent (consisting of 5 g/L p-dimethylamino benzaldehyde, 75 g/L amyl alcohol, and 25 g/L hydrochloric acid) was purchased from Sigma-Aldrich Merck (Burlington, MA, United States). 3% hydrogen peroxide was prepared in the lab from a 30% solution obtained from ACS Laboratories (Florida, USA). Calcium chloride was obtained from Thermo Fisher Scientific (New Hampshire, USA). Freeze-dried rabbit plasma was purchased from Bio-Rad Laboratories (California, USA). 1N hydrochloric acid (HCl) was prepared in the lab by dissolving 8.33 mL in 100 L of distilled water.

2.1.4. Antimicrobial susceptibility testing

The antibiotic discs were prepared in the lab using Whatman filter paper number 3. Antibiotic solutions were prepared from tablets and powders obtained from the Lebanese American University Medical Center-Rizk Hospital (LAU MCRH). The antibiotics purchased are listed below:

1. Ampicillin 500 mg, powder.
2. Amoxicillin 1 g, powder.
3. Amikacin 500 mg, powder.
4. Azithromycin 250 mg, capsule.
5. Ceftriaxone 1 g, powder.
6. Ciprofloxacin 500 mg, tablet.

7. Doxycycline 100 mg, tablet.
8. Imipenem Cilastatin 500 mg / 500 mg, powder.
9. Meropenem 1 g, powder.
10. Trimethoprim-sulfamethoxazole (TMP-SMX) 160 mg / 800 mg, tablet.

CBD dissolved in 95% ethanol at a concentration of 20 mg/mL was obtained from the School of Pharmacy at the Lebanese American University (LAU).

2.2. Bacterial culture

The unknown bacterial strain was isolated from mushroom compost in the Lebanese soil by our collaborative team. 16S rRNA gene sequencing was performed by the team prior to our receiving it, confirming the presence of a novel bacterial strain. Upon receipt, bacterial glycerol stocks were made, and the strain was preserved at -80°C for long-term storage. The addition of glycerol prevents damage to the bacterial cell membrane, thereby keeping the frozen bacteria alive and stable. To recover the bacteria from the frozen aliquots, cells were scraped off from the top of the stock using a sterile inoculating loop and streaked onto an LBA plate. The bacteria were then incubated at 36.5°C for 44 hours.

2.3. Morphological and physiological examination

2.3.1. Direct and microscopic observation of bacterial morphology

Bacteria were grown on an LBA plate and incubated at 37°C. After 24 hours, the colonies were visually inspected for shape, size, and color. A colony was then collected using an inoculating loop and placed on a glass slide. A drop of safranin O was added over the smear for 1 minute. The excess was gently washed away with an indirect stream of distilled water. The cells were then observed under light microscopy at 1,000-fold magnification (oil immersion) for bacterial morphology.

2.3.2. Aerotolerance test

Preparation of thioglycollate broth (TGB)

2.975 g of FTM was dissolved in 100 mL of distilled water and sterilized by autoclaving at 120°C for 20 minutes. The broth was distributed into 6 sterile falcon tubes, each containing a volume of 20 mL.

Inoculation of TGB

The inoculation occurred under a microbiological laminar flow hood to prevent contamination. A bacterial colony grown on an LBA plate was used to inoculate one falcon tube. The test was repeated for the 4 remaining tubes. The sixth tube served as a control, only containing the broth. Once inoculated, the tubes were incubated at 37°C for 48 hours in a horizontal position. After 48 hours, they were visually examined for oxygen diffusion. Bacterial growth in various regions of the tubes depends on their oxygen requirements, as discussed in section 1.4.2.

2.3.3. Biofilm production on air-liquid interface

TSB is the most commonly used nutritive media to assay biofilm production. 3 mL of TSB was added to two sterile test tubes and a bacterial colony inoculated each tube. A third tube only containing TSB was used as a control. The tubes were then placed vertically in the incubator at 37°C. Care was taken not to move the inoculated broth. They were then examined for biofilm formation on the A-L interface after 24, 48, and 72 hours.

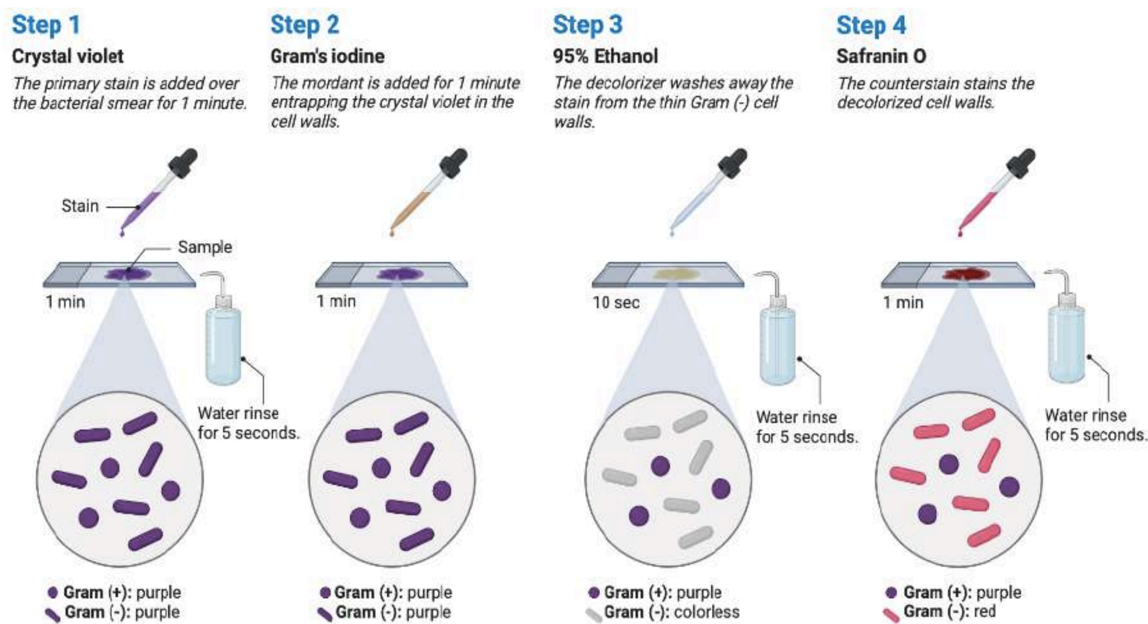
2.4. Differential staining under light microscopy

2.4.1. Gram staining

A single bacterial colony grown on LBA was placed on a microscope slide and heat-fixed by passing the slide through the Bunsen burner flame three times. The primary stain (crystal violet) was added to the slide and the sample was incubated for 1 minute. With a gentle stream of distilled water, the slide was rinsed for 5 seconds. The prepared Gram's iodine solution (the mordant) is added and incubated for 1 minute. A decolorizer (95% ethanol) was added to the slide for 3 seconds and rinsed off with distilled water. The counterstain (safranin O) is finally added and incubated for 1 minute, followed by washing with distilled water for 5 seconds. The cells are examined under light microscopy at 1,000-

fold magnification. Gram-negative cells appeared purple whereas Gram-positive cells appeared pink. The Gram stain was repeated 3 times to ensure the validity of the results.

Figure 2.1. - Gram staining protocol.



2.4.2. 3% KOH string test

Using an inoculating loop, a single bacterial colony was emulsified in 3% KOH on a microscope slide. The produced emulsion was stirred in a circular motion for 1 minute using the same loop. The loop was then pulled up to observe the production of a string. The string test was performed 3 times to guarantee the validity of the results.

2.4.3. Acid-fast stain using the Ziehl-Neelsen method

The primary stain (carbolfuchsin solution) and secondary stain (methylene blue solution) were prepared by dissolving 0.3 g of carbolfuchsin powder and 0.3 g of methylene blue, each in 100 mL of distilled water, respectively. A single bacterial colony was heat-fixed onto a glass slide by passing the slide over the Bunsen burner flame three times. A beaker containing water was placed over a hot plate and was heated until steam rose (without boiling).

The slide was placed over the beaker and a piece of absorbent paper was placed over it and saturated with the primary stain for 5 minutes. The film was then washed gently with an indirect stream of water until no color was observed. The acid-alcohol solution (decolorizing agent) containing 3 mL hydrochloric acid in 97 mL ethanol was used to wash the slide. The slide was once again washed immediately with distilled water. This step was repeated until the smear appeared faintly pink. The bacterial smear was finally saturated with the counterstain for 20-30 seconds and washed with distilled water. After gentle blotting, the cells were examined under oil immersion. AFBs appear pink over a purple-dark blue background. The acid-fast stain was repeated 3 times.

2.4.4. 20% KOH preparation

The bacterial specimen is placed on a sterile glass slide and a single drop of 20% KOH was added over the smear. A cover glass is placed on top of the slide and air bubbles were removed by adding pressure. The excess solution was blotted away using a sterile gauze and the smear was examined under light microscopy at low power (10x) to visualize any epithelial cells and/or hyphae. The 40x setting was used to further examine any fungal structures. The KOH preparation was done in a triplicate manner to ensure validity.

2.4.5. Endospore stain

A bacterial colony was heat-fixed on a glass slide which was then placed over a beaker of steaming water (without boiling). A piece of absorbent paper was placed on top of the slide. A 5% malachite green solution was prepared by dissolving 5 g of malachite green powder in 100 mL of distilled water. The smear was flooded with the primary stain (malachite green) and saturated for 5 minutes while keeping the paper moist with the dye. After 5 minutes, the paper was discarded, and the slide was washed with a gentle stream of distilled water and placed on a regular stain tray. The smear was then saturated with the counterstain (safranin O) for 1 minute. It was once again washed with distilled water and blot-dried with bibulous paper. The bacterial cells were then examined under oil immersion (1,000x). Endospores appear bright green and vegetative cells stain red to pink. The endospore stain was repeated 3 times to ensure the validity of the results.

2.4.6. Capsule stain

A bacterial colony was obtained from a 12-18-hour culture grown on an LBA plate and placed on a glass slide. The smear was air-dried (not heat-fixed) to avoid the destruction of the capsules. The smear was saturated with 1% crystal violet for 2 minutes which was then gently rinsed off using 20% copper sulfate solution. The smear was allowed to air dry (no blotting) and examined under oil immersion. The capsules appear transparent surrounding the purplish bacterial cells. The capsule stain was accomplished in a triplicate manner.

2.5. Biochemical and enzymatic reactions

2.5.1. Urease test

Preparation of the broth

2.1 g of urea agar base was dispersed in 95 mL of distilled water and soaked for 10 minutes. 2 g of urea was also dispersed in 10 mL of distilled water. Both solutions were swirled and sterilized by autoclaving at 120°C for 20 minutes. They were then allowed to cool down to 47°C before adding the sterile urea solution to the urea agar base solution. The mixture was then distributed into three sterile falcon tubes.

Inoculation of the broth

Two falcon tubes were inoculated with a bacterial smear. The third tube served as a control. The tubes were then incubated overnight at 37°C in an inclined position and the slant was observed for color change at 6 hours, 24 hours, and every day for 6 days.

2.5.2. Calcium carbonate precipitation

Preparation of the media

3 g of LBB and 1.5 g of agar were dissolved in 100 mL of distilled water. 30 mM of calcium chloride ($CaCl_2$), accounting for 0.33 g of $CaCl_2$, was then added. The mixture was then swirled and autoclaved to sterilize at 120°C for 20 minutes. Once the media has cooled down a little, 2 g of urea was incorporated and swirled to dissolve. The mixture was allowed to cool down and poured in 4 Petri dishes under sterile conditions.

Inoculation of the media

Once it has solidified, the media was inoculated with a bacterial smear heavily. The inoculated media were then incubated at 37°C for 7 days.

Calcium carbonate precipitation

After 7 days, the Petri dishes were removed from the incubator and placed under a laminar flow hood. A drop of 1N HCl was added to the inoculum. $CaCO_3$ precipitation was confirmed by the formation of effervescence.

2.5.3. Catalase test

5 drops of 3% H_2O_2 were added into three 12 x 75 mm test tubes. A bacterial colony was collected from a 24-hour culture using an inoculating loop and placed into a test tube. This step was repeated for the second tube. The third tube served as a control. The tubes were then placed against a dark/gray background and observed for immediate bubble formation.

2.5.4. Oxidase test

A Whatman number 1 filter paper was soaked with 1% Kovács oxidase reagent and allowed to dry completely. Using an inoculating loop, a bacterial colony was picked up from a fresh culture and rubbed on the filter paper. The paper was observed for a color change immediately (within 5 to 10 seconds), within 60 to 90 seconds, and longer than 2 minutes.

2.5.5. Coagulase test

Under sterile conditions, 10 mL of the diluent containing sodium oxalate was removed using a sterile pipette and added to the vial of freeze-dried rabbit plasma. The vial was then shaken gently to dissolve the plasma while preventing the formation of bubbles. 0.5 mL of 24-hour liquid culture was mixed with 0.5 mL of the reconstituted rabbit plasma and incubated at 37°C overnight vertically. The test was repeated twice to ensure the validity of the results, and a control tube was also prepared.

2.5.6. Hemolysis test

Preparation of liquid culture

15 mL of LBB was added to a sterile falcon tube and a well-isolated bacterial colony was used to inoculate the broth under aseptic conditions. The broth was then incubated at 37°C, overnight.

Inoculation of blood agar plates

A loopful of the 24-hour bacterial liquid culture was used to inoculate a blood agar plate as a small dot. The experiment was performed in a triplicate manner to ensure the validity of the results obtained. The plate was incubated at 37°C overnight and was then inspected visually for color changes.

2.6. Susceptibility to ultraviolet light

Three LBA plates were inoculated with a bacterial smear using the quadrant method. The plates were placed under ultraviolet (UV) light for 5 minutes, 15 minutes, and 30 minutes, respectively. The exposed plates were then incubated at 36.5°C for 24 hours and were then examined for bacterial growth.

2.7. Antimicrobial susceptibility testing: Kirby-Bauer method

Preparation and sterilization of filter paper discs

Whatman filter papers number 3 were used. Using a hole-punching machine, holes of 6-mm in diameter were produced to obtain the discs. They were straightened by applying pressure and placed in sterile Petri dishes. They were then exposed to UV light for 40 minutes to sterilize them. A single filter paper disc can absorb up to 15 μL of solution.

2.7.1. Susceptibility testing of antibiotics

10 antibiotics were chosen based on the Gram staining and aerotolerance results obtained initially. The antibiotics tested were:

1. Ampicillin

2. Amoxicillin
3. Amikacin
4. Azithromycin
5. Ceftriaxone
6. Ciprofloxacin
7. Doxycycline
8. Imipenem/cilastatin
9. Meropenem
10. TMP-SMX

Preparation of antibiotic stock solutions

Antibiotic tablets were crushed using a mortar and a pestle. Their weights were measured and according to the weight of the powder obtained, a stock solution was prepared using sterile distilled water. The antibiotics present as a powder formulation and of known weights were also dissolved in sterile distilled water to obtain the respective stock solutions.

Determination of antibiotic stock solution and disc concentrations

The disc concentrations were determined based on the Minimum Inhibitory Concentration (MIC) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) on the "antimicrobial wild-type distributions of microorganisms". Table 2.1 lists the stock solutions and disc concentrations. The TMP-SMX stock solution and disc concentrations calculation is provided below. All other concentrations followed the same calculations.

Table 2.1 - Antibiotic stock solution and disc concentrations.

Antibiotic	**Strength**	**Formulation**	**Stock solution**	**Disc concentration**
Ampicillin	500 mg	Powder	67 mg/mL	10 μg
Amoxicillin	1 g	Powder	67 mg/mL	10 μg

Amikacin	500 mg	Powder	20 mg/mL	30 µg
Azithromycin	250 mg	Capsule	10 mg/mL	15 µg
Ceftriaxone	1g	Powder	20 mg/mL	30 µg
Ciprofloxacin	500 mg	Tablet	30 mg/mL	5 µg
Doxycycline	100 mg	Tablet	20 mg/mL	30 µg
Imipenem Cilastatin	500 mg / 500 mg	Powder	67 mg/mL	10 µg
Meropenem	1 g	Powder	67 mg/mL	10 µg
TMP-SMX	160 mg / 800 mg	Tablet	16 mg/mL	25 µg

A single disc contains 30 µg of TMP-SMX in 15 µL of volume. Therefore, the disc contains 2 mg/mL of TMP-SMX:

30 µg / 15 µL = 2 µg/µL = 2 mg/mL

A tablet of TMP-SMX was crushed and yielded a weight of 840 mg. 840 mg of TMP-SMX powder was diluted in 42 mL of distilled water (dH_2O) to prepare a stock solution (S_0) of 20 mg/mL.

(840 mg x 1 mL) / 20 mg = 42 mL

S_0 was then diluted by a factor of 1/10 to obtain the final concentration of 2 mg/mL. Thus, 100 µL of S_0 was diluted in 900 µL of dH_2O.

Impregnation of the discs

Working under the laminar flow hood, 15 µL of each diluted solution of antibiotics was added over a paper disc, ensuring that the tip of the pipette was only in slight contact with the disc. Three discs of the same antibiotic were prepared and placed on a plate to ensure the validity of the results. The discs were then allowed to completely dry for 15-20 minutes.

Inoculation and incubation of MHA plates

10 MHA plates were prepared and inoculated with a heavy smear of a 24-hour bacterial culture. Each plate was used for a single antibiotic. Using sterile tweezers, the prepared discs were placed on the inoculated plates which were then incubated at 37°C for 24 hours.

Post-trial modifications

After the first trial, the volume of antibiotics displaying a high inhibitory effect was decreased to better observe the effects. Each plate contained 3 discs of 15 µL, 10 µL, and 8 µL. On the other hand, the concentration of the antibiotics to which the bacteria were resistant was increased. S_0 was diluted by a factor of 1/10, 1/5, and 1/2 to increase the concentrations. Similarly, each plate contained 3 discs of 3 different antibiotic concentrations. The experiment was repeated with the adjusted volumes and concentrations.

2.7.2. Susceptibility testing of CBD

The disc concentrations of CBD/ethanol were obtained by Gildea *et al.* (2022). CBD was serially diluted from a concentration of 20 mg/mL to obtain the disc concentrations of 1.25, 0.125, 0.0125, or 0.00125 µg/mL. As discussed in section 2.6.1., each disc can contain up to 15 µL of solution. Four MHA plates were inoculated with a heavy smear of a 24-hour bacterial culture. Each plate included 3 discs, two of which contained CBD with a specific concentration whereas the third served as a control containing ethanol. The plates were then incubated at 37°C for 24 hours. The results for both the antibiotic and CBD susceptibility tests were interpreted the next day. The diameter of the ZOI was measured using a ruler.

2.8. Identification of the bacterial genus

PubMed MESH indexing search was used to list all the bacterial genera based on the results of the Gram stain, oxygen requirement test, and shape of the unknown strain. All information related to the morphological, physiological, staining, and biochemical

tests were obtained from Bergey's Manual of Systematics of Archaea and Bacteria. An identification table was created on an Excel sheet summarizing each test for each bacterial genus. The genus was narrowed down by elimination.

CHAPTER THREE

RESULTS

3.1. Morphological characterization

3.1.1. Direct visualization of colony morphology grown on LB agar

Bacterial colonies grown on LBA were visually examined for colony morphology including shape, size, and color. As seen in figure 3.1, the colonies are small (around 2-mm in diameter), circular with complete edges, smooth, and have a beige to a creamy color. They become yellowish to brownish with age when placed at 4°C (figure 3.2).

Figure 3.1 - Bacteria grown on LBA for 44 hours at 36.5°C.

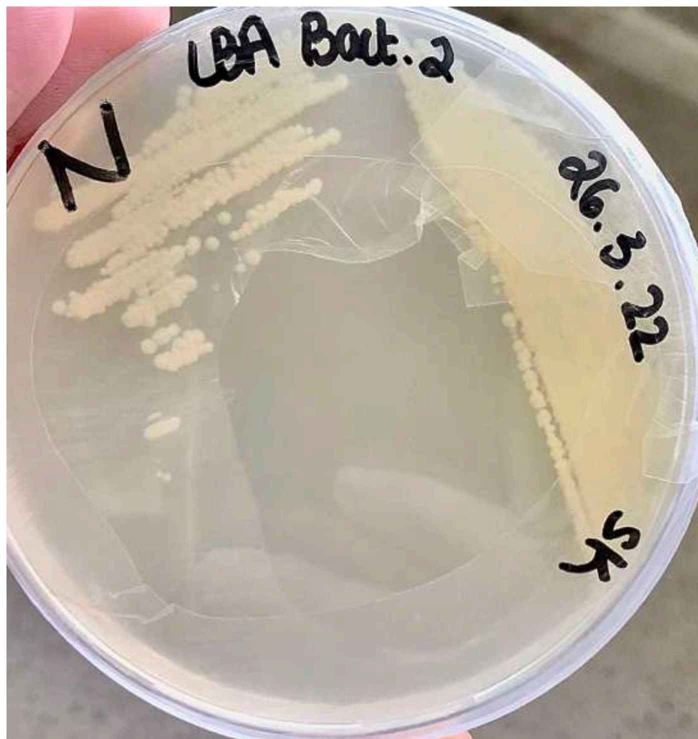
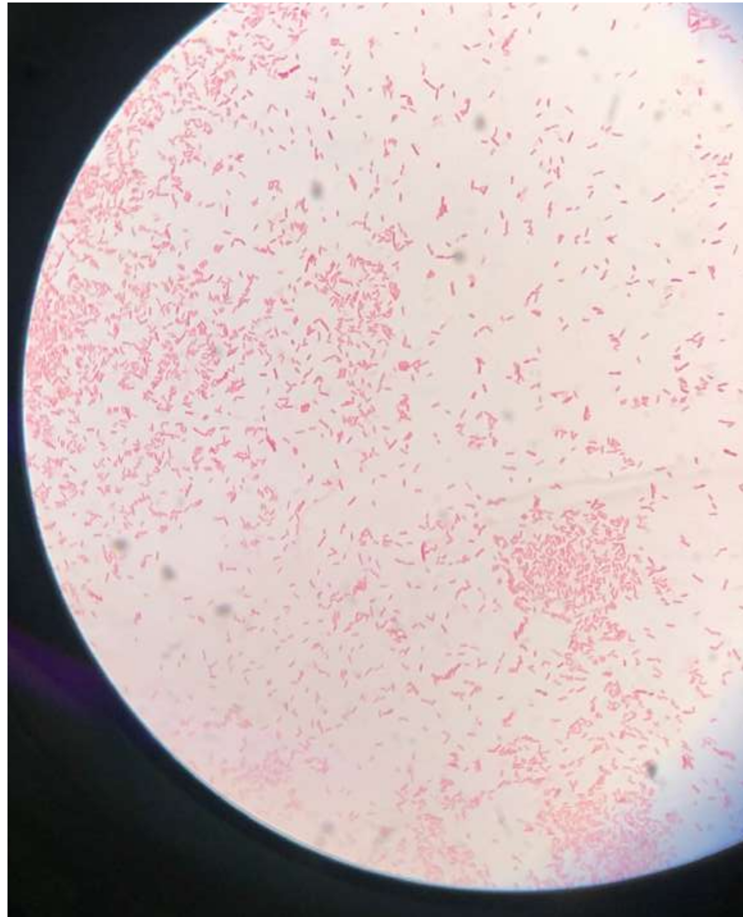


Figure 3.2 - Bacteria grown on LBA after seven days in the fridge at 4°C.

3.1.2. Examination of bacterial cell morphology under light microscopy

Initially, the staining was performed using liquid cultures. The bacterial cells were too diluted to be observed under a microscope. The following attempts placed a bacterial colony on a microscope slide with a drop of distilled water. As with the initial experiments, we were not able to observe the bacteria cells clearly. To determine whether the problem was due to the reagents, *E. coli* was used as a control. Whether we used a liquid culture or a drop of water, we were able to clearly see the *E. coli* cells. We hypothesized that the unknown strain was being too diluted. Therefore, to be able to see the cells, we placed a bacterial colony on a microscope slide without any diluent. The cells were examined under the microscope at 40x, 10x, and 100x (oil immersion). The shape of the cells along with their differentiation at 100x are displayed in figure 3.3. The bacterial cells appear to be rod-shaped, and small, with some V-shaped cells, appearing as singlets.

Figure 3.3 - Cell morphology under oil immersion.



3.1.3. 20% potassium hydroxide preparation

As the strain was obtained from mushroom compost in the soil, 20% KOH preparation was used to eliminate any fungal structures. The bacteria cells were destroyed by the action of potassium hydroxide, and we observed no fungal structure hyphae (figure 3.4).

3.2. Physiological characterization

3.2.1. Aerotolerance test

As seen in figure 3.5, the tubes inoculated formed clouding strictly at the top of the tube. Moreover, the control tube on day 3 displayed a pink color, indicating the presence of oxygen. The test tubes did not display the same pink color until day 5,

indicating the death of bacteria. These results indicate that the bacterial strain requires oxygen to grow and survive and is therefore classified as a strict or obligate aerobe.

Figure 3.4 - 20% KOH preparation demonstrating no fungal entities.

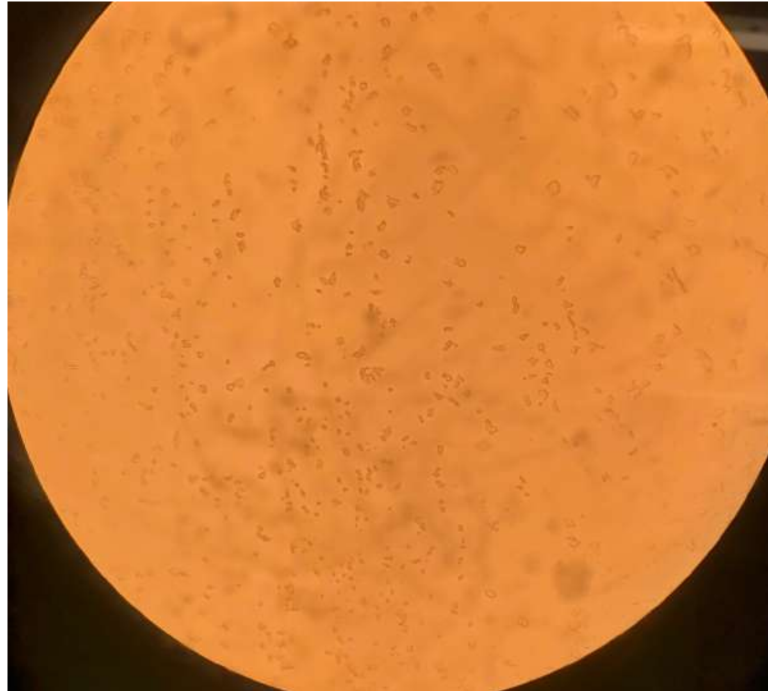
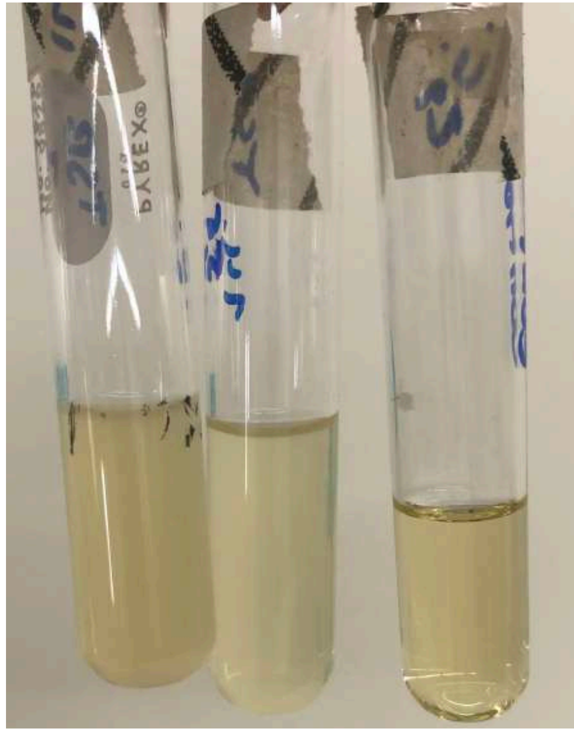


Figure 3.5 - Oxygen requirement test 48 hours after incubation.

3.2.2. Biofilm detection at the air-liquid interface

No biofilm was detected between the air and the liquid, as displayed in figure 3.6. The middle tube is the control tube whereas the two remaining tubes contain the inoculated TSB.

Figure 3.6 - Biofilm production at the air liquid interface test.

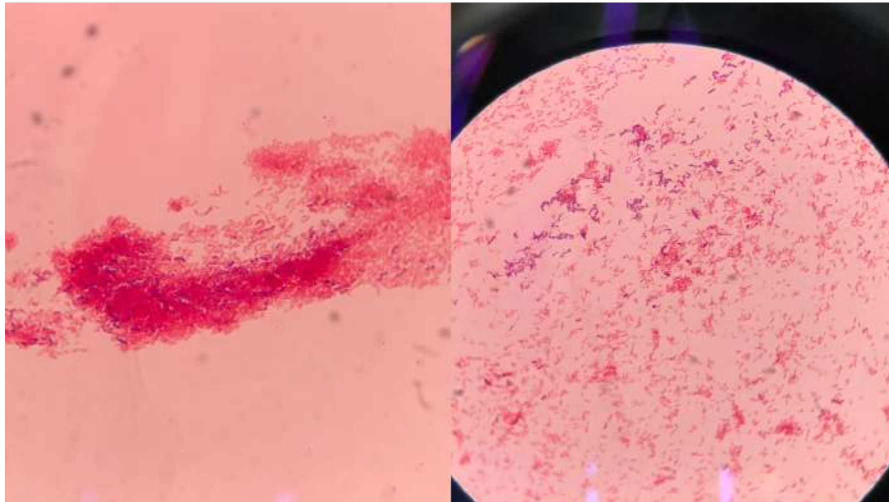


3.3. Differential staining tests

3.3.1. Gram stain

The Gram stain was repeated six times as the bacterial cells were too small to be detected. Following the same rationale as with the observation under the microscope (section 3.1.2), we placed a bacterial colony on a microscope slide and performed the Gram stain. The cells were examined under oil immersion. The first experiments yielded inconclusive results as we observed both gram-positive and gram-negative bacteria. *E. coli* was used as a control to assess if the reagents used in the Gram stain are faulty. The results showed that the cells are Gram-negative rods. Therefore, it was determined that the reagents are not defective. The stain was repeated three more times to better visualize the cells. As seen in figure 3.7, we obtained Gram-variable results, with the majority of the cells being pink (Gram-negative) and some purple cells (Gram-positive).

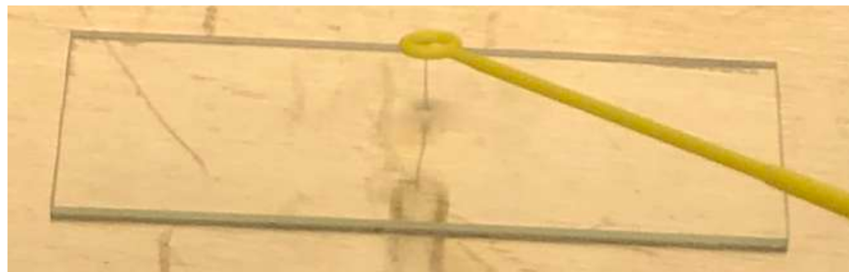
Figure 3.7 - Gram stain displaying pink and purple rods.



3.3.2. 3% potassium hydroxide string test

After obtaining Gram-variable results, the string test was used to verify the presence of Gram-negative bacteria. A string was immediately formed immediately when a bacterial colony was mixed with 3% KOH (figure 3.8), confirming that the bacteria are Gram-negative.

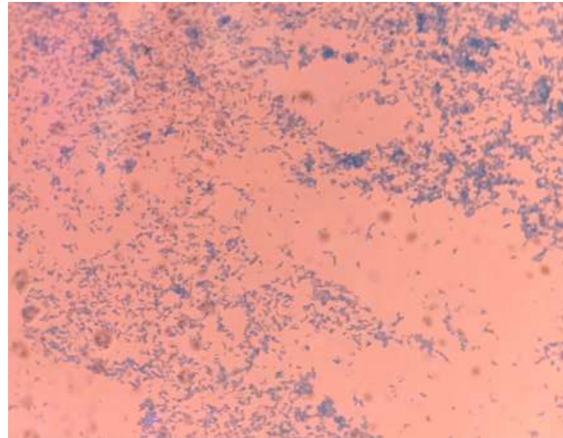
Figure 3.8 - 3% KOH string test showing the formation of a string.



3.3.3. Acid-fast stain

The acid-fast stain was performed to assess the acid-fastness of the bacterial strain. The cells were examined under oil immersion where all cells appeared blue with no red cells (figure 3.9). The absence of red cells indicates that the bacteria are non-acid-fast.

Figure 3.9 - Acid-fast test displaying blue rods under oil immersion.



3.3.4. Capsule stain and endospore stain

The capsule stain did not reveal any transparent halo surrounding the purple bacterial cells (figure 3.10). Similarly, the endospore stain only revealed pink rods stained by safranin O. No green cells were observed (figure 3.11). The results obtained prove that the bacteria are non-encapsulated and non-spore-forming.

Figure 3.10 - Capsule stain displaying purple rods with no surrounding halos.

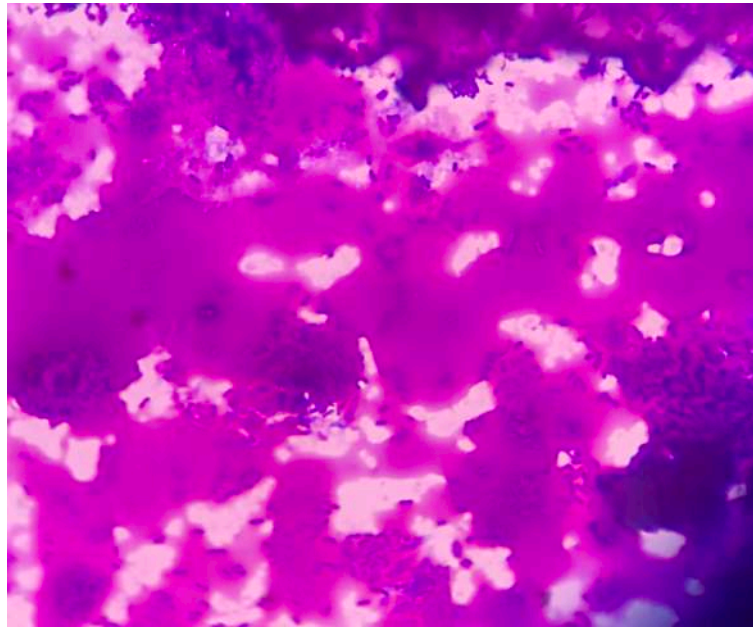
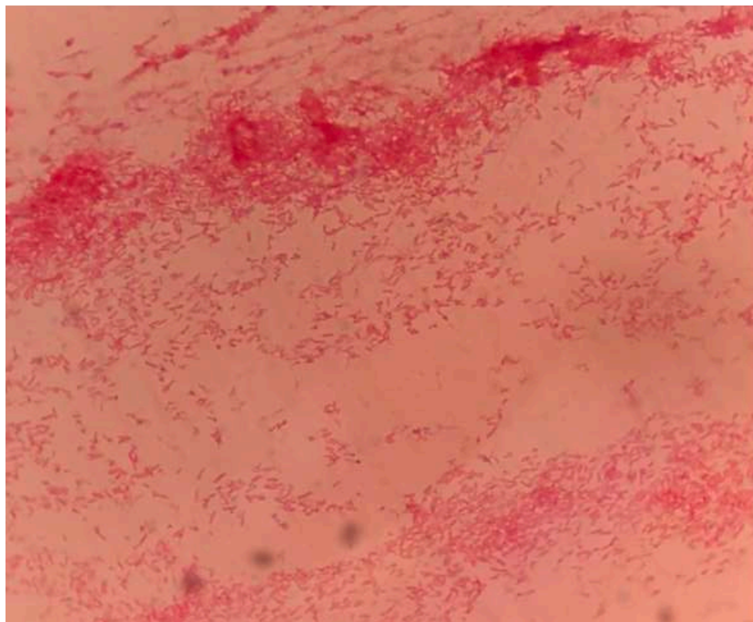


Figure 3.11 - Endospore stain only showing pink rods.



3.4. Biochemical and enzymatic tests

3.4.1. Urease test

Three tubes containing urea-agar base were utilized for the urease test. The first two tubes were inoculated with a bacterial colony and the third one served as a control (figure 3.12). The tubes were incubated for six days at 36°C and were examined every day until six days. A color shift from bright pink/fuchsia to orange was observed as soon as 12 hours for the inoculated tubes. The control tube did not display any color change. The tubes were then examined at 24 hours, 48 hours, 72 hours, and every day for the following six days. On day 2, we observed a complete color change for the two tubes inoculated (figure 3.13). Thus, we can conclude that the bacteria hydrolyze urea.

3.4.2. Calcium carbonate precipitation test

The media containing calcium chloride, urea, and agar, were inoculated by the bacterial strain. After seven days, a few drops of 1N HCl were placed on the inoculated media. Immediate bubble formation was observed (figure 3.14), suggesting that the bacteria precipitate calcium carbonate.

Figure 3.12 - Results of urease test after 12 hours of incubation.

Figure 3.13 - Results of urease test after 48 hours of incubation.

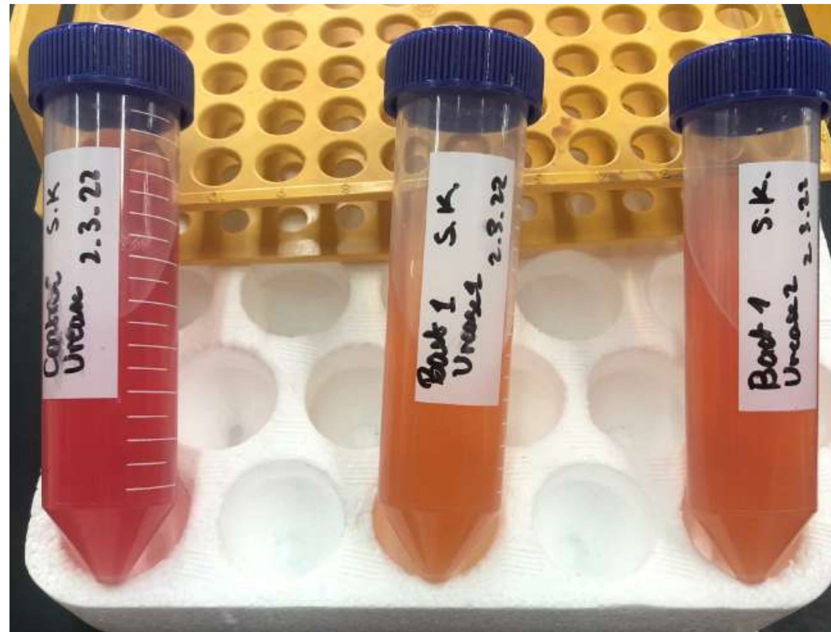
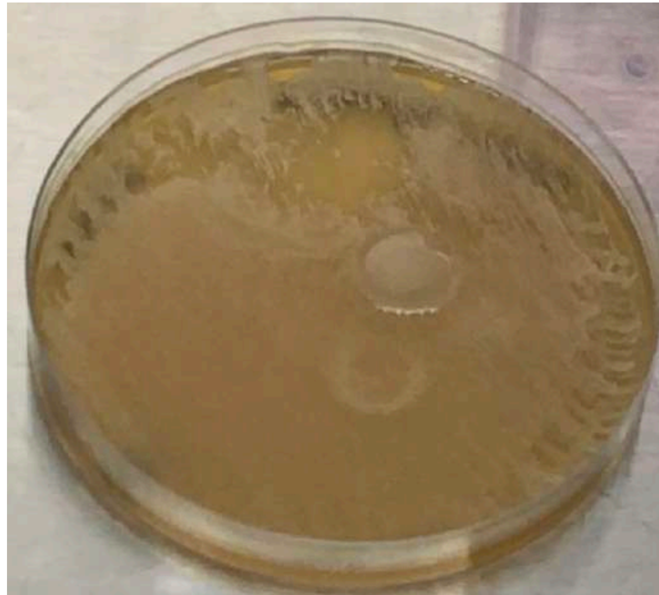


Figure 3.14 - Results of the calcium carbonate precipitation test.



3.4.3. Catalase test

The catalase test was performed to assess the ability of the bacteria to break down hydrogen peroxide into water and oxygen. Three tubes were used containing five drops of 3% H2O2. One tube served as control whereas the two other tubes were inoculated with

a bacterial colony. Immediate bubble formation was observed for the two test tubes (figure 3.15), confirming the presence of the catalase enzyme.

3.4.4. Coagulase test

The coagulase test assessed the ability of the bacterial strain to clot plasma. After inoculating the diluted rabbit plasma with a bacterial colony, the tubes were placed in an incubator overnight. After 24 hours, we observed a film at the bottom of the tube as displayed in figure 3.16, which is not present in the control tube. The bacteria are therefore coagulase positive.

3.4.5. Oxidase test

The oxidase test was performed and timed to assess the presence of cytochrome c oxidase. No color change was observed on the filter paper disc for six minutes. At 6.3 minutes, a faded purple color was observed (figure 3.17). This delayed reaction indicates that the bacteria is oxidase negative.

Figure 3.15 - Results of the catalase test.

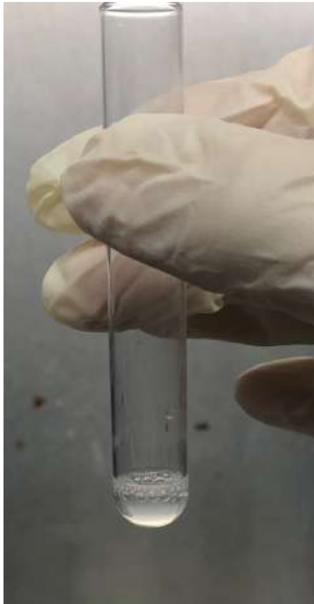
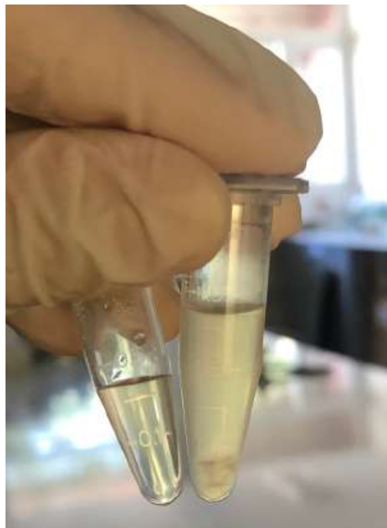


Figure 3.16 - Results of the coagulase test.



3.4.6. Hemolysis test

A single bacterial colony was placed as a dot on the plate in a triplicate manner on the blood agar plate. The inoculated plate was placed in the incubator at 36.5°C for 24 hours. The following day, the plate was visually observed for a color change. As displayed in figure 3.18, a clear, white, zone appeared where the bacterial colony was placed,

suggesting that the bacteria possess a β-hemolytic activity. However, the diameter of hemolysis is relatively small (around 0.3-0.5 mm in diameter).

Figure 3.17 - Results of the oxidase test at 6.3 minutes.

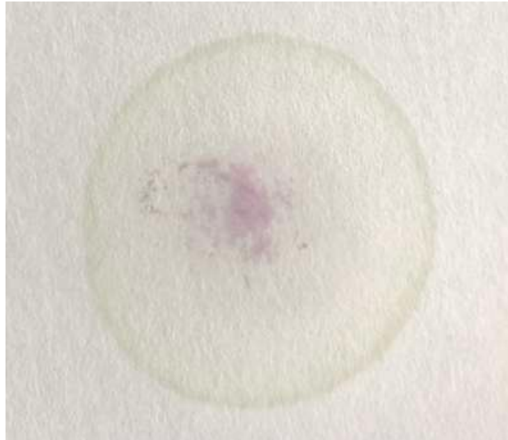
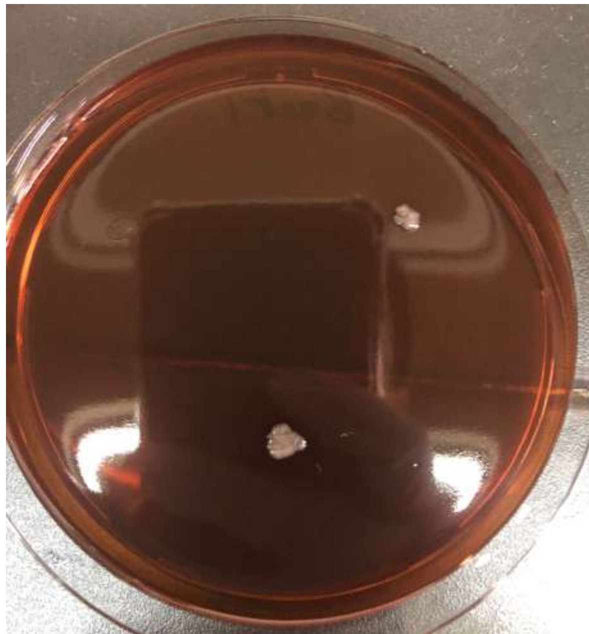


Figure 3.18 - Results of the hemolysis test.



3.5. Antimicrobial susceptibility test

3.5.1. Susceptibility test of antibiotics

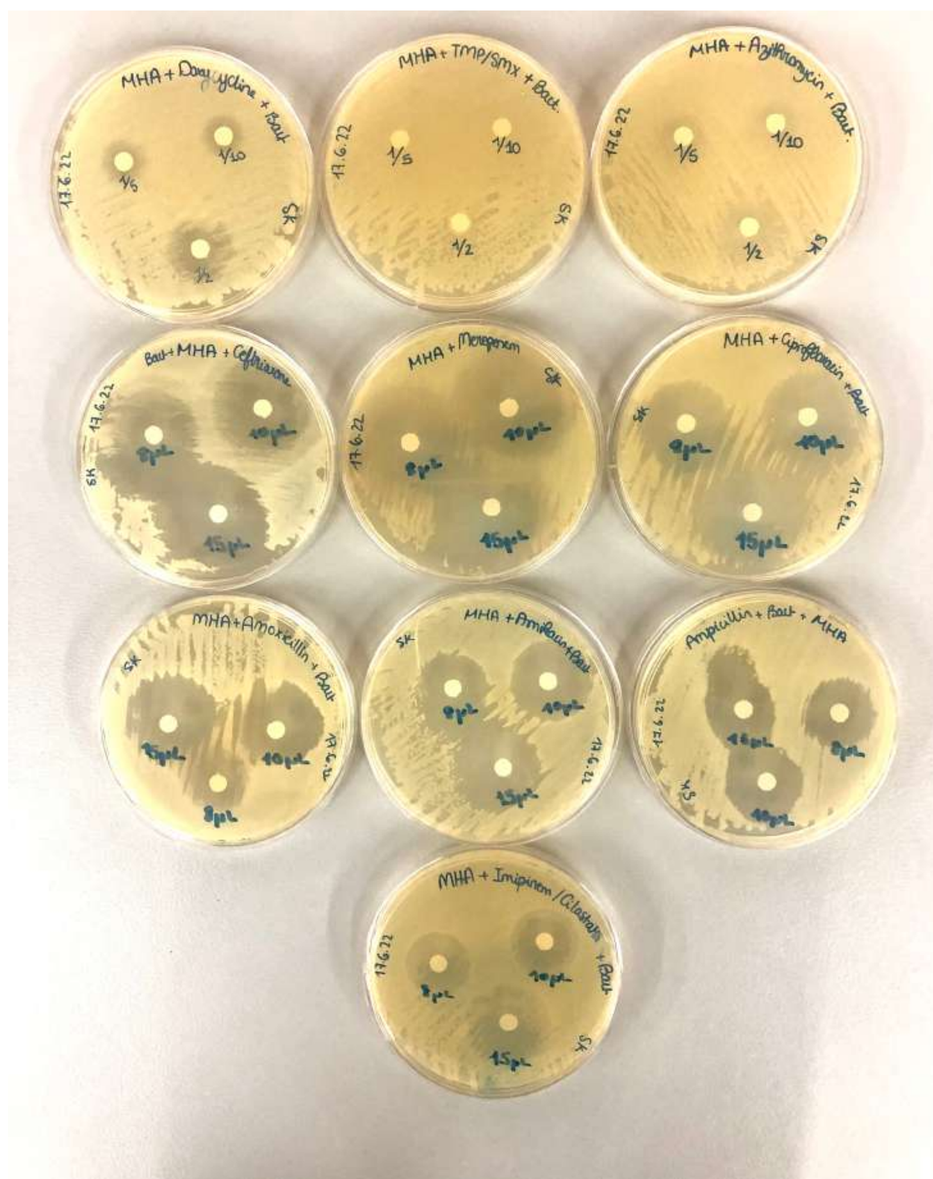
24 hours after incubating the inoculated plates, we observed and measured the zone of inhibition of each antibiotic at different concentrations. The results are displayed in figure 3.19. Based on the ZOI, we determined that the bacteria strain is resistant to TMP-SMX,

doxycycline, and azithromycin. It is susceptible to amikacin, amoxicillin, ampicillin, ceftriaxone, ciprofloxacin, imipenem/cilastatin, and meropenem. The largest ZOI was produced by ceftriaxone (ZOI diameter = 3.9 cm), followed by meropenem (ZOI diameter = 3.7 cm), ciprofloxacin (ZOI diameter = 3.6 cm), ampicillin (ZOI diameter = 3.5 cm), amoxicillin (ZOI diameter = 3.4 cm), and amikacin (ZOI diameter = 3 cm). Interestingly, any imipenem/cilastatin produced a lower ZOI of 2.7 cm. On the other hand, TMP-SMX did not have any effect on the bacteria (ZOI diameter = 0 cm). Azithromycin and doxycycline only produced small ZOI (0.6 cm and 0.9 cm, respectively). Table 3.1 summarizes the results of the antibiotics susceptibility test.

Table 3.1 - Table displaying the results of the antibiotic susceptibility test.

Antibiotic	**Concentration and volume per disc**	**Results**	**Diameter of zone of inhibition (ZOI)**
Amikacin	10 µg (15 µL)	Susceptible	3 cm
Ampicillin	10 µg (15 µL)	Susceptible	3.5 cm
Amoxicillin	30 µg (15 µL)	Susceptible	3.4 cm
Azithromycin	15 µg (15 µL)	Resistant	0.6 cm
Ceftriaxone	30 µg (15 µL)	Susceptible	3.9 cm
Ciprofloxacin	5 µg (15 µL)	Susceptible	3.6 cm
Doxycycline	30 µg (15 µL)	Resistant	0.9 cm
Imipenem / Cilastatin	10 µg (15 µL)	Susceptible	2.7 cm
Meropenem	10 µg (15 µL)	Susceptible	3.7 cm
Trimethoprim - Sulfamethoxazole	25 µg (15 µL)	Resistant	0 cm

Figure 3.19 - Results of the antibiotic susceptibility test.



3.5.2. Susceptibility test of CBD oil dissolved in ethanol

The test was performed in a duplicate manner, where two discs were impregnated with CBD dissolved in ethanol and the third one denoted as "E" was impregnated with ethanol. The results of the CBD/ethanol susceptibility test are displayed in figure 3.20. Based on visual observation, we determined that the bacterial strain is susceptible to lower doses of CBD, whereas it is resistant to higher doses. The strain did not produce a ZOI for the concentration of 1.25 μg/mL (C1). The largest ZOI was observed for plate C3

containing 0.0125 µg/mL, followed by C2 (concentration = 0.125 µg/mL). We also observed that the ZOI of C3 covered also the area where the ethanol disc was placed. C4 (concentration = 0.00125 µg/mL) produced a smaller ZOI compared to C3. Moreover, ethanol did not appear to have any effect on the growth of the bacteria, as seen on plates C1 and C4, making it a suitable control. We can also note that for all 4 concentrations, one of the CBD discs did not produce any effect.

Figure 3.20 - Results of the CBD/ethanol susceptibility test.

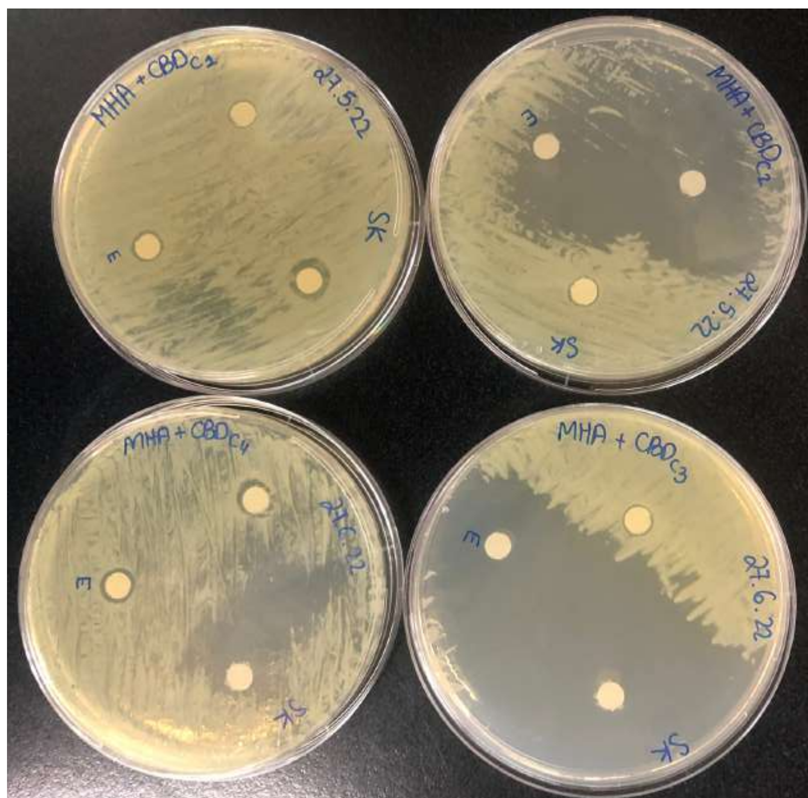


Table 3.2 - Table displaying the results of the CBD/ethanol susceptibility test.

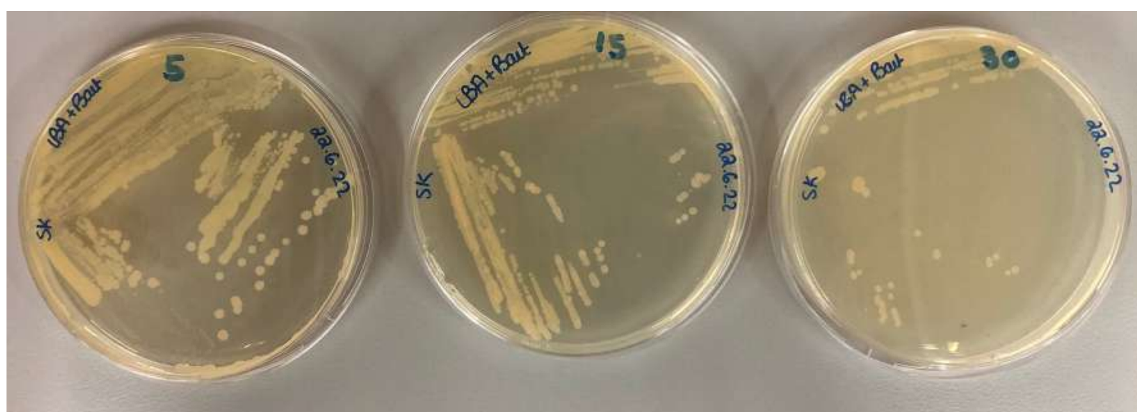
CBD/ethanol concentration	Results	Diameter of zone of inhibition (ZOI)
C1 1.25 µg/mL	Resistant	0.1 cm
C2 0.125 µg/mL	Susceptible	6 cm
C3 0.0125 µg/mL	Susceptible	8 cm

C4 0.00125 µg/mL	Susceptible	4 cm

3.5.3. Susceptibility to UV light

The inoculated plates were exposed to UV light for t5, 15, and 30 minutes, and incubating them for 24 hours. The results are displayed in figure 3.21 below. We can note that the bacteria grew completely when exposed to 5 and 15 minutes of UV light. The growth was decreased for the plate exposed for 30 minutes.

Figure 3.21 - Results of the susceptibility test to UV light.



3.6. Identification of the bacterial genus

The PubMed indexing search focused on Gram-negative aerobes based on the differential staining and physiological tests obtained. The search identified 76 bacterial genera. Based on the results of the search and based on the results of our study, we can eliminate the following genera:

- *Bdellovibrio,* *Beggiatoa*, *Caulobacter*, *Gallionella*, *Herbaspirillum*, *Magnetospirillum*, *Neisseria*, *Rhodospirillum*, and *Vitreoscilla* species as they are not rod-shaped.

- *Acidiphilium*, *Acidobacteria*, *Azospirillum*, *Bradyrhizobium*, *Brucella*, *Cellvibrio*, *Chromohalobacter*, *Coxiella*, *Francisella*, *Moraxella*, *Nitrobacter*, *Ornithobacterium*, *Rhodopseudomonas*, *Rhodothermus*, *Riemerella*,

Sphingobacterium, *Sphingomonas*, *Taylorella*, *Tenacibaculum*, and *Thiothrix* species as they are not strict aerobes.

- *Afipia*, *Agrobacterium*, *Alcaligenes*, *Alteromonas*, *Azorhizobium*, *Azotobacter*, *Chryseobacterium*, *Comamonas*, *Cupriavidus*, *Cytophaga*, *Delftia*, *Flavobacterium*, *Halomonas*, *Herbaspirillum*, *Kingella*, *Leptospira*, *Lysobacter*, *Methylobacillus*, *Methylococcus*, *Methylomonas*, *Methylophilus*, *Nitrosomonas*, *Ochrobactrum*, *Paracoccus*, *Pedobacter*, *Pseudoalteromonas*, *Pseudomonas*, *Psychrobacter*, *Rhizobium*, *Sinorhizobium*, *Thermus*, *Thiobacillus*, *Xanthobacter*, and *Zoogloea* species as they are oxidase positive.

- *Achromobacter*, *Acidithiobacillus*, *Acinetobacter,* *Bordetella*, *Flexibacter,* *Halothiobacillus,* *Xanthomonas*, and *Xylella* species as they are encapsulated.

- *Legionella* species as they are endospore-forming.

- *Acetobacter* and *Gluconobacter* as their colonies are large (more than 3-mm in diameter), slimy, and pale. Moreover, *Gluconobacter* species have a milky white to yellowish color.

After elimination, two bacterial genera remain: *Gluconacetobacter* and *Sphaerotilus*. *Sphaerotilus* colonies are rough and possess a filamentous appearance whereas *Gluconacetobacter* colonies are small (1 to 2-mm in diameter), smooth, circular, and have a beige color. Based on colony morphology, the closest genus to the unknown strain is *Gluconacetobacter*.

CHAPTER FOUR

DISCUSSION

The increase in resistant bacteria poses a major obstacle to healthcare systems, rendering both prevention and treatment difficult. Antimicrobial resistance (AMR) represents one of the major public health issues of the twenty-first century (Jindal *et al.,* 2015). While AMR is an inevitable part of evolution, it has been hastened by the overuse and misuse of antibiotics. Bacteria continuously employ a variety of long-term strategies to counteract the effects of antibiotics (Burmeister, 2015). Moreover, due to diminished economic incentives and harsher regulatory barriers, the pipeline for novel drugs is drying up. In the past three decades, only two new antibiotic classes with new target sites have been developed: oxazolidinones (linezolid) and cyclic lipopeptides (daptomycin) (Gupta and Nayak, 2014). Thus, the need for new and undiscovered antimicrobial agents has never been more pronounced. The vast majority of known antibiotics originate from a small number of culturable soil microbes; however, little research has been done on the biosynthetic potential of most soil bacteria (Cragg and Newman, 2013). As soil microbial communities are very diverse, they supply a wealth of novel secondary metabolites and allow the discovery of new antibiotic classes (Sharrar *et al.*, 2020). On the other hand, with the emergence of highly resistant novel and ancient bacteria from the soil (Frindte *et al.*, 2020), it is crucial to identify and characterize those with potential diagnostic or therapeutic implications.

The bacterial strain isolated from the soil in Lebanon has demonstrated inconclusive results upon 16S rRNA gene sequencing, indicating that it is absent from the current databases. In addition to providing preliminary results to our collaborative team regarding its use in biocement applications, identifying and characterizing the novel bacterial species is crucial from a therapeutic and pharmaceutical perspective. Our study has determined that the bacterial strain has a Gram-variable aspect, such as most of the cells are Gram-negative rods with some Gram-positive ones. It is non-acid fast, non-endospore forming, non-encapsulated, and does not produce a biofilm on the air-liquid

interface. Physiological tests have determined that it is strictly aerobic, requiring oxygen to survive and proliferate. The negative oxidase test determined that the bacteria do not have cytochrome C oxidase as part of its electron transport chain. This suggests that the bacteria respire using other oxidases. On another note, culturing bacteria on LBA plates was very difficult. The colonies only grew when they were incubated for a long period (around 40 to 48 hours). This implies that the bacterial species requires specific nutrients for adequate growth. The colonies grown on LBA are small, circular, creamy in color, and smooth. Based on the results presented in chapter 3 and appendix 1, we determined that the strain belongs to the genus *Gluconacetobacter*. *Gluconacetobacter* belongs to the *Acetobacteraceae* family, a group of Gram-negative to Gram-variable, strictly aerobic, acetic acid bacteria. *Acetobacteraceae* includes ten genera; the major ones are *Gluconacetobacter*, *Acetobacter*, and *Gluconobacter* (Gomes *et al.*, 2018). In most cases, strains of *Acetobacter* and *Gluconacetobacter* are co-isolated. They can be differentiated based on their physiological and morphological characteristics (Matsutani *et al.*, 2011). Sugarcanes are the biggest habitats of *Gluconacetobacter* species. As alternatives to agrochemicals, they have been employed as bioinoculants to boost plant development and growth (Saravanan *et al.,* 2007). On the other hand, *Acetobacter* is one of the most common bacterial species found in mushroom cultivation by-products (Suwannarach *et al.,* 2022). *Gluconacetobacter* is nutritionally demanding and difficult to cultivate on common media such as LBA (Mizzi *et al.*, 2022). This phenomenon was observed with the strain investigated as it required a longer time of incubation to produce full colonies, indicating that it needs specific nutrients for growth.

The strain does possess some pathogenic and virulence traits. It is urease, catalase, and coagulase positive, and has a β-hemolytic activity. Urease activity enables bacteria to acclimate to the acidic environment of the human stomach and colonize it (Weeks *et al.*, 2000). *H. pylori*, a bacterial species known to establish infection in the stomach, use a proton-gated urea channel called UreI. This channel brings in urea in the bacterial cytosol and the present urease hydrolyzes it. Ammonia and bicarbonate are produced, which buffer the protons within the periplasm of the bacteria (Marcus *et al.*, 2005). Ammonia damages the epithelial lining of the stomach (Smoot *et al.*, 1990) as well as the

glycosaminoglycan surface of the urinary tract which protects it from bacterial infections (Parsons *et al.*, 1984). The damage to different organ cell linings and the changes in the pH make ureolytic bacteria pathogenic. Catalase is also known to be a virulent enzyme. The phagocytes of immune cells produce hydrogen peroxide to destroy microbes. Bacterial catalase protects the intraphagocytic bacteria from phagocytosis through the action of catalase (Mandell, 1975). Being involved in oxidative stress resistance, catalase is an important virulent factor of certain bacteria. Similarly, coagulases contribute to the pathogenicity of bacteria. *Staphylococcus aureus* is one of the major bacteria that caused severe infections in humans (such as infective endocarditis and bacteremia) and has a high mortality rate (Diekema *et al.*, 2001). Coagulase is one of the main bacterial factors that interact with the human coagulation system. Research conducted *in vitro* and animal models has shown that bacteria with the coagulase gene have a considerable effect on the onset and progression of infective endocarditis (Mancini *et al.*, 2018). Moreover, coagulase acts in synergy with the van Willebrand factor binding protein. This synergistic effect converts fibrinogen to fibrin, causing severe endocarditis (Claes *et al.*, 2017). β-hemolysin is also regarded as an important virulence factor of bacteria, notably for *Streptococcus agalactiae* (*S. agalactiae*). Hemolysins also release iron from erythrocytes to be consumed by the invading bacteria. Iron is important for proper bacterial physiology as it is a fundamental component of metabolic enzymes and proteins (Balashova *et al.*, 2001). The complete lysis of red blood cells contributes to invasive disease. Moreover, it destabilizes the membranes of brain endothelial cells (Doran *et al.*, 2003) and lung epithelial cells (Nizet *et al.*, 1996), causing a more severe type of infection. A recent study published in 2020 has found that the expression of the β-hemolytic gene increases the resistance to specific antibiotics such as clindamycin and cefoxitin (Nasaj *et al.*, 2020).

Since the unknown bacterial strain is Gram-variable to Gram-negative, we selected the major classes of antibiotics that target Gram-negative bacteria. As the antimicrobial susceptibility test has shown, the bacteria are resistant to 3 out of 10 antibiotics tested belonging to the following classes: tetracyclines (doxycycline), macrolides (azithromycin), and sulfonamides (TMP-SMX). As of 2022, there are no CLSI or EUCAST MIC breakpoints for *Gluconacetobacter* species. A major reason for that is that

this genus is considered a plant pathogen. To the best of our knowledge, only two cases of *Gluconacetobacter* infections have been reported. The first case reported lymphadenitis caused by *Gluconacetobacter sacchari*. The patient was suffering from chronic granulomatous disease, causing immunosuppression (Greenberg *et al.,* 2006). The second case report was published in 2020 describing an infection caused by *Gluconacetobacter liquefaciens* (*G. liquefaciens*) following the ingestion of sugarcane juice. The patient, suffering from advanced liver cirrhosis, developed recurrent bacteremia. The antimicrobial susceptibility test resistance to chloramphenicol (MIC ≥ 256 µg/mL) followed by ciprofloxacin, TMP-SMX, and meropenem, all three having a MIC ≥ 32 µg/mL. *G. liquefaciens* were susceptible to gentamicin (MIC ≥ 0.125 µg/mL) and tetracycline (MIC ≥ 1 µg/mL) (Olenski *et al.*, 2020). Our results alongside these two case reports show the resistance of this genus to antibiotics and the possible emergence of *Gluconacetobacter* species as a human pathogen, specifically in immunocompromised patients.

Moreover, and as previously discussed in section 1.4.2, *Cannabidiol* oil has only been shown to be effective at killing a selected number of Gram-negative bacteria. The strain was susceptible to CBD oil which has shown to be a more effective bactericidal agent than the tested antibiotics. At a concentration of 0.0125 µg/mL, CBD has produced a ZOI of 8 cm. The largest ZOI produced by antibiotics was 3.9 cm for ceftriaxone. Interestingly, the strain was resistant to higher doses of CBD (1.25 µg/mL) and susceptible to lower doses (C3 = 0.0125 µg/mL). This effect was not seen for the different concentrations of the antibiotics tested. It appears as though, at a certain concentration, the bacteria developed a defense mechanism preventing CBD from penetrating the cells or utilized efflux pumps propelling it back out. One main issue was encountered with the Kirby-Bauer disc diffusion method. As discussed in section 2.7.2, the test was performed in a duplicate manner where two discs were impregnated with CBD/ethanol. The same issue was noticed for all four plates prepared where one of the desks did not produce an effect. This could be interpreted as a result of the quick evaporation of ethanol. A possible way to avoid the evaporation of the agent is by performing the test faster and covering the plates between each loading. The results presented show the possible use of CBD oil as

an antimicrobial agent for resistant bacteria. Yet, animal models are essential to test this hypothesis as CBD is highly attracted to proteins. Studies have shown that 86-90% are bound to human plasma, leaving 10-14% free to attack bacterial cells. Once the CBC reaches the bacterial cytosol, it might be lured away by bacterial proteins (Blaskovich *et al.*, 2021).

From a biocementation perspective, the strain did produce calcium carbonate precipitate through its urease activity. In the bio-cementation process, the precipitated CaCO3 crystals could serve as solid bridges to bind dispersed particles together and improve the mechanical properties of the ground and soil (Xu *et al.*, 2021). Ideally, bacteria should possess a high urease activity that can also be induced regardless of the environmental conditions (i.e., the presence of urea) (Chaparro-Acuña *et al.*, 2017). Generally, urease-producing bacteria belonging to the *Bacillacae* family (i.e., *Bacillus*) are used in MICP as most are considered to be non-pathogenic (Stocks-Fischer *et al.*, 1999). However, there are a few studies related to the isolation of the strain. Many researchers around the world have isolated different organisms from different areas. For instance, 12 bacterial strains were isolated from different soil samples, gardens, landfills, and cement and calcite residues of a calcification reactor. The strains were urease positive and were phylogenetically related to *Bacillus sphaericus* (Hammes *et al.*, 2003). While MICP has a lot of advantages economically and environmentally, there are numerous factors to consider after the isolation of ureolytic bacteria, including:

1) The generation of ammonia following ureolysis can be toxic and may pose a risk to human health (Harkes *et al.*, 2010).
2) Microbial activity depends on specific factors including pH, temperature, and concentration of nutrients, making MICP a slow and complex process (Ivanov *et al.*, 2008).

Therefore, although the investigated strain produced calcite and hydrolyzed urea quickly (as the color change was noted after 12 hours), additional studies about its pathogenicity and urease activity have to be performed.

CHAPTER FIVE

CONCLUSION

Soil is an essential part of the human habitat as it provides food, recreation, and space for living. Its microbiota is widely diverse, affecting microbial ecology, soil health, and human health. The alarming rise of antimicrobial resistance and resistance genes triggers an urgency for the discovery of new antimicrobial agents. Soil microorganisms provide essential by-products and secondary metabolites that may aid in the advancement of drug development. However, the destruction of the soil ecosystem and heavy human interference has increased the emergence of novel resistant pathogens and the re-emergence of ancient deadly bacteria.

The strain isolated yielded important features related to its biochemical and morphological structures and to its resistance to antimicrobials. Based on morphological, physiological, staining, and biochemical tests, we determined that the strain belongs to the *Gluconacetobacter* genus. As our study provides important data to our collaborative team aiming to use the bacteria in biocementation, future *in vivo* studies are required to assess the pathogenicity of the strain. From a biochemical point of view, the strain appears to have virulence traits such as positive urease, catalase, and coagulase activities, and completely destroys red blood cells. The strain also confers resistance to three main antibiotic classes and interestingly is resistant to higher doses of *Cannabidiol* but susceptible to lower doses. Before we are able to provide a scientific recommendation to our collaborative team, additional studies are required. In addition to animal studies, whole genome sequencing should be performed to detect and examine resistance genes, virulence factors, and clusters for the production of biocement. From a biocementation perspective, the bacteria must be able to survive at a basic pH of 12 to 14. Therefore, it is essential to measure the optimum, minimum, and maximum pH and temperature for growth and survival. Moreover, the time for $CaCO_3$ formation should be assessed and the produced crystals should be quantified. Their morphological characteristics must also be described and analyzed under various conditions.

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