MOLECULAR INVESTIGATION OF *P. aeruginosa* IN THE PRESENCE OF 4-HBA

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ABSTRACT

MOLECULAR INVESTIGATION OF *P. aeruginosa* IN THE PRESENCE OF 4-HBA

The escalating threat to human health posed by bacterial pathogens is increasingly attributed to the growing prevalence and distribution of antibiotic-resistant bacteria. In response to antibiotics, microorganisms have developed resistance mechanisms to elude and survive the impacts of these drugs. Phenolic acids have emerged as potent candidates in the battle against bacterial infections due to their unique property of not inducing resistance. This study focuses explicitly on 4-hydroxybenzoic acid (4-HBA), a type of phenolic acid, as an effective antimicrobial agent. Proteomics research has become an indispensable tool in the fight against antimicrobial resistance. *Pseudomonas aeruginosa*, a bacterium capable of existing in both planktonic and biofilm states and known to cause numerous human diseases, is of particular significance in this context. Furthermore, the study explores the molecular aspects of *P. aeruginosa* when exposed to 4-HBA through proteomic analysis, revealing a significant impact on protein biosynthesis as a predominantly affected function. Additionally, *P. aeruginosa*, the study investigates the effects of phenolic acid and antibiotic-loaded bone cements on Methicillin-sensitive *Staphylococcus aureus*, Methicillin-resistant *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, and *P. aeruginosa*. Remarkably, 20 percent inhibition rate is achieved after 48 hours of treatment. Moreover, the study examines biofilm produced by *P. aeruginosa* in the presence of 4-HBA, utilizing both the Crystal Violet assay and Scanning Electron Microscopy. Significantly, the biofilm formation is observed to be disrupted by these methodologies. Overall, this study underscores the effectiveness of 4-HBA as antimicrobial compound against diverse range of pathogens, as demonstrated through both phenotypic and proteomic approaches.

Keywords: *Antimicrobial resistance, Pseudomonas aeruginosa, 4-HBA, Proteomics, Bone cement*

ÖZET

4-HBA'NIN VARLIĞINDA *P. aeruginosa'*NIN MOLEKÜLER İNCELEMESİ

Bakteriyel patojenlerin insan sağlığına yönelik artan tehdidi, giderek artan sıklıkta ve dağılımda görülen antibiyotik dirençli bakterilerin büyümesine atfedilmektedir. Antibiyotiklere karşı, mikroorganizmalar bu ilaçların etkilerini atlatabilmek ve hayatta kalabilmek için direnç mekanizmaları geliştirmişlerdir. Fenolik asitler, direnç oluşturmayan benzersiz bir özelliğe sahip oldukları için bakteriyel enfeksiyonlarla mücadelede etkili adaylar olarak ortaya çıkmıştır. Bu çalışma özellikle etkili bir antimikrobiyal madde olarak 4-hidroksibenzoik asit (4-HBA) adlı bir fenolik asite odaklanmaktadır. Proteomik araştırma, antimikrobiyal dirence karşı savaşta vazgeçilmez bir araç haline gelmiştir. *Pseudomonas aeruginosa,* hem planktonik hem de biyofilm durumlarında var olabilen ve birçok insan hastalığına neden olduğu bilinen bir bakteridir ve bu bağlamda özellikle önemlidir. Ayrıca, çalışma *P. aeruginosa*'nın 4-HBA'ya maruz kaldığında protein biyosentezi üzerindeki etkilerini proteomik analiz yoluyla ortaya koymaktadır ve bu etkinin belirgin olduğunu göstermektedir. *P. aeruginosa*'nın yanı sıra, çalışma fenolik asit ve antibiyotik yüklü kemik çimentosunun Metisilin-duyarlı *Staphylococcus aureus,* Metisilin-dirençli *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium* ve *P. aeruginosa* üzerindeki etkilerini araştırmaktadır. Şaşırtıcı bir şekilde, 48 saatlik muamele sonrasında yüzde 20 inhibisyon oranı elde edilmiştir. Ayrıca, çalışma 4-HBA'nın varlığında *P. aeruginosa* tarafından üretilen biyofilmi Crystal Violet testi ve Taramalı Elektron Mikroskopi kullanarak incelemektedir. Önemli bir şekilde, bu yöntemlerle biyofilm oluşumunun bozulduğu gözlemlenmektedir. Genel olarak, bu çalışma, hem fenotipik hem de proteomik yaklaşımlarla 4-HBA'nın çeşitli patojenlere karşı etkili bir antimikrobiyal bileşik olarak etkinliğini vurgulamaktadır.

Anahtar Kelimeler: *Antimikrobiyal direnç, Pseudomonas aeruginosa, 4-HBA, Proteomik, Kemik çimentosu*

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LIST OF ABBREVIATIONS

4-HBA	4-Hydroxybenzoic Acid
BSA	Bovine Serum Albumin
CDC	Centers for Disease Control and Prevention
CFU	Colony Forming Unit
ddH2O	Double-distilled water
ddPCR	Droplet Digital Polymerase Chain Reaction
ESI	Electrospray Ionization
g	Gram
Genta	Gentamicin
h	Hours
HCl	Hydrochloric Acid
HPLC	High-Pressure Liquid Chromatography
LC	Liquid Chromatography
LC-ESI-MS/MS	Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry
M	Molar
MDR	Multidrug-resistant
mg	Milligram
mL	Milliliter
mM	Millimolar
MRSA	Methicillin-resistant *Staphylococcus aureus*
MSSA	Methicillin-sensitive *Staphylococcus aureus*
nm	Nanometer
O.D.	Optical Density
PBP	Penicillin Binding Proteins
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
PMMA	Polymethylmethacrylate
ROS	Reactive Oxygen Species
SEM	Scanning Electron Microscopy

STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
T3SS	Type III Secretion System
T6SS	Type VI Secretion System
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
v/v	Volume/Volume
WHO	World Health Organization
x g	Gravity
µl	Microliter

CHAPTER 1

LITERATURE REVIEW

1.1. Overview of Pathogens

Bacteria are a diverse group of microorganisms that belong to the domain Bacteria. They are prokaryotic organisms that lack a defined nucleus and other membrane-bound organelles. Bacteria are characterized by their small size, simple cell structure, and lack of organelles. They can be found in nearly every habitat on Earth, including soil, water, and the human body (Baron et al., 1996). Bacteria are essential in many ecological processes, such as nutrient cycling, soil formation, and plant growth. They also break down organic matter and recycle crucial elements like nitrogen and carbon. In addition, many bacteria have mutualistic relationships with other organisms, such as in the gut of animals, where they aid digestion (Lladó et al., 2017).

Bacteria are also used in many industrial applications. Microorganisms are vital in producing fermented foods like yogurt, cheese, and pickles. Additionally, they find extensive applications in the biotechnology sector, primarily for generating enzymes, antibiotics, and various chemical compounds. (Anand et al., 2022). Nevertheless, certain bacteria can potentially cause diseases in both humans and animals. These pathogenic bacteria have the capability to cause various infections, including strep throat, urinary tract infections, and foodborne illnesses such as food poisoning. It is important to understand the different types of bacteria and how to prevent the spread of pathogenic strains through good hygiene practices and the use of antibiotics when necessary (Balloux et al., 2017).

The human body is a dynamic and intricate ecosystem comprising approximately 10^{13} human cells and a vast population of microbial species, including bacteria, yeast, and protozoa, amounting to around 10^{14} cells. These microorganisms, known as normal flora, typically inhabit specific body regions, such as the skin, oral cavity, large intestine, and vagina. Furthermore, individuals consistently harbor viral infections, with the majority of them remaining asymptomatic. (Alberts et al., 2002)

Microorganisms that normally exist in our body do not make people sick, but if there is a weakness in the immune system or if they reach normally a sterile place, as in the case of intestinal perforation, the intestinal flora enters the abdominal peritoneal cavity and causes peritonitis. However, the host doesn't need to be injured or immune-compromised for pathogenesis. Because pathogens have evolved mechanisms to circumvent biochemical barriers and elicit specific responses in the host that will contribute to their survival and proliferation. There are a series of steps a p athogen must undergo in order to survive and reproduce in a host. These:(1) colonize the host, (2) find a compatible nutritional niche in the host body, (3) overcome the host's immune system, (4) reproduce using the host's resources, (5) emerge and spread to a new host (Alberts et al., 2002)

Pathogenic bacteria refer to bacteria that elicit disease in humans, animals, or plants. Several instances of pathogenic bacteria encompass *Salmonella*, *Escherichia coli* (*E. coli*), *Streptococcus*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*. These bacteria can spread through contaminated food and water, physical contact with infected individuals, or inhaling respiratory droplets. Pathogenic bacteria are responsible for a broad spectrum of diseases, spanning from minor ailments like food poisoning to severe and potentially life-threatening conditions such as pneumonia, meningitis, and sepsis. Treatment for bacterial infections may involve antibiotics. However, it is important to note that the overuse of antibiotics can lead to antibiotic resistance, making treatment more difficult (Zourob et al., 2008).

According to the World Health Organization (WHO), pathogenic bacteria cause a significant number of deaths each year globally. In 2016, it was estimated that bacterial infections caused 7% of all deaths worldwide, which amounts to approximately 517,000 deaths. Some specific examples of bacterial diseases that cause significant morbidity and mortality include tuberculosis (TB), sepsis, and bacterial diarrhea. Tuberculosis alone is estimated to cause approximately 1.4 million deaths annually, while sepsis and diarrhea combined contribute to 1.3 million fatalities each year. It is important to note that these are rough estimates, and the actual number of deaths caused by pathogenic bacteria may be higher, as some deaths may go unreported or misclassified (WHO, 2019).

Multidrug-resistant (MDR) *Pseudomonas aeruginosa*, a type of bacteria, is particularly prevalent among individuals with Cystic Fibrosis. In the United States alone, it is responsible for over 50,000 healthcare-associated infections annually, leading to

approximately 440 deaths. Among these infections, more than 13% (around 6,700 cases) are caused by MDR *Pseudomonas*. The Centers for Disease Control and Prevention (CDC) has classified MDR *Pseudomonas aeruginosa* as a significant threat in its Antibiotic Resistance (CDC AR) report. Furthermore, the World Health Organization's (WHO) 2017 list of priority antibiotic-resistant pathogens, which encompasses 12 bacterial families, identifies carbapenem-resistant *Pseudomonas aeruginosa* as the most severe menace to human health. (Azam and Khan, 2019).

As the Centers for Disease Control and Prevention (CDC) states, bacterial infections continue to present a significant public health concern in the United States, contributing to substantial rates of illness and death. Some of the most common bacterial infections in the U.S. include *Streptococcus pneumoniae* (pneumonia), Clostridium difficile (C. difficile) infection, and Methicillin-resistant *Staphylococcus aureus* (MRSA). In 2017, the CDC reported that approximately 3 million cases of invasive bacterial infections occurred in the U.S., leading to an estimated 38,000 deaths. Additionally, the CDC estimates that *C. difficile* infection alone causes nearly 500,000 illnesses and at least 15,000 deaths yearly in the U.S. These numbers highlight the continued threat posed by pathogenic bacteria and the need for continued efforts to prevent and treat bacterial infections (CDC, 2019).

Opportunistic bacteria are normally harmless microorganisms that can cause disease when the host's immune system is weakened or compromised. These bacteria take advantage of the weakened immune system to cause infection, hence the term "opportunistic". Examples of opportunistic bacteria include *Escherichia coli (E. coli)*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Streptococcus pneumoniae*. Although these bacteria are commonly found in the environment and on the skin and mucous membranes of healthy individuals, they can cause infections in people with weakened immune systems, such as those with HIV/AIDS, cancer, organ transplant patients, and those receiving long-term treatment with antibiotics or steroids. Opportunistic bacterial infections can range from mild conditions, like skin and urinary tract infections, to severe diseases, such as pneumonia and sepsis. Early diagnosis and prompt treatment of these infections are crucial in preventing severe complications and improving outcomes for patients with weakened immune systems (Brown, Cornforth, and Mideo, 2012).

Some of the dangerous and opportunistic pathogens are the subject of this thesis. These pathogens described below are *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Enterococcus faecium*, and *Pseudomonas aeruginosa*.

1.1.1. *Staphylococcus aureus*

Staphylococcus aureus (*S. aureus*) is a Gram-positive bacterium associated with various clinical diseases. The infection can be transmitted in both healthcare settings and the community (Lowy et al., 1998). Since there is a development of multi-drug resistant strains, for instance, Methicillin-Resistant *Staphylococcus aureus* (MRSA), treatment of the infections remains problematic (Centers for Disease Control and Prevention (CDC); Boucher and Corey, 2008). The bacteria do not normally infect healthy skin tissue; however, if they can get into the bloodstream, a wide range of serious infections can emerge (Lowy et al., 1998). Transmission of the bacteria happens typically through direct contact. Other than being in the environment, it can be found in the healthy human flora.

Colonies of the bacteria can be found as yellow or golden (aureus means yellow or golden) on media, and with up to 10% salt concentration, they can grow. They could grow from 18 °C to 40 °C at anaerobic and aerobic conditions. The bacteria can be distinguished by being catalase-positive (all pathogenic *Staphylococcus* species), coagulase-positive (discrimination of *S. aureus* from the other *Staphylococcus* species), novobiocin sensitive (to discriminate from *Staphylococcus saprophyticus*), and mannitol fermentation positive (to differentiate from *Staphylococcus epidermidis*) (Rasigade and Vandenesch, 2014). The *mec* gene, which is found in the Staphylococcal chromosomal cassette *mec* (SCC*mec*) part, is carried by MRSA strains. Depending on the SCC*mec* type, multiple antibiotic resistance is deliberated by the situation (CDC, 2003). The gene encodes the penicillin-binding protein 2a (PBP-2a). PBP-2a is one type of penicillin-binding protein (PBP) that plays a crucial role in catalyzing the production of peptidoglycan in the bacterial cell wall. As an important enzyme involved in bacterial cell wall synthesis, it is significant for the structural integrity of the cell. Unlike other PBPs, PBP-2a exhibits a lower affinity for binding to beta-lactam antibiotics. Consequently, even in the presence of several antibiotics, PBP-2a allows for the continued synthesis of the bacterial cell wall. This characteristic makes MRSA strains resistant to oxacillin, methicillin, cephalosporins, and nafcillin. (CDC, 2003; Rasigade and Vandenesch, 2014).

The predominant sources of *S. aureus* strains are humans, such as mucous membranes and skin (Boucher and Corey, 2008) (Chambers et al., 2005). Colonization of these organisms has been estimated to occur in approximately half of the adult population, with 15% of adults carrying *S. aureus* constantly in their anterior nostrils. It has been found that hospitalized patients, healthcare workers, and immunocompromised individuals are particularly prone to carry the bacteria at higher ratios. The transmission of bacteria can occur via fomites or from person to person (Tong et al., 2015; Rasigade and Vandenesch, 2014).

S. aureus causes several diseases, including infective endocarditis, bacteremia, pulmonary infections, gastroenteritis, and skin and soft tissue infections (Tong et al., 2015). Strain differences lead to different serious infections and/or toxin-mediated diseases (DeLeo et al., 2009). Formation of biofilm, antigen masking using Protein A, production of an antiphagocytic capsule, and inhibiting leukocytes chemotaxis are the ways to avoid host immune response (Foster et al., 2005). During infectious endocarditis, bacteria have the ability to attach themselves to fibronectin and extracellular matrix proteins. This attachment is facilitated by specific bacterial cell wall-associated proteins, such as teichoic acids, clumping factors, and fibrinogen-binding proteins. These bacterial proteins play a role in mediating the binding of bacteria to fibronectin and other components of the extracellular matrix (DeLeo et al., 2009). Additionally, in infectious endocarditis, toxic shock syndrome, and sepsis, Staphylococcal superantigens (TSST-1 or toxic shock syndrome toxin 1) are significant for evasion of the host cells (Salgado-Pabón et al., 2013; Musser et al., 1990). Since *S. aureus* strains produce biofilms and use quorum sensing in a bacterial cell density-dependent manner, they cause infections of prosthetic devices (Le and Otto, 2015).

The type of infection and the presence or absence of drug-resistant strains determine the treatment of *S. aureus* infections (Tong et al., 2015). If isolates are sensitive, penicillin would be used for treatment; it is vancomycin for MRSA strains (Boucher and Corey, 2008). There may be cases where alternative treatment is required in addition to antimicrobial therapy (Tong et al., 2015). In situations involving toxin-mediated diseases, prosthetic endocarditis, or catheter-related infections, fluid replacement management is frequently necessary to remove foreign devices. MRSA (methicillin-resistant *Staphylococcus aureus*) infections pose a significant threat in both

hospital and community settings due to their resistance to multiple antibiotics. (Boucher and Corey, 2008; Chambers et al., 2005).

Treating implant-associated infections (IAIs) caused by *S. aureus* is a pressing medical requirement for effective therapeutic interventions. (Darouiche et al., 2004). IAIs originating from implant-associated infections account for approximately half of the 2 million nosocomial cases reported annually. (Darouiche et al., 2004). Treating this medical complication poses significant challenges, resulting in elevated morbidity and mortality rates. (Kapadia et al., 2016). *S. aureus* is a prominent causative agent of IAI, exhibiting a remarkable ability to infect foreign objects present in the human body. (Del Pozo and Patel, 2009). *S. aureus* can establish persistence on implant surfaces through the formation of biofilms. Biofilms are structured communities of microcolonies surrounded by an extracellular matrix, allowing them to adhere to biomedical implants. (Bjarnsholt et al., 2013). Treating infections associated with biofilms is challenging because biomaterials provide a conducive environment for *S. aureus*, reducing the necessary inoculum size for infection by a factor of more than 100,000 (Ari-Pekka et al., 2014).

1.1.2. *Staphylococcus epidermidis*

The common bacterial colonizers of humans and other mammals' skin and mucous membranes are *Staphylococci* species (Kloos and Schleifer, 1986). Since *Staphylococcus epidermidis* (*S. epidermidis*) colonizes head, nares, and axillae, it is the most commonly isolated species from human epithelia (Kloos and Musselwhite, 1975). Studies have demonstrated that *S. epidermidis* possesses genes that confer protection against harsh environments. For example, the bacteria possess eight sodium ion/proton exchangers and six transport systems for osmoprotectants, enabling them to effectively manage extreme osmotic pressure and salt concentration. (Rogers et al., 2009; Chu et al., 2008). Other than coagulase-positive Staphylococci, for instance, *S. aureus*, *S. epidermidis* does not contain coagulase enzymes and is in the group of coagulase-negative Staphylococci (CoNS). The species demonstrate a high degree of variety by 74 identified sequence types (STs) (Miragaia et al., 2007).

S. epidermidis, in its natural environment, does not cause infection; however, it can invade the human body with prosthetic devices. The situation leads to the entering of

the microbes into the bloodstream. Then, *S. epidermidis* could produce biofilm for protection from the antimicrobials and host defense mechanism (Otto et al., 2009). Biofilm formation is another way for coagulase-negative species to protect from harsh environments. Once the biofilm is formed by protective exopolymers called poly-y-glutamic acid, biofilm provides protection against innate host defense mechanisms (Otto et al., 2009).

1.1.3. *Enterococcus faecium*

Enterococci were initially identified in the human fecal microbiota in 1899. However, they remained classified within the *Streptococcus* genus until 1984. (Murray et al., 1990). *Streptococcus faecalis* (*S. faecalis*) was initially characterized in 1906 following its isolation from an individual with endocarditis. On the other hand, *Streptococcus faecium (S. faecium)* was first recognized in 1919. Subsequently, Streptococci belonging to serogroup D were further classified into two groups based on investigations involving DNA-rRNA homology and 16S rRNA sequence studies. These studies revealed notable biochemical properties and nucleic acid composition distinctions among the two groups. (Schleifer and Kilpper-Balz, 1984). *S. faecalis* and *S. faecium* have been classified within the *Enterococcus* group, which encompasses over 50 species in total (Parte et al., 2013).

Within the Enterococci group, *Enterococcus faecalis* (*E. faecalis*) and *Enterococcus faecium* (*E. faecium*) are the primary pathogens responsible for human infections. In the 1970s, Enterococci emerged as the predominant cause of infections acquired within hospital settings. (Gilmore et al., 2013). Over the past twenty years, *E. faecium* has undergone significant evolutionary changes, establishing itself as a prominent nosocomial pathogen globally. It has demonstrated remarkable adaptability to the conditions prevalent in healthcare facilities and has acquired resistance to glycopeptides. (Top et al., 2008; Bonten et al., 2001). Genes associated with glycopeptide resistance are governed by *van* operons found on mobile genetic elements (MGEs). These operons consist of regulatory genes that oversee the expression of ligase genes, which confer resistance to glycopeptides. Among these ligase genes, *vanA* and *vanB* are the most prevalent. (Arias and Murray, 2012).

1.1.4. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa (*P. aeruginosa*) is a Gram-negative, aerobic, and rod-shaped bacterium with measurements of 0.5 to 0.8 μm by 1.5 to 3.0 μm (Baron and Iglewski, 1996; Tang et al., 2015). Single polar flagellum is found in almost all of them, whereas two or three flagella can be seen in some strains (Baron and Iglewski, 1996). Although the temperature between 25 °C and 37 °C is proper for its growth, at 42 °C, it can also grow, which is a distinctive feature different than other *Pseudomonas* species (Tang et al. 2015). Different environments provide suitable conditions for its growth, which induces disease in humans, animals, and plants (Tang et al., 2015).

Including *P. aeruginosa*, more than 25 *Pseudomonas* species lead to diseases in humans that are related to opportunistic infections. Since it causes several human diseases, it is crucial to highlight *P. aeruginosa*. It is considered one of the biggest threats for hospitalized patients because of their weakened immune defenses and resistance to a broad range of antibiotics (Baron and Iglewski, 1996; Moradali et al., 2017). The bacterium can occupy immunodeficient tissues; meanwhile, the situation isn't seen in healthy tissues (Wu and Li, 2015). Cystic fibrosis and cancer-diagnosed patients and ones with burns in hospitals have shown that 50% of fatalities are caused by *P. aeruginosa* infection (Wu and Li, 2015).

In nature, *P. aeruginosa* exhibits two forms: biofilm and planktonic. A complex nutrition supply is not required for these bacteria, as distilled water alone would be sufficient for their growth. Additionally, media containing ammonium sulphate and acetate provide conditions for the good growth of the bacteria (Wu and Li, 2015). *P. aeruginosa*'s strains produce one or more pigments, including the fluorescent, green-yellow pyoverdine, and green-blue pyocyanin. Pyochelin, a derivative of pyocyanin, has the ability to sequester iron from *P. aeruginosa* from the host. Regular work of the human respiratory system and nasal cilia could be disorganized by pyocyanin (Wu and Li, 2015). Pyoverdine also plays a crucial role in the colonization and biofilm formation of *P. aeruginosa* (Alonso et al., 2020)

P. aeruginosa strains possess extensive genomes, typically ranging from approximately 5 to 7 megabase pairs (Mbp) in size. These strains exhibit a considerable metabolic capacity, as evidenced by their ability to synthesize numerous secondary metabolites. (Moradali et al., 2017). The genes of *P. aeruginosa* exhibit a significant

degree of conservation and comprise a rich repertoire of regulatory genes and networks, surpassing those observed in other well-studied bacterial genomes. These genes enable the bacterium to effectively respond to and adapt to various environments. (Moradali et al., 2017).

1.1.4.1. Pathogenesis of *P. aeruginosa*

Escape from innate immunity mechanisms and harm to host cells can be conducted by toxins and extracellular enzymes. Additionally, host-killing mechanisms, for instance, phagocytosis and complement, can be inhibited by alginate or bacterial capsules (Wu and Li, 2015).

In the initial invasion process, virulence consists of alkaline protease and elastase, two extracellular proteins. Fibrin is lysed, and fibrin formation is disorganized by alkaline protease. IgG, IgA and collagen are cut by elastase. Furthermore, two hemolysins and a cytotoxin protein are produced by *P. aeruginosa* and have a role in invasion. Different types of cells, including neutrophils, can be eliminated by the cytotoxin; lipids and lecithin in host cell membranes can be eliminated by phospholipase and lecithinase, which are the two hemolysins (Wu and Li, 2015).

Exotoxin A and exoenzyme S are extracellular protein toxins generated by *P. aeruginosa* (Wu and Li, 2015). To inhibit the synthesis of proteins in host cells and thus lead to cell death, elongation factor 2 (EF2) is ADP ribosylated by exotoxin A, which is a mono-ADP-ribosyltransferase (Tang et al., 2015; Wu and Li, 2015). Burned tissue has been found to contain exoenzyme S, which is another ADP-ribosyltransferase (Wu and Li, 2015).

P. aeruginosa also has a systemic effect on organisms. Since it has LPS and capsule, serum bactericidal activity and phagocytosis are not usable for *P. aeruginosa.* Inflammatory cytokines can be stopped, and the proteases can cut IgG antibodies. Hypotension, fever, and intravascular coagulation are examples of Gram-negative septicemia's common diseased characteristics provided by LPS's lipid A moiety. Eventually, dissemination is advocated by cytotoxicity that is generated by exotoxin A (Wu and Li, 2015).

1.1.4.2. Colonization of *P. aeruginosa*

P. aeruginosa commonly threatens hospitalized patients because it may prefer damaged tissue for respiratory track colonization, for instance, respiratory track infected by tuberculosis or AIDS. Pili of *P. aeruginosa* may use sialic acid (*N*-acetylneuraminic acid) as the tracheal epithelial cells' receptor. Additionally, respiratory cells' glycoproteins can be served by exoenzyme S in the same manner to appear to be adhesion. *P. aeruginosa* needs pili to adhere to epithelial cells, which is conducted via receptors of sialic acid, galactose, or mannose on epithelial cells after providing fibronectin degradation using a protease enzyme (Wu and Li, 2015).

Alginate, a repeating polymer of guluronic and mannuronic acid found in *P. aeruginosa's* mucoid exopolysaccharide and matrix of biofilm of the *Pseudomonas,* is made by the alginate. Cells are anchored to the environment by the alginate, and bacteria are defended from host protection by that structure (Wu and Li, 2015).

1.1.4.3. Biofilm of *P. aeruginosa*

Several different bacteria have the ability to attach to distinct surfaces and form biofilms (Donlan et al., 2002). A biofilm is a sophisticated bacterial community enclosed within a self-generated matrix composed of extracellular polymeric substances (EPS). When confronted with swift alterations in environmental factors like nutrient availability and temperature fluctuations, the formation of a biofilm represents a crucial survival strategy for the species involved (Rollet et al., 2008). Biofilms offer a significantly enhanced capacity, approximately 1000 times greater, to evade the host immune system and exhibit resistance against antimicrobial agents compared to their planktonic counterparts (Lewis et al., 2001). Due to its well-established ability to form biofilms, *P. aeruginosa* is a valuable model for investigating the process of biofilm formation (Crespo et al., 2018; Ghafoor et al., 2011). A robust biofilm plays a crucial role in *P. aeruginosa*, enabling it to effectively compete, persist, and establish dominance within the complex polymicrobial environment of the cystic fibrosis lung (Oluyombo et al., 2019). *P. aeruginosa* has the ability to colonize various surfaces, such as medical materials, including contact lenses, implants, and urinary catheters. (Ghafoor et al., 2011).

Therefore, it is of utmost importance to comprehend the architectural characteristics of biofilms and the underlying molecular mechanisms governing the antimicrobial tolerance displayed by bacteria within biofilms. This knowledge is crucial for developing strategies to prevent or eradicate biofilm-associated infections (Thi et al., 2020). Considering that biofilm takes up a vital place in pathogenesis, its composition enlightened us about the importance of biofilm. Biofilm is a formation that allows bacteria to survive against sudden changes such as the presence of food or temperature and is a complex bacterial community consisting of extracellular polymeric substances (EPS) (Moradali and Rehm, 2020; Rehm et al., 2010).

The biofilm matrix of *P. aeruginosa* consists of polysaccharides, extracellular DNA, lipids, and proteins. (Strempel et al., 2013). The biofilm matrix, comprising polysaccharides, extracellular DNA, lipids, and proteins, contributes to approximately 90% of the biofilm biomass. It serves as a structural framework for attachment to living and non-living surfaces. Additionally, it provides protection to bacteria against antibiotics and host immune responses. The matrix is also crucial in facilitating nutrient exchange, enzyme activity, and intercellular communication within the biofilm community. (Strempel et al., 2013; Jackson et al., 2004; Ryder et al., 2007). The molecules Psl, Pel, and alginate have significant roles in the processes of adhesion, formation, and stability of biofilms. They are essential for the initial attachment of bacteria to surfaces, as well as for the development and structural integrity of the biofilm community. These molecules contribute to the overall architecture and cohesion of the biofilm, ensuring its robustness and persistence over time. (Billings et al., 2013).

Psl is a type of pentasaccharide found in neutral form, consisting predominantly of d-glucose, d-mannose, and l-rhamnose components. (Ma et al., 2009; Byrd et al., 2009). This exopolysaccharide is essential for facilitating the attachment of sessile cells to surfaces and promoting cell-to-cell interactions during the initiation of biofilms in both non-mucoid and mucoid strains. (Ma et al., 2012; Jones and Wozniak, 2017). The characteristics of Psl can be summarized as follows: (i) Psl confers advantages to biofilm communities rather than unattached populations; (ii) mixed biofilms with Psl-producing cells exhibit improved growth compared to non-Psl-producing cells; (iii) Psl-positive populations tend to dominate over Psl-negative populations during biofilm development; and (iv) non-Psl producers may not benefit from the presence of Psl producers (Irie et al., 2017). Psl contributes to the structural integrity of mature biofilms by surrounding the

mushroom-shaped architecture. (Ma et al., 2009). Heightened Psl expression is linked to the formation of cell aggregates in a liquid medium, a characteristic observed in the sputum of individuals with cystic fibrosis (CF) (Irie et al., 2012; Staudinger et al., 2014). Psl is a signaling molecule that stimulates the synthesis of c-di-GMP (bis-(3'-5')-cyclic dimeric guanosine monophosphate). Elevated levels of c-di-GMP lead to the development of denser and more resilient biofilms (Staudinger et al., 2014). Moreover, Psl also serves as a protective barrier, shielding biofilm bacteria from antimicrobial agents and preventing their engulfment by neutrophils through phagocytosis. This attribute makes Psl an effective defensive molecule, enabling the establishment of persistent infections. (Mishra et al., 2011).

Pel is a polysaccharide polymer with a positive charge. It is composed of N-acetyl-D-glucosamine and N-acetyl-D-galactosamine, which have undergone partial deacetylation. It serves as a crucial component of the biofilm matrix in non-mucoid strains, contributing to both the initial adhesion to surfaces and the overall integrity of the biofilm structure. Its properties are comparable to those of Psl in these aspects (Colvin et al., 2013; Jennings et al., 2015). Pel is accountable for the formation of a membrane-like biofilm that develops at the interface between air and liquid in a stationary broth culture (Friedman and Kolter, 2003). The production of Psl and Pel is specific to certain strains and can be modified in response to changes in the surrounding environment (Colvin et al., 2011). Pel enhances the resistance of bacteria residing within the biofilm to aminoglycoside antibiotics (Yang et al., 2011). Furthermore, biofilms containing Pel demonstrate resistance against the antibiotic colistin and exhibit decreased susceptibility to neutrophil-mediated killing initiated by human HL-60 cell lines. (Baker et al., 2016). In contrast to Psl, Pel is not readily available to Pel-negative cells, both in the biofilm and planktonic populations (Irie et al., 2017).

In the biofilm of *Pseudomonas* strains, the presence of alginate mucoid is observed due to a mutation in the mucA22 allele. The shift from an acute to a chronic infection is frequently linked to the appearance of mucoid phenotypes, particularly in isolates obtained from individuals with cystic fibrosis. (Ciofu et al., 2015; Folkesson et al., 2012). Alginate is a polymer with a negative charge composed of acetylated residues of mannuronic acid and guluronic acid (Evans and Linker, 1973). Alginate has been extensively studied and found to serve numerous crucial functions, including the maturation of biofilms, shielding against phagocytosis and opsonization, and limiting the

diffusion of antibiotics within the biofilm (Tseng et al., 2013; Hay et al., 2013). The ratio of mannuronic acid to guluronic acid in alginate has an impact on the viscoelastic properties of biofilms. This, in turn, can lead to impaired cough clearance in the lungs of individuals with cystic fibrosis who are infected with *P. aeruginosa*. (Gloag et al., 2018; Rehm and Valla, 1997).

Exposure to environmental stress, including antimicrobial treatment, can trigger the release of DNA from disrupted cells, referred to as extracellular DNA (eDNA). eDNA plays a crucial role in the formation of biofilms. The endolysin Lys, encoded within the R- and F-pyosin gene cluster, contributes to cell disruption, which can occur during both the initial stages of biofilm formation and the planktonic phase. Environmental stress leads to structural damage to the cell wall, causing rod-shaped bacteria to transform into round cells and ultimately undergo lysis. The resulting eDNA, along with cytosolic proteins and RNA, is enclosed within membrane vesicles (MVs) that form from fragments of the membranes of lysed cells (Turnbull et al., 2016). Extracellular DNA (eDNA) can also be found in specific locations, such as the surface and the stem regions of mushroom-like micro-colonies (Allesen-Holm et al., 2006). eDNA serves multiple functions within the biofilm environment. Firstly, it acts as a source of nutrients for bacteria residing in the biofilm. Secondly, it aids in cellular organization and alignment through twitch motility. Thirdly, eDNA acts as a chelator for divalent cations on the outer membrane, such as Mg^{2+} and Ca^{2+}. This chelation subsequently activates the type VI secretion system and facilitates the diffusion of virulence factors within the host. Fourthly, eDNA contributes to the acidification of the biofilm environment and infection sites. This acidification limits the penetration of antimicrobial agents. Lastly, eDNA has the potential to influence the inflammatory response initiated by neutrophils in the presence of *P. aeruginosa* biofilms. (Wilton et al., 2016; Wilton and Wong et al., 2016; Gloag et al., 2013; Fuxman Bass et al., 2010).

Polyhydroxyalkanoate (PHA) is a carbon and energy storage biopolymer synthesized within the cells present in a biofilm. While it does not directly contribute to the structural composition of the biofilm matrix, PHA serves important functions. It provides stress tolerance to the cells and facilitates adhesion to abiotic surfaces like glass (Pham et al., 2004).

Gaining insights into the stages of biofilm formation is crucial for studying the impact of antimicrobial agents. *P. aeruginosa* has been found to exhibit slow growth

under hypoxic and anoxic conditions, providing a basis for comparison with the growth patterns observed in patients with cystic fibrosis (CF) and chronic wounds (Sønderholm et al., 2017). The reduced growth rate of *P. aeruginosa* in oxygen-limited environments is associated with its antibiotic resistance. This bacterium forms biofilms on abiotic surfaces like industrial equipment and medical implants. The process of biofilm development can be described through five distinct steps (Figure 1.1). Step 1: Bacterial cells attach to surfaces through the presence of cellular appendages such as flagella and type IV pili, facilitating their adhesion (O'Toole et al., 2017; Klausen et al., 2003). Restricted flagellar movement mediates twitch motility and the biosynthesis of exopolysaccharides, which are necessary for surface aggregation during biofilm formation (Hickman and Harwood, 2008). It's a reversible adherence. Bacterial responses and biofilm formation of wild-type PAO1 were shown to be surface-specific in a proteomics study. This situation can be understood by the records of *P. aeruginosa, which* detects a different surface and adjusts other proteins and the amounts of these proteins accordingly (Guilbaud et al., 2017). Step 2: Transition of bacterial cells from reversible to irreversible attachment occurs. Step 3: The gradual spread of attached bacteria into a more structured architecture is achieved at this stage, and this structure is called micro-colonies. Step 4: These micro-colonies evolve into three-dimensional mushroom-like structures, a hallmark of biofilm maturation. Step 5: In the center of the micro-colony, matrix space is disrupted by cell autolysis to release dispersed cells, followed by a transition from sessile to planktonic growth mode to seed non-colonized areas (Step 6), allowing the biofilm cycle to repeat (Rasamiravaka et al., 2015). It has recently been presented that the endonuclease EndA via eDNA degradation is required for the dispersal of existing biofilm (Cherny and Sauer, 2019).

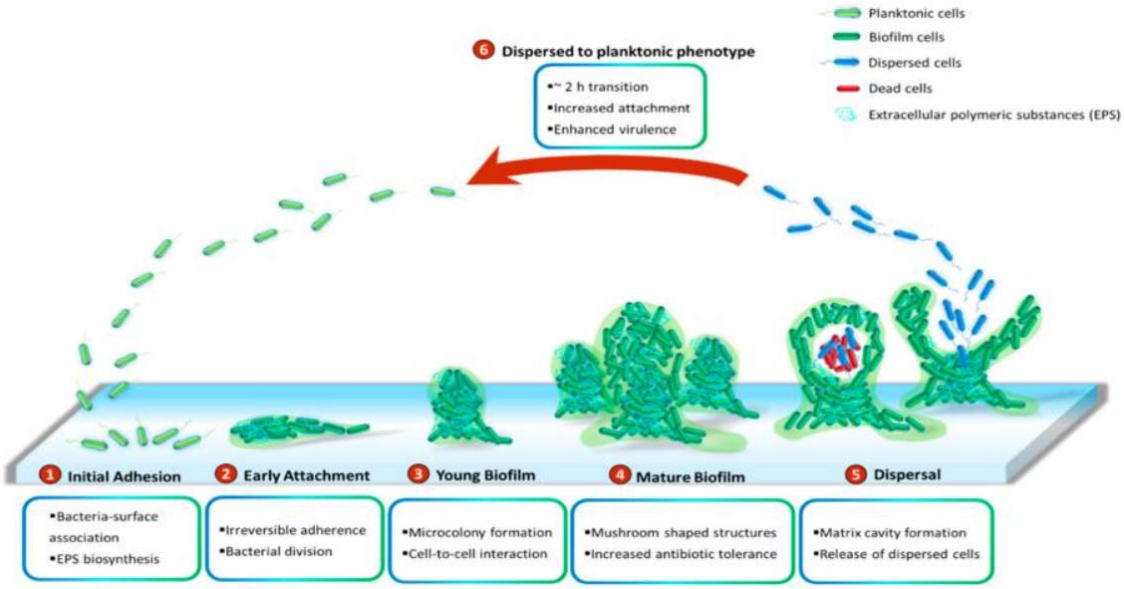

Figure 1.1. *P. aeruginosa*'s biofilm development cycle is divided into six steps. At first, bacteria settle on the surface and produce extracellular polymeric substances (EPS), including lipids, eDNA, polysaccharides, and proteins. After that, cell division and the evolution of reversible binding to irreversible binding occurs. The following steps are the formation of microcolonies and the transformation of these colonies into mushroom-shaped structures. Production of virulence factors and cell-cell interaction play a vital role in the maturation and robustness of biofilms. A matrix cavity is created in the center of the micro-colony through cell autolysis to disrupt the matrix to release the dispersed population. Finally, the released cells undergo a transition to planktonic phenotypes of about 2 hours, during which planktonic phenotypes will then invade non-colonized areas (Moradali and Rehm, 2020).

Recent studies have revealed that cells within biofilms exhibit distinct physiological characteristics compared to planktonic cells and display enhanced resistance to antibiotic treatments (Rollet et al., 2008). Despite advancements in understanding the physiological differences between attached and free-swimming cells within biofilms, there remains limited knowledge regarding the intermediate forms that bridge the gap between these two lifestyles. Recent evidence suggests that the transition from detachment to planktonic growth represents a unique stage in the development of biofilms (Step 6) (Figure 1). Distinct from both planktonic and sessile cells, these cells exhibit altered physiology and mark the transition from chronic infections to acute infections. After a 2-hour lag phase, during which pyoverdin and intracellular c-di-GMP levels decrease, these cells undergo a phenotypic shift towards a planktonic phenotype (Chua et al., 2013; Chua et al., 2015). In the dispersed population, a decrease in the expression of iron-uptake genes and an increase in the expression of virulence-encoding

genes were detected (Chua et al., 2014). Results from both laboratory experiments (in vitro) and studies involving live organisms (in vivo) demonstrated that the dispersed cells exhibited high cytotoxicity toward macrophages, increased vulnerability to iron deficiency, and greater lethality toward nematode hosts compared to the planktonic bacteria (Chua et al., 2014). A separate study demonstrated that dispersed bacteria obtained from biofilms treated with glycoside hydrolase exhibited rapid spread in a mouse model of chronic wound infection, ultimately leading to fatal septicemia (Fleming and Rumbaugh, 2018).

1.2. Antimicrobial Resistance

Antimicrobial resistance (AMR) poses a substantial public health challenge and imposes a significant economic burden on healthcare systems worldwide. The World Health Organization (WHO) has identified certain bacteria as priority threats to human health, with a significant number belonging to the Gram-negative bacteria category. This includes *P. aeruginosa*, *Acinetobacter baumannii*, and Enterobacteriaceae (WHO, 2017; Willyard et al., 2017). Recent findings indicate the emergence of resistance against antibiotics that were once considered last-line treatments, such as colistin and polymyxin B (Zavascki et al., 2007; Liu et al., 2016).

Most of the antibiotics currently used in clinical practice were identified and developed during the period known as the "golden age" of antibiotic discovery, which spanned from the 1940s to the 1960s. Subsequently, new drugs were developed by chemically modifying existing scaffolds to create analogs with improved properties (Coates, Halls, and Hu, 2011). The issue is further compounded by the phenomenon that bacteria evolve resistance more rapidly than the development of new antibiotics (Martínez and Baquero, 2014; Bonomo et al., 2016). The most recent class of antibiotics, designed to specifically target gram-negative bacteria, are synthetic fluoroquinolones, introduced to medical practice almost five decades ago (Lundquist et al., 2020; Lewis et al., 2020).

Antibiotic resistance is an inherent process that arises when microorganisms come into contact with antibiotic medications. When exposed to antibiotics, susceptible bacteria are either eliminated or suppressed, while bacteria that possess inherent resistance or have acquired resistance mechanisms have a higher likelihood of survival and proliferation.

The rise of antibiotic resistance is not only attributed to the excessive use of antibiotics but also to their inappropriate use, which includes improper selection, inadequate dosing, and inadequate adherence to treatment guidelines (Francesco, Pezzotti, and Pantosti, 2015).

1.3. Phenolic Acids

Plant usage for significant needs, such as food, medicine, and clothes, started from thevery beginning of human history. Several types of diseases have been observed with decreased risk during the consumption of a diet rich in plant-derived foods. One of the substances found in the cures is polyphenols, which have roles in disease healing and biological activities (Kumar and Goel, 2019).

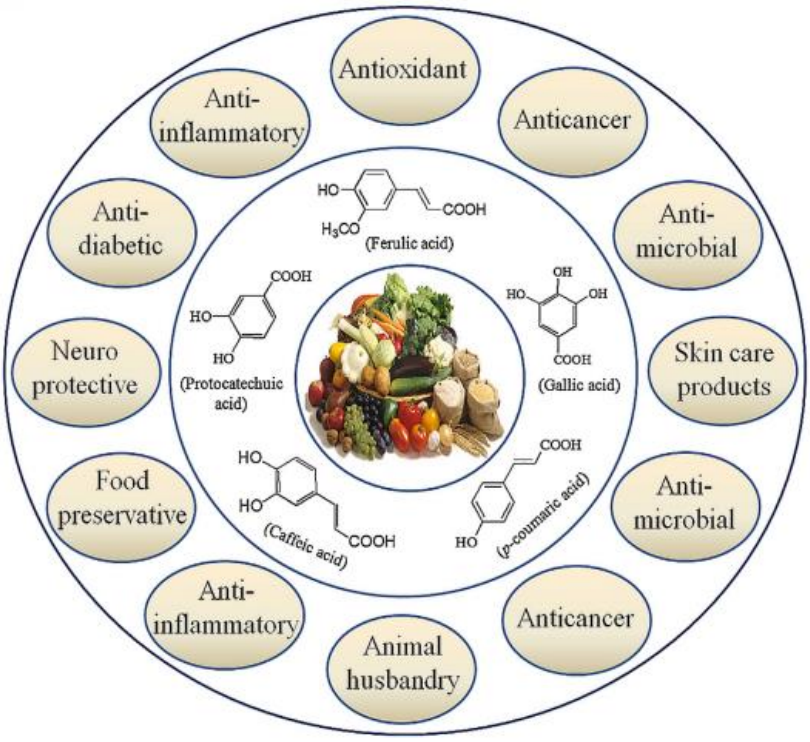

Figure 1.2. Different applications of phenolic acids (Kumar and Goel, 2019).

Plant phenolics, composed of an aromatic ring that is attached to one or more hydroxyl groups, and are generated through a shikimate pathway from L-phenylalanine and L-tyrosine, are the secondary metabolites mostly abundant and have types of phenolic

acids, simple phenols, flavonoids, stilbenes, and coumarins, etc. Those secondary metabolites are derived mainly for defense against insects, viruses, and bacteria. Flavor, color, and smell production is another role of these metabolites. Besides having functions in plant defense, development, and growth, they also have beneficial properties for humans since they are considered powerful antioxidants with antimicrobial, anticancer, and anti-inflammatory features (Figure 1.2) (Kumar and Goel, 2019).

Free phenolic acids have the ability of high solubility in water and increased levels of bioavailability compared to flavonoids. Additionally, other than flavonoids, phenolic acids can be absorbed in the stomach. Phenolic acids, like flavonoids, can alleviate chronic diseases and body damage caused by excessive free radicals, and thus, they are recognized as exceptional antioxidants (Chen et al., 2020).

Phenolic compounds, including one carboxyl group, are generally known as phenolic acids. The highest concentrations of these substances are typically found in various plant-based foods, including seeds, fruit skins, and vegetable leaves. They are commonly present in bound forms such as esters, glycosides, or amides and are less frequently encountered in their free form. (Pereira et al., 2009). Hydroxycinnamic acid and hydroxybenzoic acid are two subtypes of phenolic acids (Kumar and Goel, 2019). It has been presented that the in vitro antioxidant activity of phenolic acids is much higher than acknowledged antioxidant vitamins (Tsao and Deng, 2004). Hydroxycinnamic acids are commonly found in food and are derived from cinnamic acid. They are often present as simple esters with glucose or quinic acid. Among these compounds, chlorogenic acid is the most abundant soluble-bound hydroxycinnamic acid. It is composed of a combination of quinic acid and caffeic acid. Caffeic acid, sinapic acid, ferulic acid, and p-coumaric acid are typical examples of hydroxycinnamic acids. On the other hand, hydroxybenzoic acids include p-hydroxybenzoic acid, gallic acid, vanilic acid, protocatechuic acid, and syringic acid. Hydroxybenzoic acids have a C6-C1 structure and are derived from benzoic acids. By conjugating organic acids and sugars, they are in soluble form and found in cell wall arrangements' integral section as lignin (Kumar and Goel, 2019). Compared with hydroxycinnamic acids, hydroxybenzoic acids are often found in onions, black radishes, red fruits, etc., found in low concentration (Griffiths et al., 1996). The most found hydroxybenzoic acids are vanillic, p-hydroxybenzoic, syringic acid, and protocatechuic (Kumar and Goel, 2019).

The precise role of phenolic acids in plants is still not fully comprehended, but it is recognized that they contribute to enzyme activity and nutrient uptake. Phenolic acids are the primary polyphenols synthesized by plants and serve as precursors for bioactive compounds widely utilized in the cosmetic, food industries and therapeutics. They have been extensively studied for their diverse biological applications. (Croft et al., 1998). Phenolic acids can be metabolized by natural microbes, which is the most important advantage of using them. In that way, an important alternative to human-made environmentally harmful chemicals is provided. Moreover, plant phenolics have the role of signaling molecules, for instance, in the initiation of legume rhizobia and arbuscular mycorrhizal symbiosis production (Mandal, Chakraborty, and Dey, 2010).

1.3.1. Phenolic Acids as Antimicrobial Agents

In fecal water, phenolic acids are the most abundant type compared to other phenolic compounds (Cueva et al., 2010). They operate as food preservatives and show antimicrobial activity. The antimicrobial properties of phenolic acids are influenced by their chemical structure, particularly the position of saturated chains, the number of substitutions in the benzene ring, and the chain length. Increased length of the alkyl chain has been found to enhance the antimicrobial activity. For example, phenolic acid oligomers exhibit greater activity than monomers (Elegir et al., 2008). The antimicrobial activity of compounds, such as hydroxybenzoic and hydroxycinnamic acids, is influenced by the presence of hydroxyl (−OH) and methoxy (−OCH3) functional groups. However, the relationship between their structure and function does not fully explain the diverse range of antimicrobial activities observed in these compounds (Sánchez-Maldonado, Schieber, and Gänzle, 2011). Similar to other weak organic acids, hydroxybenzoic acids exert their antimicrobial activity by diffusing across the cell membrane in their undissociated acid form. This acidification of the microbial cytoplasm disrupts cell function and ultimately leads to cell death. The antimicrobial effectiveness of phenolic acids, determined by their solubility in the microbial membrane, is influenced by factors such as pKa (acid dissociation constant) and lipophilicity. (Campos et al., 2009). Since pH provides a charge on the ring substitutions (methoxy and hydroxyl groups), the saturation of the side chain and the carboxyl (-COOH) group shows a significant part.

There is a contrary interaction between the change in pH and phenolic acids' antimicrobial activity (Almajano et al., 2007).

The emergence of drug-resistant bacterial pathogens poses a growing challenge, and there is a need to discover compounds with novel antibacterial mechanisms to address this issue. Plant phenolic acids, which are naturally occurring compounds, present a promising reservoir of diverse chemical structures that have the potential to inhibit bacteria through distinct mechanisms. However, the investigation of novel antibacterial action mechanisms associated with plant phenolic compounds is not widely practiced and can pose challenges. The use of systems biology approaches can aid in evaluating antibacterial action mechanisms without the bias of targeted bioassays, enabling the discovery of novel mechanisms against drug-resistant microorganisms. As natural products, including plant-derived compounds, exhibit a range of bioactive properties, they have gained renewed interest in this context (Rempe et al., 2017). Multi-drug resistant bacteria can be defeated by using phenolic acids derived from plants; however, to use them as antibacterial curing, the description of their action mechanism needs to be thoroughly investigated (Cristina, McBain and Simões, 2012).

Because conclusive evidence of protein-ligand binding is available, proteomic approaches have been prevalent for discovering small molecule binding targets. For small molecule binding targets isolation for the phage display of peptides and protein microarrays for separation, affinity chromatography was utilized, and then mass spectrometry was used for identification. Broad research has been conducted on techniques of proteomics (Wong et al., 2008; Ziegler et al., 2013). Proteomic approaches by differential expression of proteins can also determine mechanisms of actions of antimicrobial therapy. Research shows mechanistic determinations can be provided by expression proteomics. For instance, the approach provided evidence for a second mechanism of action of atypical tetracycline chelocardin, which leads to membrane depolarization at high concentrations and inhibits peptidyl transferase at low concentrations and in *B. subtilis* (Stepanek et al., 2016).

1.3.2. 4-Hydroxybenzoic Acid

4-hydroxybenzoic acid, also referred to as p-hydroxybenzoic acid (PHBA), is a monohydroxybenzoic acid derived from benzoic acid. It contains a hydroxy substituent at the C-4 position of the benzene ring. It is an organic compound with a chemical formula of C7H6O3 and a molecular weight of 138.12 g/mol. PHBA is a white crystalline solid with a 214-216 °C melting point range. The hydroxyl group on the fourth carbon imparts important properties to 4-hydroxybenzoic acid, such as solubility in water and stability towards oxidation (Figure 1.3). The compound is soluble in water and polar organic solvents such as ethanol, methanol, and acetone but is insoluble in nonpolar solvents like benzene and ether (PubChem, 2020).

4-Hydroxybenzoic acid is found in various plant species, including rose, carnation, clove, etc. It functions as a secondary metabolite in plants, serving as a protective agent against environmental stress and pathogenic attacks. It also plays a role in plant growth and development and has been reported to have antifungal, antibacterial, and antiviral properties (Kim et al., 2020).

In the chemical industry, 4-hydroxybenzoic acid serves as a crucial precursor for the synthesis of diverse derivatives. For instance, it is utilized in the production of salicylic acid, which possesses analgesic properties and finds application as a pain reliever. Additionally, 4-hydroxybenzoic acid is an essential compound in the synthesis of various fragrances, flavorings, preservatives, and pharmaceuticals, including anti-inflammatory drugs, anti-tumor drugs, and anti-viral drugs (Wang et al., 2018).

In conclusion, 4-hydroxybenzoic acid is a versatile and important organic compound with diverse applications in the chemical and pharmaceutical industries and the natural world. Its hydroxyl group gives it solubility in water and stability towards oxidation, making it an ideal starting material for synthesizing a wide range of compounds.

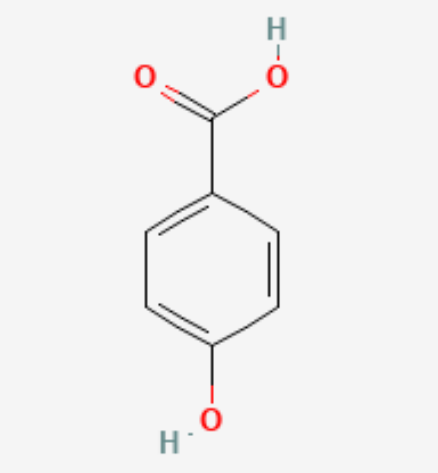

Figure 1.3. 4-hydroxybenzoic acid's chemical structure (PubChem, 2020).

1.4. Bone Cement

Bone cement is a medical adhesive used to fix artificial joints, such as hip or knee replacements, to the bones in the body. The cement is a mixture of a powder and a liquid that hardens quickly when mixed. It is applied to the surface of the artificial joint and the bone, and then the joint is pressed into place. Bone cement is used to fill bone voids, and as a bone graft substitute (Vaishya, Chauhan, and Vaish, 2013).

Antibiotic-loaded bone cement, also known as antibiotic-impregnated bone cement (AIB cement), is a medical adhesive used in orthopedic surgeries, such as joint replacements and spinal fusions. It is the antibiotics added type of traditional bone cement. The antibiotics are released gradually, providing a sustained release of the drugs to the surgical site. This can help to prevent or reduce the risk of infection in the implanted joint (Bistolfi et al., 2011).

Staphylococcal species, such as *S. aureus* and *S. epidermidis*, are common causes of infection in orthopedic surgeries. These bacteria are often found on the skin and can easily pass to the surgical site during the procedure. When they infect a replaced joint, they can cause prosthetic joint infection (PJI). PJI is a serious complication that can occur after joint replacement surgery, and it's one of the most common reasons for revision surgery (Cara et al., 2021).

AIB cement can help reduce the risk of PJI by providing a sustained release of antibiotics to the surgical site. Antibiotics, such as gentamicin and vancomycin, can help to kill or inhibit the growth of the Staphylococcal bacteria, thus reducing the risk of

infection. In-vitro studies have shown that using AIB cement can significantly reduce the bacterial load on the replaced joint and decrease the incidence of infection (Ghosh et al., 2022).

However, the use of AIB cement should be used with caution. Excessive utilization of antibiotics can result in the development of antibiotic resistance, a phenomenon that can pose challenges in the effective treatment of bacterial infections in subsequent instances. Additionally, the use of AIB cement can also lead to an increased risk of implant failure and aseptic loosening, particularly in cemented total hip arthroplasty. In conclusion, Antibiotic-loaded bone cement is an option in orthopedic surgeries that can help to reduce the risk of prosthetic joint infection caused by Staphylococcal species. Therefore, the use of AIB cement should be based on a balance between the potential benefits and risks and should be reserved for high-risk patients or cases with a high risk of infection.

P. aeruginosa can be responsible for prosthetic joint infections (PJI), and it is known to be one of the most virulent and difficult-to-treat pathogens in orthopedic surgery. AIB cement can be used as an adjunctive measure in preventing *P. aeruginosa* infections, but it's not considered the main line of defense. The antibiotics used in AIB cements, such as gentamicin and vancomycin, are ineffective against *P. aeruginosa*. Therefore, AIB cement alone may not be enough to prevent PJI caused by *P. aeruginosa* (Scott & Higham, 2003). A comprehensive strategy is necessary to prevent *P. aeruginosa* infections in orthopedic surgery. The antibiotics used in AIB cement are not very effective against *P. aeruginosa*. Therefore, AIB cement alone may not be enough to prevent PJI caused by this bacterium.

E. faecium can cause infections in various contexts, including orthopedic surgical procedures. It can be responsible for prosthetic joint infections (PJI), and it is known to be a nosocomial pathogen that can cause nosocomial infections (Baldassarri et al., 2005). AIB cement can be used as an adjunctive measure in preventing *E. faecium* infections. However, it should not be relied upon as the main line of defense. The antibiotics used in AIB cements, such as gentamicin and vancomycin, exhibit activity against *E. faecium*, but their efficacy may be limited by the emergence of antibiotic resistance. Therefore, AIB cement alone may not be enough to prevent PJI caused by *E. faecium*. The prevention of *E. faecium* infections in orthopedic surgery requires a multifaceted approach, including prophylactic antibiotics, appropriate surgical techniques, and implant coatings designed

to minimize bacterial colonization. While considering the inclusion of AIB cement as an adjunct measure, it should be viewed as part of a broader preventive regimen, complementing these other preventive measures.

In conclusion, AIB cement can be used as an adjunctive measure in preventing infections caused by opportunistic bacteria such as *P. aeruginosa* and *E. faecium* infections in orthopedic surgery, but it should be used in conjunction with other preventive measures such as prophylactic antibiotics, appropriate surgical techniques, and implant coatings.

1.5. Aim

This study aims to investigate the alterations in the overall protein profile of *Pseudomonas aeruginosa* in the presence of 4-HBA using LC-ESI-MS/MS analysis. Furthermore, the protein data obtained will be validated at the transcript level using droplet digital PCR. Additionally, the research aims to examine the impact of gentamicin, 4-HBA, and a combination of gentamicin and 4-HBA loaded bone cement on various bacterial strains, including MSSA, MRSA, *S. epidermidis*, *E. faecium*, and *P. aeruginosa*. Furthermore, the study assesses the phenotypic changes in the biofilm formation of *P. aeruginosa* after the 4-HBA exposure.

CHAPTER 2

MOLECULAR INVESTIGATION OF *Pseudomonas aeruginosa* IN THE PRESENCE OF 4-HYDROXYBENZOIC ACID

2.1. Introduction

Proteomics is the large-scale study of proteins and their functions within an organism. It has become an essential field of study in various biological and medical research areas due to its importance in understanding biological processes, identifying disease biomarkers, and developing new therapeutic targets (Aslam et al., 2016). The study can be conducted to do protein expression analysis because it allows researchers to study the expression of proteins in different biological systems. By identifying changes in protein expression levels, researchers can gain insights into cellular responses to stimuli, such as drugs or environmental factors. Post-translational modifications can be found in the study. Proteins can undergo various post-translational modifications, such as phosphorylation, acetylation, and glycosylation, affecting their function. Proteomics allows researchers to study these modifications and their impact on protein activity and cellular signaling. Protein-protein interactions can be investigated by the method, as well. Proteins often work together in complex networks to carry out biological processes. Proteomics can be used to identify protein-protein interactions, which can provide insights into cellular signaling pathways and protein function. Proteomics can be utilized to identify disease biomarkers proteins present in different disease states. By analyzing the protein profiles of different disease samples, researchers can identify proteins specific to a particular disease, which can be used for early detection and diagnosis. Proteomics can also be used in drug discovery to identify potential targets and to develop more effective drugs. By studying the proteins involved in disease processes, researchers can design drugs that target specific proteins and pathways (Cho et al., 2007)

In summary, proteomics is a powerful tool that allows researchers to study the complex world of proteins and their role in biological processes. The information

obtained through proteomics can lead to new insights into disease mechanisms, better diagnostics and treatments, and improved patient outcomes.

Mass spectrometry (MS) is an extensively utilized analytical technique in proteomics research. It is a method that assesses the mass-to-charge ratio (m/z) of ions to ascertain the identity, abundance, and structure of molecules. In the field of proteomics, Mass Spectrometry (MS) is employed for the identification and quantification of proteins and peptides, as well as post-translational modifications.

The basic principle of MS is to ionize molecules, separate the ions based on their mass-to-charge ratio, and detect the resulting ion currents. There are different types of ionization techniques used in MS, such as electrospray ionization (ESI), matrix-assisted laser desorption/ionization (MALDI), and gas-phase ionization. ESI and MALDI are the most commonly used techniques in proteomics (Büyükköroğlu et al., 2018). In the field of proteomics, Mass Spectrometry (MS) serves various purposes, including protein identification. It achieves protein identification by creating peptide fragments from protein digestion and matching these fragment ions with a protein database. Additionally, in quantitative proteomics, MS plays a crucial role in measuring protein abundance within a sample, allowing researchers to compare protein levels across different samples or conditions. Moreover, MS is instrumental in the identification and quantification of post-translational modifications on proteins, encompassing phosphorylation, acetylation, and glycosylation in post-translational modification analysis. Furthermore, MS can be harnessed for identifying protein-protein interactions by detecting proteins that co-purify with a bait protein in protein-protein interaction analysis.

Overall, mass spectrometry is a critical technique in proteomics research that allows researchers to identify, quantify, and characterize proteins and their modifications. It is a highly sensitive and accurate technique that can provide detailed information about protein structure, function, and interactions.

The rising threat to human health from bacterial pathogens is increasingly attributed to the growing number and distribution of antibiotic-resistant bacteria. At the same time, the rate of discovery of new antimicrobial drugs has been diminishing. As a consequence of using antibiotics to combat infectious diseases, microorganisms have developed resistance mechanisms to evade and survive the effects of these drugs. Efforts are underway to identify these mechanisms and develop strategies to counter the steady rise in treatment failures caused by multidrug-resistant microbes. Antimicrobial

resistance (AMR) poses a significant global threat to public health, progressively emerging as a prominent hurdle in the effective management of infectious diseases. Antimicrobial agents, such as antibiotics, are becoming increasingly ineffective against certain pathogens due to the development of resistance mechanisms. According to the Centers for Disease Control and Prevention (CDC), over 2.8 million individuals are impacted annually by antibiotic-resistant infections, leading to a minimum of 35,000 fatalities (CDC, 2019). In Europe, there is a significant prevalence of illnesses and fatalities attributed to multidrug-resistant bacteria, with an estimated 670,000 infections and 33,000 deaths reported in 2015 (Cassini et al., 2019). Furthermore, it is projected that the number of fatalities attributed to antimicrobial resistance (AMR) could rise to approximately 10 million annually by 2050. This figure surpasses the projected burden of other significant health conditions, such as cancer and diabetes, when combined (Balouiri, Sadiki, and Ibnsouda, 2016).

Proteomics research has become an important tool in the fight against AMR by helping to identify new drug targets, understand resistance mechanisms, and develop more effective treatments. Proteomics plays a crucial role in this field of research by elucidating the molecular mechanisms of bacterial pathogenesis and identifying factors that contribute to disease outcomes. By uncovering physical interactions, proteomics provides valuable insights for developing targeted treatment approaches that can curb the spread of antibiotic resistance. With the recent advancements in whole-genome sequencing, proteomic technologies are extensively employed to investigate microbial gene expression. Consequently, proteomics has emerged as a dependable tool for studying bacteria (Khodadadi et al., 2020).

Proteomics techniques play a pivotal role in comprehensively studying the entire proteome of pathogenic microorganisms, including the proteins involved in resistance mechanisms. By identifying proteins that are essential for the survival of the pathogen, researchers can develop novel drug targets and therapeutic strategies.

Proteomics can also be used to study the proteome of host cells during infection. This can provide insights into the mechanisms used by pathogens to evade the host immune response and cause disease. By understanding these mechanisms, researchers can develop new treatments that target both the pathogen and the host response.

One of the critical challenges in antimicrobial research is the development of rapid diagnostic tests that can accurately identify the pathogen and determine its susceptibility

to different antimicrobial agents. Proteomics can be used to develop new diagnostic tests based on detecting specific proteins or peptides unique to a particular pathogen or strain. These tests can be faster and more accurate than traditional culture-based methods and can provide information on the pathogen's resistance profile.

Another important area of antimicrobial research in proteomics is the study of post-translational modifications (PTMs) on proteins. PTMs are chemical modifications that can alter the function, stability, and localization of proteins. Many pathogenic bacteria and fungi use PTMs to evade the host immune response and resist antimicrobial agents. Proteomics can be used to identify and quantify PTMs and to understand their role in resistance mechanisms.

Proteomics can serve as a valuable tool for investigating the interactions between antimicrobial agents and their respective targets. The analysis of protein-protein interactions involving antimicrobial agents and their targets makes it possible to develop novel drugs with enhanced effectiveness and reduced side effects, as stated by Tsakou et al. in 2020.

Finally, proteomics can be used to develop new vaccines against pathogenic bacteria and fungi. By identifying the proteins essential for the survival and virulence of the pathogen, researchers can develop new vaccine targets. Proteomics can also be used to develop new adjuvants to enhance the immune response to vaccines.

2.2. Materials and Methods

The antimicrobial activity of 4-HBA on *P. aeruginosa* at the protein level was determined through LC-ESI-MS/MS analysis and Protein Discoverer software, utilizing the *P. aeruginosa* database in UniProt for reference. Gene Ontology (GO) analysis was employed to analyze the downregulated proteins. The proteins that exhibited downregulation or upregulation following treatment were categorized based on their functions using Venn diagram analysis. These proteins were further grouped according to the biological processes they are involved in, as determined by UniProt. To gain insights into the effects of 4-HBA on *P. aeruginosa* at the proteomic level, the STRING database was utilized to visualize the interaction network among these groups of proteins. Afterward, ddPCR analysis was used to observe the transcriptomic level of changes in selected genes.

2.2.1. Growth Conditions of *P. aeruginosa* and 4-HBA Treatment

Pseudomonas aeruginosa (ATCC 27853) that were maintained at -80 °C, TSB included 20% of glycerol has been inoculated to fresh TSA plate every four weeks and during the period kept at 4 °C, a single colony was passaged to new agar plate and incubated at 37 °C in each week.

A single colony of *P. aeruginosa* was inoculated to 4 ml of TSB media and incubated at 37 °C for 18 hours without shaking. Spectrophotometric analysis at 600 nm of the overnight bacterial cultures was conducted to determine optical density (OD). Phenolic acid 4-HBA was purchased commercially (Sigma-Aldrich). 1.6 mg/ml of 4-HBA was found as the sub-inhibitory concentration in previous research of the Soyer Laboratory (Ozdemir and Soyer, 2020). Therefore, 10^6 CFU/ml of bacteria was inoculated to 400 ml of TSB for large-scale growth, including 1.6 mg/ml of 4-HBA. The same amount of bacteria was inoculated to 400 ml of TSB without 4-HBA as a control group. The control and treated groups were incubated without shaking at 37 °C and for 18 hours. Then, the cultures were used for total protein isolation.

2.2.2. Total Protein Isolation

The following protocol was followed to isolate total proteins from the 4-HBA treated and control groups: A culture volume of 200 ml was subjected to centrifugation at 10,000 x g for 20 minutes at 4 °C. The supernatant was discarded, and the remaining 200 ml of the culture was added to the same tube and centrifuged under the same conditions. After removing the supernatant, the harvested cells were washed twice with 20 ml of 0.85% NaCl and centrifuged at 17,000 x g for 20 minutes at 4 °C. The resulting pellet was dissolved in 2.5 ml PBS (pH 7.4). Sonication was performed for 15 minutes for the control group and 10 minutes for the 4-HBA treated groups, with 9-second intervals of sonication followed by 9 seconds of rest. The samples were then centrifuged at 17,000 x g for 20 minutes at 4 °C, and the supernatant was collected in Lo-bind Eppendorf tubes. The protein samples were stored at -80 °C until peptide sample preparation.

2.2.3. Preparation of Peptide Samples

To perform acetone precipitation, ice-cold acetone in a volume four times that of the protein samples was added to the samples and vortexed. The samples were then kept at -20 °C overnight. Protein concentrations of the samples were determined using the Bradford Assay. Bovine serum albumin (BSA) standards and protein samples were dissolved in a resuspension buffer containing 7 M urea and 2 M thiourea in pH 7.8 0.1 M Tris-HCl. The standards and unknown protein samples were subjected to spectrophotometric measurement at 595 nm, and a graph was plotted using the OD values versus concentrations (Appendix A). The equation derived from the graph (R^2>0.98) was used to determine the concentrations of the protein samples. After the Bradford assay, the protein sample concentrations were adjusted to 0.1 mg for the LC-ESI-MS/MS analysis. Before the addition of trypsin, a solution digestion procedure was employed. The protein samples were treated with DTT to achieve a final concentration of 5 mM. Subsequently, iodoacetamide was added to attain a final concentration of 10 mM, followed by the addition of DTT again to achieve a final concentration of 10 mM. These steps were performed in 50 mM Tris-HCl (pH 7.8), and the samples were kept in the dark at room temperature for 40 minutes during each step. The urea content was reduced to less than 1 M by adding 50 mM Tris-HCl (pH 7.8). Trypsin, at a concentration of 0.04 µg/ml (in 50 mM Tris-HCl (pH 7.8)), was added to each protein sample. The trypsin digestion was carried out overnight at 37 °C. The next day, samples were acidified (pH 3 or less) with 2% of TFA. Stage Tip assembly was prepared. A syringe needle was used to remove a piece of filter paper from the Empore disk (0.24 µm). The needle was placed into a 200 µl pipette tip, and cored disk pieces were pushed into the tip. The process was repeated three times for each pipette tip to increase loading capacity. The estimated binding capacity of the one core was 2 µg. A hole was poured on the top of the Eppendorf tubes to provide Stage Tip/Tube assembly by fitting tips, including Empore disk cores. For each sample, an assembly was prepared. For the desalting procedure, three solutions were prepared. Solution 1 (Wash Solvent) was prepared from 98:2:0.1% of water:acetonitrile: trifluoroacetic acid (TFA), solution 2 (Wetting Solvent) was prepared from 80:20:0.1% of acetonitrile:water: trifluoroacetic acid and solution 3 (Elution Solvent) was prepared from 60:40:0.1% of acetonitrile:water: trifluoroacetic acid. A 100 µl Wash solvent was

added to the tips and then centrifuged at 6000 rpm for 5 minutes. 60 µl of 100% acetonitrile was used for the wetting process. The wetting process was repeated by adding 100 µl of wetting solvent to the tips, and centrifugation was applied for 5 minutes at 6000 rpm. 100 µl of wash solvent was added to the tips and centrifuged for 5 minutes at 6000 rpm. The liquids in the bottom of the Eppendorf tubes were discarded. 10 µg of acidified samples were added to tips and centrifuged for 20 minutes at 4000 rpm. Then, the washing procedure was applied by 100 ml of wash solvent and centrifuged for 10 minutes at 4000 rpm. Cap/Stage tip assembly was removed to new Eppendorf tubes, and the elution process was conducted. 50 µl of elution solvent was added to the tips and centrifuged for 10 minutes at 2000 rpm, and the procedure was repeated three times to collect all of the samples. Finally, a speed vacuum was applied to the desalted peptide mixture to dry out. Then, the samples dissolved in a mobile phase solution, and nanodrop measurements were taken to load between 200-500 ng of peptide samples to the mass spectrometry equipment.

2.2.4. SDS Protocol

The SDS-PAGE analysis was conducted using the Mini-PROTEAN Tetra Cell (Bio-Rad). The samples were mixed with an equal amount of 2X Laemmli Buffer, which consisted of 1.0 ml of 0.5 mM Tris-HCl (pH 6.8), 2.0 ml of 25% glycerol, 0.08 ml of 1.0% bromophenol blue, 1.6 ml of 10% SDS, 0.4 ml of β-mercaptoethanol, and 2.92 ml of ddH2O. The mixture was heated at 95 °C for 5 minutes before loading into the wells. A pre-stained protein marker (Applichem Protein Marker VI) was used to determine the molecular weights of the proteins.

For the resolving gel (12%) preparation, 2 ml of 30% acrylamide-bisacrylamide solution (19T:1) was mixed with 1.675 ml of ddH2O. Then, 1.25 ml of 1.5 M Tris (pH 8.8) and 50 µl of 10% SDS were added to the acrylamide solution. Then, 25 µl of 10% freshly prepared ammonium persulfate (APS) and 2.5 µl of tetramethylethylenediamine (TEMED) were added. The gel was poured into the cast, and 200 µl of ethanol was layered on top for faster polymerization. After complete solidification of the gel, the ethanol was removed, and the top of the gel was washed with distilled water. The stacking gel was prepared separately and added on top of the resolving gel. For the stacking gel

(4%) preparation, 0.325 ml of 30% acrylamide-bisacrylamide solution was mixed with 1.525 ml of ddH2O. Then, 0.625 ml of 0.5 M Tris (pH 6.8) and 25 µl of 10% SDS were added to the solution. Additionally, 12.5 µl of 10% APS and 2.5 µl of TEMED were added. The prepared gel was poured onto the resolving gel, and a comb was placed.

Once the stacking gel polymerized, 1X Run Buffer was added, and the combs were removed. A protein marker (3 µl) was added to one well, and the prepared protein samples (40 µg per well) were loaded into the remaining wells.

The 5X Run Buffer (1X: 25 mM Tris, 192 mM glycine, 0.1% SDS) was prepared by dissolving 15 g of tris and 72 g of glycine in 600 ml of ddH2O. Then, 5 g of SDS was added, and the volume was brought up to 1 liter. The Run Buffer was stored at +4°C and warmed to room temperature before use. Electrophoresis was performed at 100 V for 90 minutes after adding 1X run buffer. The gel was placed into a staining solution to visualize protein bands.

Staining Solution 'Coomassie Blue R-250' (500 ml): 1.0 g of Coomassie Brillant Blue-R-250 dissolved in 150 ml of ethanol. Then, 300 ml of ddH2O and 150 ml of glacial acetic acid were added. De-staining Solution (1000 ml): 100 ml of methanol and 800 ml of dH2O were mixed, and then 100 ml of glacial acetic acid was added.

2.2.5. Isolation of Total RNA

The Roche High Pure RNA Isolation Kit was employed for RNA isolation. Initially, 1 mL of *Pseudomonas aeruginosa* cultures (both control and 4-HBA treated) with a concentration of 1 x 10^9 CFU/mL was collected through centrifugation at 2,000 x g for 5 minutes, and the resulting pellet was resuspended in 200 µl of 10 mM Tris (pH 8.0). 4 µl of lysozyme with a 50 mg/mL concentration was added to the samples, followed by incubation at 37 °C for 10 minutes. Subsequently, 400 µl of Lysis/-Binding buffer was added and thoroughly mixed. The samples were then transferred to the upper reservoir of a combined high pure filter tube and collection tube. Centrifugation was performed for 30 seconds at 8,000 x g, and the flow-through was discarded. Next, a mixture of 90 µl of DNase incubation buffer and 10 µl of DNase I was added to the filter tube, and incubation was carried out for 60 minutes at 20 °C. Following this, 500 µl of wash buffer I was added to the upper reservoir of the filter tube and centrifuged for 30 seconds at 8,000 x g. Again,

the flow-through was discarded. The same procedure was repeated using 500 µl of wash buffer II. Subsequently, 200 µl of wash buffer II was added to the upper reservoir of the filter tube, and centrifugation was performed for 2 minutes at 13,000 x g to eliminate any residual wash buffer. The collection tube was discarded, and the filter tube was inserted into a sterile 1.5 ml centrifugation tube. To elute the RNA, 50 µl of elution buffer was used, and the tube assembly was centrifuged for 1 minute at 8,000 x g. The resulting RNA samples were subsequently employed for cDNA preparation.

2.2.6. cDNA Preparation

The SensiFast cDNA Synthesis Kit (Meridian Bioscience) was utilized for cDNA synthesis. Firstly, the master mix was prepared on ice. For this, total RNA (up to 1 µg), 4 µl of 5X TransAmp Buffer, 1 µl of reverse transcriptase, and the reaction mix was brought up to 20 µl by addition of DNase/RNase water were mixed by gentle pipetting. Then, the following program was set: 20 °C for 10 minutes, 42 °C for 15 minutes, 85 °C for 5 minutes and 4 °C for hold. Afterward, the cDNA product was stored at -20 °C.

2.2.7. LC-ESI-MS/MS (Liquid Chromatography-Electrospray Ionization-Tandem Mass Spectrometry)

The analysis was conducted by the İzmir Institute of Technology EAS specialists in Orbitrap Fusion Tribrid (Thermo Scientific).

2.2.8. Droplet Digital PCR

A PCR mix of 20 µL was prepared for analysis. The mix included 10 µL of 2X ddPCR EvaGreen Supermix (Bio-Rad, cat. no. 1864034), 6 µL of water free of nucleases, 1 µL each of forward and reverse primers and 10 ng of cDNA from each sample. The ddPCR assay mixture was loaded into a disposable DG8 Cartridge (Bio-Rad, cat. no. 1864008) and placed in a cartridge holder (Bio-Rad, cat. no. 1863051). Droplet generation oil for EvaGreen (Bio-Rad, cat. no. 1864005) was added to each of the eight

oil wells, totaling 70 µL. The cartridge was covered with a DG8 Gasket (Bio-Rad, cat. no. 1863009) and inserted into the QX200 Droplet Generator (Bio-Rad, cat. no. 1864002). Once droplet generation was completed, the droplets were carefully transferred to a new ddPCR 96-well PCR plate kit (Bio-Rad, cat. no. 10023379). The plate was sealed with a pierceable aluminum foil (Bio-Rad, cat. no. 1814040) using the PX1 PCR Plate Sealer (Bio-Rad, cat. no. 1814000) and placed in a thermal cycler. The thermal cycling conditions were an initial step at 95 °C for 5 min, followed by 50 cycles of 95 °C for 30 s and 58 °C for 1 min. Then, two steps at 98 °C for 10 seconds and a continuous hold at 4 °C. After PCR, the sealed plate was transferred to the plate holder of the QX200 Droplet Reader (Bio-Rad, cat. no. 1864003). Using the QuantaSoft software (Bio-Rad), droplet analysis was set up to detect the droplets optically. Once the plate reading was complete, the resulting data were analyzed using QuantaSoft software v1.7. Specifically, the positive droplets in each well were identified from the 2D amplitude plot. Finally, the recQ, pilM, and deaD mRNA quantities were determined as copies/µL.

The primers in this experiment were designed for the genes *recQ*, *pilM,* and *deaD*. The primer sequences are presented in Table 2.1.

Table 2.1. Designed Primers for ddPCR.

Targeted Gene	**Primers**	
recQ	**Forward**	5’ AAGAGCGGGAAATGTGGGAG 3’
	Reverse	5’ GGGAAGATCACATAGGGCGG 3’
pilM	**Forward**	5’ GCTGAACGGCAAGGTGAATG 3’
	Reverse	5’ AGTCGAA ACTCCTCAACGCC 3’
deaD	**Forward**	5’ GGCAAACCCATGACTCAGGA 3’
	Reverse	5’ TTGAGCCTGGATAGGCGAAG 3’

2.2.9. Data Analysis

The Gene Ontology tool was used to interpret downregulated proteins. STRING 11.5 version was used to interpret both upregulated and downregulated proteins. In STRING tools, different colors were used to represent specific types of evidence. A red

line represents fusion evidence, indicating the presence of fusion events between proteins. A green line represents neighborhood evidence, indicating that the proteins are found in close proximity to each other in the genome. A blue line represents co-occurrence evidence, suggesting that the proteins are frequently found together across different organisms. A purple line indicates experimental evidence, meaning the protein-protein interaction has been experimentally validated. A yellow line represents text-mining evidence, indicating that the interaction was inferred from the text-mining of scientific literature. A light blue line represents database evidence, indicating that the interaction is supported by information in databases. Lastly, a black line represents co-expression evidence, indicating that the proteins are co-expressed across different conditions or tissues.

2.3. Results and Discussion

In this chapter, the proteomics approach was utilized to analyze the functional effects of 4-HBA on *P. aeruginosa*. For this reason, Gene Ontology and STRING tools were used to interpret the results obtained from the proteomics data. Validation of the proteomics data was confirmed by ddPCR analysis on selected genes.

2.3.1. Quality Control of Isolated Proteins

The SDS-PAGE method was conducted to perform quality control of total isolated proteins from 4-HBA treated and untreated *P. aeruginosa* cultures. It was observed that proteins on the gel were found to be separated with clear bands (Figure 2.1). Additionally, smear formation was not observed, meaning isolated proteins were not degraded.

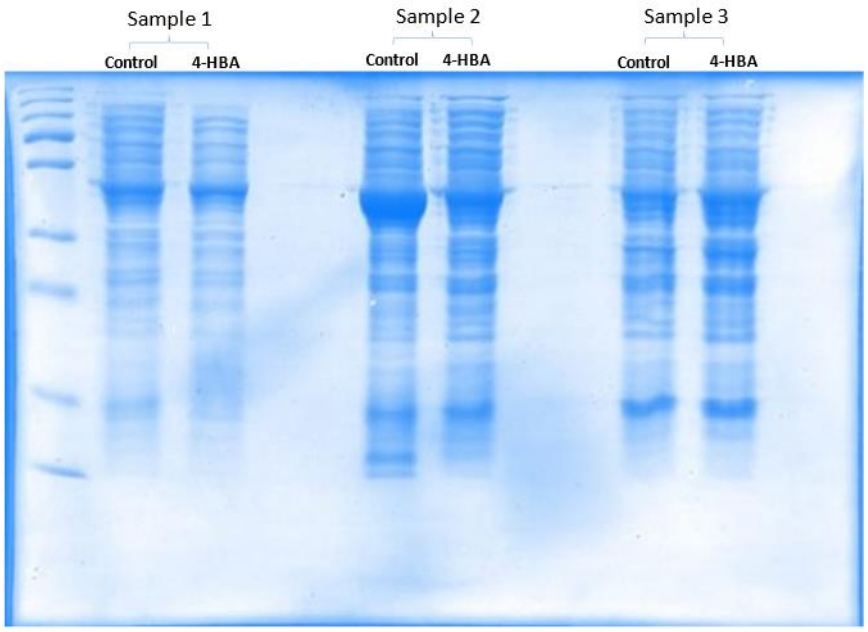

Figure 2.1. SDS-PAGE result of total protein isolation of *P. aeruginosa* untreated and 4-HBA treated samples. Protein samples were run on the gel as three replicates: Sample 1, 2, and 3. The first well includes the protein ladder, which shows the protein molecular weight ranging between 250 kDa and 10 kDa.

2.3.2. Gene Ontology (GO) Analysis of the Differentially Expressed Proteins

Through proteomic analysis, a total of 1701 proteins were found. The criteria, which is a p-value smaller than 0.05 and a q-value smaller than 0.01, were taken into consideration to select the differentially expressed proteins. According to these criteria, it was found that 58 proteins were downregulated, whereas 40 proteins were upregulated after 4-HBA treatment on *P. aeruginosa*. Gene Ontology analysis was conducted on downregulated proteins to examine the related biological functions on the number of genes, FDR value, and fold enrichment levels (Figure 2.2).

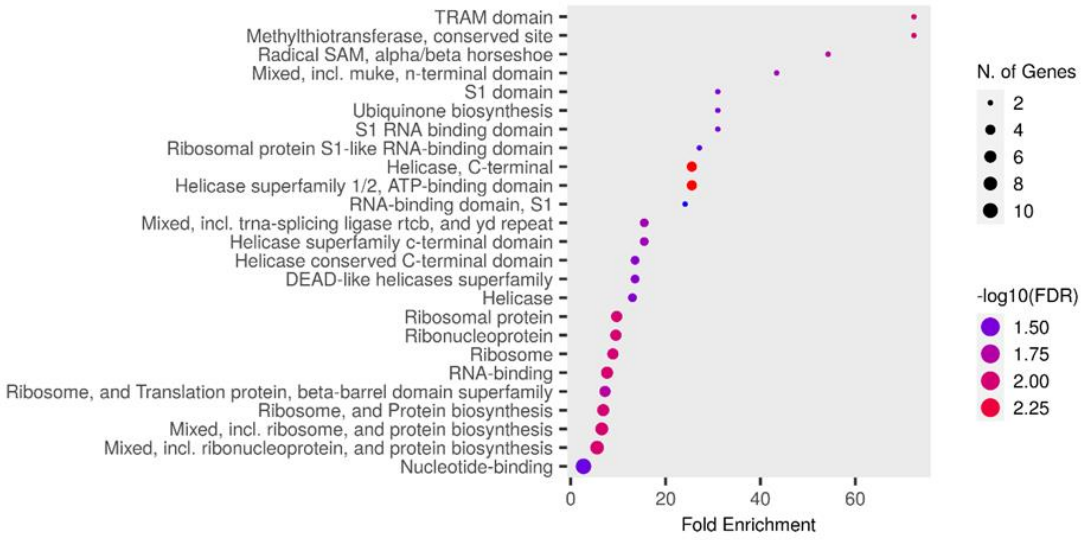

Figure 2.2. Gene Ontology (GO) analysis of differentially expressed proteins between 4-HBAtreated and control groups. The top 30 pathways were utilized based on the fold enrichment ranging from 0 to 80, the number of genes ranging between 2 and 10, and the FDR value ranging from 1.50 to 2.25 as log10 fold.

The top 30 pathway enrichments of downregulated proteins are all available gene sets based on GO analysis (Figure 2.2). The result showed that the TRAM domain, methylthiotransferase, conserved site proteins give the highest fold enrichment value of 60. Helicase, C-terminal, and helicase superfamily 1/2 and ATP binding domain proteins showed the highest FDR value of 2.1. Nucleotide-binding and protein biosynthesis proteins show the highest number of genes throughout the downregulated proteins. Throughout the analysis, it was observed that mostly ubiquinone biosynthesis, helicase, ribosomal protein, ribonucleoprotein, RNA-binding, and nucleotide-binding were downregulated, respectively. The downregulation of ubiquinone biosynthesis, helicase, ribosomal protein, ribonucleoprotein, RNA-binding, and nucleotide-binding proteins in *P. aeruginosa* may have various impacts on the organism. It may affect the synthesis of ubiquinone, which is important in electron transport and ATP production. The downregulation of helicases may affect the separation of double-stranded DNA during replication, transcription, and DNA repair. The downregulation of ribosomal proteins and ribonucleoproteins may affect protein synthesis and cellular processes. Similarly, the downregulation of RNA- and nucleotide-binding proteins may affect RNA processing and other cellular functions that depend on nucleotide binding. The specific impact of these changes would depend on the context and the specific roles of these proteins in *P. aeruginosa*.

The TRAM, methylthiotransferase, and S1 domains play crucial roles in protein biosynthesis. The TRAM domain in tRNA-modifying enzymes aids tRNA binding and delivers RNA-modifying enzymatic domains to their targets. Methylthiotransferases, the radical SAM superfamily, add methylthio groups to compounds like tRNA and proteins through Fe-S clusters (Anantharaman, Koonin, and Aravind, 2001; Arragain et al., 2010). The S1 domain interacts with the ribosome and messenger RNA, binding RNA in a sequence-specific manner during translation. These proteins are essential for proper protein synthesis, ensuring accurate modification and translation of tRNA and maintaining cellular processes (Young and Karbstein, 2011).

Helicase proteins were found to have the highest FDR value, with 2.1 throughout the chart. Helicases can modify and unwind nucleic acid or nucleic acid-protein complexes. They can bind to and separate double-stranded DNA during DNA replication, allowing each strand to be copied. This separation requires energy, which is provided by ATP. Helicases also play a role in other cellular processes, such as DNA repair and transcription. RNA helicases, on the other hand, are involved in shaping RNA molecules during various processes involving RNA, including transcription, splicing, and translation (Raney, Byrd, and Aarattuthodiyil, 2012).

As a result, after 4-HBA treatment on *P. aeruginosa*, it has been found that through the GO analysis, protein translation machinery was mostly affected. Therefore, it would mean that the mechanism of action of 4-HBA within the cell may target protein biosynthesis at the molecular level directly or indirectly. On the other hand, its impact on helicases may affect replication machinery. In parallel to this, bacteria wouldn’t survive under these conditions. Hence, protein translation machinery was blocked by the indirect effect of 4-HBA.

2.3.3. The Protein Profile in the Presence of 4-HBA

The proteomics analysis revealed that, upon treatment with 4-HBA, 58 proteins were identified as downregulated, while 40 proteins were found to be upregulated (Figure 2.3).

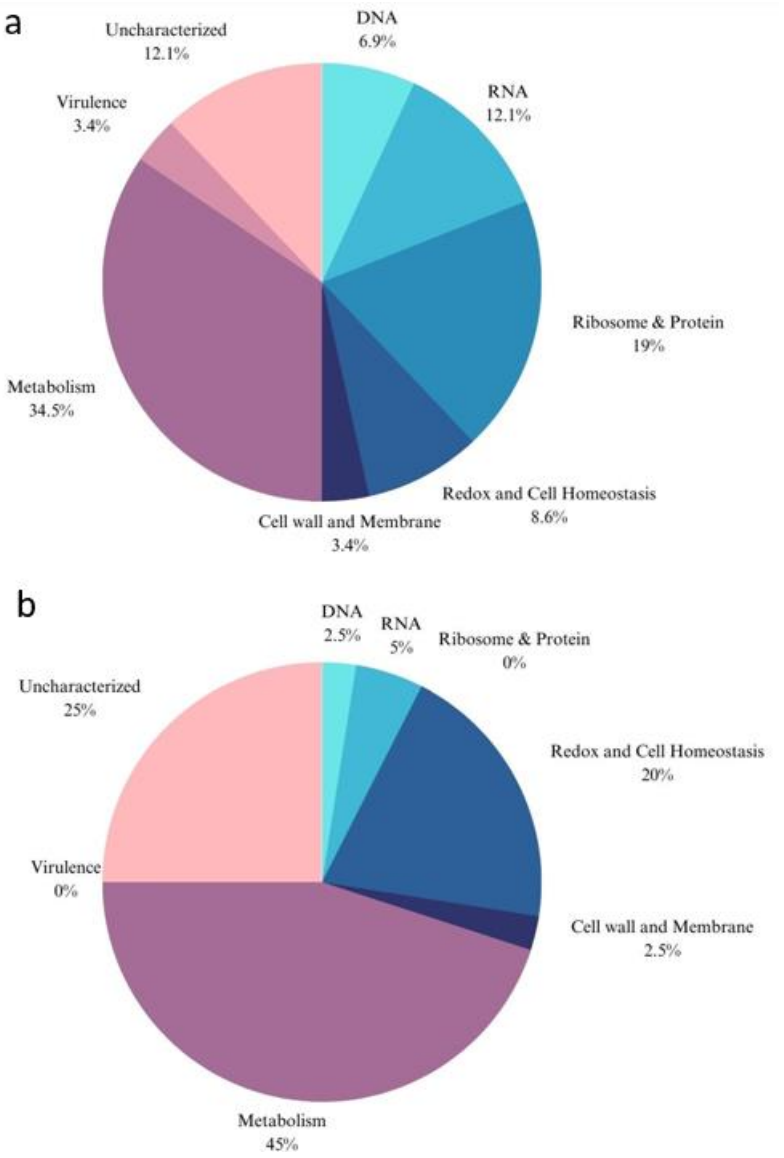

Figure 2.3. The percentages of proteins for each group of function in the presence of 4-HBA. a) Downregulated proteins b) Upregulated proteins.

The proteins that showed downregulation and upregulation were categorized based on their functions by using the UniProt database. These proteins were then grouped into eight main categories: DNA, RNA, ribosomes and proteins, cell wall and membrane, metabolism, redox and cell homeostasis, virulence, and uncharacterized proteins. The percentages of downregulated proteins in these groups were 6.9%, 12.1%, 19%, 8.6%, 3.4%, 34.5%, 3.4%, and 12.1%, respectively. On the other hand, the percentages of upregulated proteins in the same groups were 2.5%, 5%, 0%, 20%, 2.5%, 45%, 0%, and 25%, respectively. Notably, metabolism-related proteins showed the highest percentages in both downregulated and upregulated proteins, with 45% upregulated and 34.5%

downregulated. This suggests that the treatment with 4-HBA led to an overall increase in protein expression in metabolism-related proteins rather than a decrease.

Among the downregulated proteins, the least affected ones were observed in the categories of virulence and cell wall and membrane proteins, accounting for 3.4% of the total. This suggests that the impact of 4-HBA treatment was primarily focused on the intracellular environment rather than the outer membrane. Additionally, the proteins related to virulence and ribosomes and proteins did not show any upregulation during 4-HBA treatment. This finding suggests that the bacteria were unable to compensate for the downregulation of these functions by producing more proteins.

2.3.3.1. DNA-Related Proteins of 4-HBA Treatment

The STRING database was employed to visualize the interaction network of DNA-related proteins based on their gene names. The network of downregulated proteins was depicted in Figure 2.4.a, while the network of upregulated proteins was illustrated in Figure 2.4.b. Furthermore, a comprehensive interaction network encompassing both downregulated and upregulated proteins in response to 4-HBA treatment was presented in Figure 2.4.c. Additionally, Table 2.2 provides an overview of the functions associated with the downregulated and upregulated proteins.

The findings showed that four proteins were downregulated after the treatment of 4-HBA, whereas one protein was upregulated. The results demonstrated that DNA polymerase III subunit alpha, DNA helicase, Recombination-associated protein, and NalD were downregulated during the 4-HBA treatment; meanwhile, STAS domain-containing protein was found to be upregulated.

DNA polymerase III is a complex enzyme responsible for bacterial DNA replication. Its alpha chain, DnaE, acts as the DNA polymerase and exhibits 3' to 5' exonuclease activity. Downregulating DnaE in bacteria can have several consequences. Firstly, impaired DNA replication and slower synthesis may occur since DnaE is the primary polymerase component. Secondly, an increased error rate in DNA replication can result from impaired proofreading abilities. Thirdly, sensitivity to DNA damage may rise due to reduced efficiency in repair. Lastly, reduced cell viability or death may occur due to defects in DNA replication. Downregulating DnaE significantly impacts bacterial DNA replication, growth, and survival.

RecQ helicase, a conserved protein from bacteria to humans, plays a critical role in genome stability and DNA repair. It processes stalled replication forks and participates in SOS signaling. Downregulating RecQ protein can have several consequences. Firstly, increased sensitivity to DNA damage may occur. Secondly, impaired replication fork restart can lead to DNA damage or cell death. Thirdly, defective SOS signaling may make cells less capable of responding to DNA damage. Lastly, genome instability and increased mutation frequency could result from RecQ downregulation. Overall, downregulating RecQ protein significantly impacts DNA repair, genome stability, and cell survival, affecting the ability of cells to respond to DNA damage effectively (Berstein, Gangloff, and Rothstein, 2010; Hishida et al., 2004).

NalD protein is downregulated during 4-HBA treatment in *P. aeruginosa*. The major contributor to multidrug resistance in this bacterium is the MexAB-OprM efflux system, which exports various antimicrobials. The study revealed that disruption of the *nalD* increases MexAB-OprM expression and multidrug resistance, indicating that NalD negatively regulates this efflux system. Conversely, introducing the *nalD* reduces resistance to MexAB-OprM substrates. This suggests that bacteria downregulate *nalD* expression to maintain their drug resistance and protect themselves (Sobel et al., 2005).

The RdgC protein, downregulated during treatment, binds double-stranded DNA (dsDNA) through a central hole. Mutagenesis studies indicate that positively charged residues surrounding the hole are crucial for DNA binding. Downregulating RdgC could have two main consequences: decreased DNA binding ability and increased sensitivity to DNA damage. Reduced DNA binding may affect processes reliant on RdgC-mediated DNA binding, such as DNA repair and transcriptional regulation. Moreover, decreased levels of RdgC could make cells more vulnerable to DNA damage, compromising their ability to repair DNA and maintain genome stability. Overall, the downregulation of RdgC has significant implications for DNA-related processes and cellular survival (Ha et al., 2007).

The upregulation of the PA3347 protein in *Pseudomonas aeruginosa* after 4-HBA treatment has significant implications. PA3347, a STAS domain-containing protein, is involved in regulating gene expression and swarming motility (Aravind and Koonin, 2000). Its upregulation could lead to increased swarming motility, facilitating colonization and infection. It may also result in altered gene expression patterns, affecting various cellular processes and pathways. The phosphorylation of PA3347 by the

neighboring response regulator PA3346 plays a crucial role in its regulatory function (Hsu et al., 2008). The dysregulation of sigma factors, which bind RNA polymerase and control transcription, could occur due to the upregulation of PA3347. These consequences may impact *P. aeruginosa*'s ability to regulate gene expression and carry out essential cellular processes (Bhuwan et al., 2012). Understanding the regulatory mechanisms involving PA3347 provides insights into the complex behavior and virulence of *P. aeruginosa*, contributing to the development of targeted interventions for combating *Pseudomonas* infections.

Table 2.2. The proteins related to DNA in the presence of 4-HBA.

Accession	**Protein ID**	**Protein Name**	**Localization**	**q-Value**	**Downregulation/Upregulation**
PA3640	DnaE	DNA polymerase III subunit alpha	Cytoplasm	0.000	Downregulated
PA3344	RecQ	DNA helicase	Cytoplasm	0.000	Downregulated
PA3263	RdgC	Recombination-associated protein	Periplasm	0.000	Downregulated
PA3574	NalD	NalD	Cytoplasm	0.002	Downregulated
PA3347		STAS domain-containing protein	Unknown	0.004	Upregulated

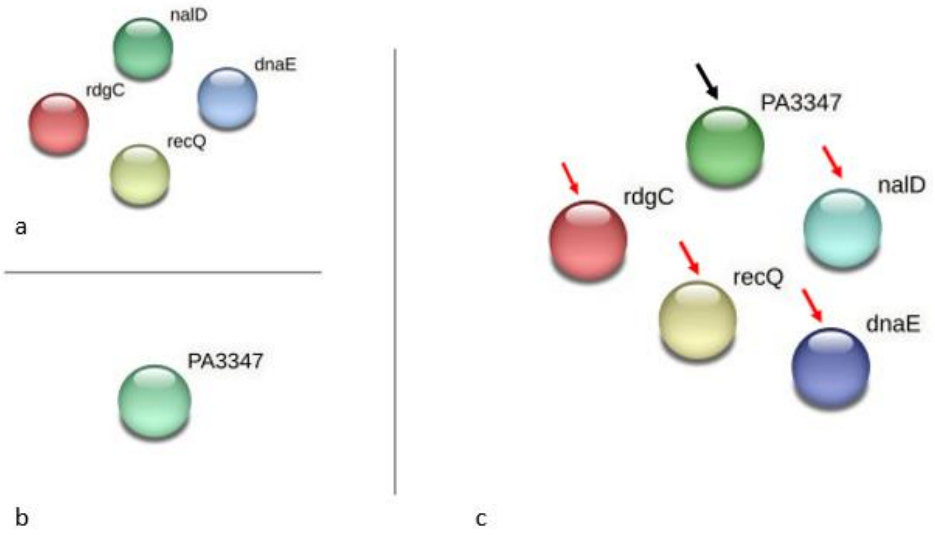

Figure 2.4. String representation of DNA-related proteins in the presence of 4-HBA. a) Downregulated proteins, b) Upregulated proteins, c) Both downregulated and upregulated proteins. The red arrows indicate the downregulated proteins, and the black arrows indicate the upregulated proteins.

2.3.3.2. RNA-Related Proteins of 4-HBA Treatment

The visualization of the interaction network of RNA-related proteins based on gene names was performed using the STRING database. Figure 2.5.a presented the network of downregulated proteins, while Figure 2.5.b depicted the network of upregulated proteins. The complete interaction network of these proteins, with a focus on downregulation and upregulation, was demonstrated in Figure 2.5.c. Additionally, Table 2.3 showcased the functions of the downregulated and upregulated proteins.

The findings showed that seven proteins were downregulated after the treatment of 4-HBA, whereas two proteins were upregulated. The results demonstrated that tRNA pseudouridine synthase B, tRNA-2-methylthio-N(6)-dimethylallyladenosine synthase, HTH-type transcriptional regulator, tRNA sulfurtransferase, RNA polymerase-associated protein RapA, another HTH-type transcriptional regulator and S1 motif domain-containing protein downregulated during the 4-HBA treatment meanwhile an endoribonuclease and a transcriptional regulator have been found as upregulated.

The downregulation of tRNA pseudouridine synthase B (TruB) after treatment has significant consequences for cellular processes. TruB functions as an RNA chaperone,

aiding in the folding of the tRNA molecules necessary for proper protein synthesis. Impaired tRNA folding due to TruB downregulation can disrupt protein synthesis and impact cellular functions. TruB is also responsible for adding pseudouridine modifications to tRNA molecules, and its downregulation may lead to altered RNA modification patterns, affecting the stability and function of modified RNAs (Keffer-Wilkes, Govardhan Veerareddygari, and Kothe, 2016). The study suggests that TruB's role in tRNA folding is crucial for cellular survival, and its downregulation could result in decreased survival under conditions where protein synthesis is vital. Additionally, mutations in tRNA-modifying enzymes, including TruB, have been associated with impaired biofilm formation, indicating a potential impact on the virulence of bacterial pathogens (Schinner et al., 2020). Understanding the consequences of TruB downregulation provides insights into the importance of tRNA maturation, modification, and folding for cellular processes, highlighting potential therapeutic targets.

In a recent study, researchers discovered that the tRNA modification enzyme PA3980, also known as MiaB, plays a crucial role in regulating the expression of Type III secretion system (T3SS) genes in *P. aeruginosa*, a pathogenic bacterium. MiaB modifies a base called isopentenyladenosine (i6A) in tRNA molecules, and its downregulation can lead to several consequences (Koshla et al., 2019; Griffiths et al., 1978; Durand et al., 1997; Lamichhane et al., 2013). Firstly, the decreased expression of MiaB results in reduced T3SS gene expression impairs the bacterium's ability to infect host cells and cause acute infections (Lin et al., 2022). Secondly, the altered i6A modification patterns due to MiaB downregulation can affect the stability and function of modified tRNAs, potentially impacting protein synthesis accuracy. Thirdly, MiaB is critical for inducing cytotoxicity in human lung epithelial cells, so its downregulation may result in impaired cytotoxicity, reducing the bacterium's ability to cause damage to host cells. Lastly, MiaB's role in regulating T3SS gene expression suggests it may play a part in the bacterium's response to changing environmental conditions, and its downregulation could lead to altered environmental responses and impact the bacterium's ability to survive and cause infections. Overall, the downregulation of MiaB in *P. aeruginosa* can significantly affect T3SS gene expression, i6A modification, cytotoxicity, and environmental responses, influencing the bacterium's virulence and pathogenicity.

In 1998, a protein called RapA was discovered in *E. coli*, which interacts with RNA polymerase (RNAP) and functions as an ATPase. RapA forms a stable complex with

RNAP, and its ATPase activity is stimulated by binding to RNAP. Interestingly, RapA is a homolog of eukaryotic SWI/SNF proteins involved in transcription activation and DNA repair (Sukhodolets and Jin, 1998). A study identified a mutant *E. coli* strain with a *rapA* gene mutation, causing lower resistance to penicillin G in biofilms but no effect on planktonic cells. The mutant biofilms exhibited reduced polysaccharide quantities and coverage, important components of the biofilm matrix (Lynch et al., 2007). Although no study has been conducted on RapA in *P. aeruginosa*, its effects can be discussed in relation to *E. coli*. Downregulation of RapA may result in decreased ATPase activity associated with RNAP, leading to reduced transcription efficiency and impaired adaptability to environmental changes. This could have implications for bacterial biofilm formation, antibiotic resistance, and stress response.

The GntR is a regulator protein found in bacteria that controls glucose metabolism in *P. aeruginosa* (Suvorova, Korostelev, and Gelfand, 2015). If the GntR protein is downregulated, it can result in increased expression of target genes, such as GntP, which encodes the gluconate permease. This would enhance gluconate uptake and lead to a shift in carbon metabolism towards its utilization. GntR binds to specific promoters, and the operator sequence for GntR has been identified. The binding of gluconate or 6-phosphogluconate to the GntR/DNA complex induces the release of GntR from the promoter, enabling gene expression. GntR and the related protein PtxS may have evolved from a common ancestor (Daddaoua et al., 2017). While the physiological relevance of GntR's negative feedback loop is not fully understood, its downregulation would likely alter the metabolic profile of *P. aeruginosa*, potentially impacting its growth, survival, and virulence. Understanding the regulation of glucose metabolism by GntR and other regulatory systems in *P. aeruginosa* can provide insights into complex regulatory circuits in bacteria.

The enzyme ThiI is responsible for catalyzing the transfer of sulfur from ATP to tRNA, resulting in the formation of 4-thiouridine in position 8 of tRNAs. This modification acts as a photosensor sensitive to near-UV light. In certain types of bacteria, ThiI serves a dual function, as it is necessary for both the production of the 4-thiouridine modification in tRNA and the formation of the thiazole component in the biosynthesis pathway of thiamine (Martinez-Gomez et al., 2011).

The research findings highlight the crucial role of the YbeY gene in *E. coli* and *P. aeruginosa*. In *E. coli*, YbeY is involved in ribosome assembly and the maturation of

ribosomal RNAs (rRNAs), particularly at the ends of 16S, 23S, and 5S rRNAs (W. Davies et al., 2010). In *P. aeruginosa*, YbeY is essential for rRNA processing and contributes to the bacterium's virulence. Knocking out the ybeY gene in *P. aeruginosa* leads to downregulation of oxidative stress response genes and increased vulnerability to oxidative stress and neutrophil killing (Xia et al., 2020). Therefore, upregulating the YbeY protein in *P. aeruginosa* could enhance ribosome assembly, translation accuracy, and overall protein synthesis capabilities. This may result in increased production of virulence factors and improved resistance to oxidative stress and immune defense mechanisms. However, the precise effects of upregulating YbeY may depend on specific conditions, and further research is required to understand its consequences fully.

Overall, via the STRING database, the main interaction has been observed in the axis of downregulated proteins TruB, MiaB, and ThiI and upregulated protein YbeY. The downregulation of TruB, MiaB, and Thil proteins in *Pseudomonas aeruginosa* may affect the modification of tRNAs, which could lead to a decrease in protein synthesis and affect the overall fitness of the bacteria. On the other hand, the upregulation of YbeY and Hfq proteins may play a role in the regulation of gene expression and the processing of small noncoding RNAs, respectively. These changes in gene expression could potentially affect the virulence, antibiotic resistance, and adaptive responses of *P. aeruginosa* to different environmental conditions. Considering that 4-HBA treatment has a detrimental effect on the bacteria, it could try to survive by compensating affected mechanisms in terms of RNA-related proteins.

Table 2.3. The proteins related to RNA in the presence of 4-HBA.

Accession	Protein ID	Protein Name	Localization	q-Value	Downregulation/Upregulation
PA4742	TruB	tRNA pseudouridine synthase B	Cytoplasm	0.007	Downregulated
PA3980	MiaB	tRNA-2-methylthio-N(6)-dimethylallyladenosine synthase	Cytoplasm	0.002	Downregulated
PA5374	BetI	HTH-type transcriptional regulator	Unknown	0.000	Downregulated

PA5118	ThiI	tRNA sulfurtransferase	Cytoplasm	0.000	Downregulated
PA3308	HepA	RNA polymerase-associated protein RapA	Cytoplasm	0.000	Downregulated
PA2320	GntR	HTH-type transcriptional regulator	Cytoplasm	0.002	Downregulated
PA5201		S1 motif domain-containing protein	Cytoplasm	0.000	Downregulated
PA3982	YbeY	Endoribonuclease	Cytoplasm	0.000	Upregulated
PA0155	PcaR	Transcriptional regulator	Cytoplasm	0.000	Upregulated

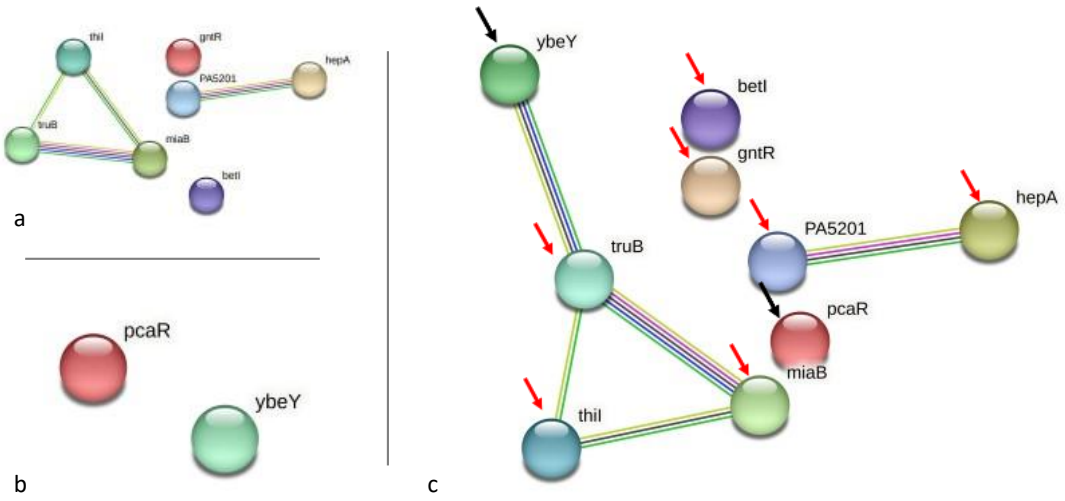

Figure 2.5. String representation of RNA-related proteins in the presence of 4-HBA. a) Downregulated proteins, b) Upregulated proteins, c) Both downregulated and upregulated proteins. The red arrows indicate the downregulated proteins, and the black arrows indicate the upregulated proteins.

2.3.3.3. Ribosome and Protein-Related Proteins of 4-HBA Treatment

The visualization of the interaction network of ribosome and protein-related proteins was carried out by utilizing the STRING database. Figure 2.6 displays the network of downregulated proteins. Furthermore, Table 2.4 presents the functions of the downregulated.

Throughout the 4-HBA treatment, 11 proteins were detected as downregulated, and no protein was found as upregulated. Specifically, DeaD, SecA, and a heat shock protein were found to be downregulated. Remarkably, all proteins have a q-value of 0, which is found explicitly in the ribosome and protein-related proteins. The result of found proteins by the Proteome Discoverer tool indicates there is no false hit possibility of the proteins.

The DeaD protein, also known as CsdA protein, was extensively studied in *E. coli* and *P. aeruginosa*, uncovering its diverse functions in these bacteria. In *E. coli*, DeaD is involved in the biogenesis of the large ribosomal subunit, playing a direct role in its assembly. Its absence leads to the formation of misassembled subunits and impairs ribosome activity, affecting translation accuracy. Additionally, the DeaD/CsdA protein in *E. coli* has been associated with RNA remodeling and is implicated in various cellular processes (Charollais et al., 2004). In *P. aeruginosa*, DeaD/CsdA protein has been linked to the regulation of the type III secretion system (T3SS), a critical virulence factor. It promotes the synthesis of ExsA, a key T3SS regulator, by facilitating the relaxation of secondary structures in its mRNA. This mechanism ensures proper T3SS gene expression and contributes to the bacteria's ability to cause disease (Intile et al., 2015). Furthermore, studies have shown that the DeaD/CsdA protein in *P. aeruginosa* affects cell size, replication origins, and growth rate at low temperatures. Its absence results in larger cells with more replication origins and slower growth, accompanied by significant changes in gene expression. DeaD/CsdA coordinates the cell cycle and growth by stabilizing mRNA for essential metabolic and cell division genes while degrading mRNA for nucleotide metabolic genes (Lv et al., 2022). Understanding the functions of DeaD/CsdA protein in both *E. coli* and *P. aeruginosa* provides valuable insights into bacterial physiology, ribosome assembly, translation regulation, and virulence mechanisms. Further research on this protein may uncover new targets for therapeutic interventions, as its diverse roles make it a potential candidate for the development of antimicrobial strategies.

SecA, a protein component of the Sec translocase, has been observed to be downregulated in *P. aeruginosa*. The Sec translocase is a complex machinery responsible for transporting secretory proteins across the bacterial plasma membrane. It involves the trimeric SecYEG protein, which forms a membrane-embedded "clamp," and the dimeric SecA ATPase, which acts as a "motor" for protein translocation (Vrontou and Economou, 2004). Recent studies have highlighted the role of the Sec translocon in facilitating protein

movement across lipid bilayers and the importance of SecA as an ATPase and a potential ribosome binder (Cranford-Smith and Huber, 2018). The Sec machinery is vital for the translocation of soluble secreted proteins and outer membrane proteins in bacteria (Zhu, Wang and Shan, 2022). SecA, working in coordination with SecYEG, recognizes substrate proteins and ensures their unfolding before translocation (Gupta, Toptygin, and Kaiser, 2020). Downregulation of SecA in *P. aeruginosa* is likely to have significant consequences for protein secretion across the plasma membrane, leading to reduced efficiency in the translocation and secretion of soluble and outer membrane proteins. This could impact bacterial physiology, including a decrease in virulence and pathogenicity due to the impaired secretion of virulence factors. Additionally, it may affect the bacterial cell envelope's response to environmental stressors, such as antibiotics. The downregulation of the SecA protein in *P. aeruginosa* highlights its essential role in bacterial physiology, emphasizing the significance of this protein for bacterial fitness, survival, and adaptation to different environments. Further research on the regulation and functions of SecA could provide valuable insights into bacterial protein secretion mechanisms and potential targets for therapeutic interventions.

A probable heat shock protein also has been downregulated during the 4-HBA treatment. Heat shock proteins (HSPs) play essential roles in various biological processes, including bacterial responses to environmental stress and the pathogenesis of infections. In the case of *P. aeruginosa*, however, the specific role of HSPs has yet to be fully understood. In a study, the unique role of the HSP DnaJ in biofilm formation and pathogenicity in *P. aeruginosa* was investigated. A mutant strain lacking *dnaJ* was found to produce very little pyocyanin and form significantly fewer biofilms, resulting in decreased pathogenicity, as demonstrated by a reduced mortality rate in a *Drosophila melanogaster* infection model. The decrease in pyocyanin production in the mutant strain was due to a reduction in the transcription of the phenazine synthesis operons, including *phzA1*, *phzA2*, *phzS*, and *phzM*. The reduced biofilm formation and initial adhesion in the *dnaJ* mutant could be restored by the addition of exogenous pyocyanin or extracellular DNA (eDNA). The absence of *dnaJ* was also found to reduce the release of eDNA in *P. aeruginosa* significantly, and the addition of exogenous pyocyanin could restore eDNA release. These findings suggest that DnaJ is required for pyocyanin production and full virulence in *P. aeruginosa*, and it affects biofilm formation and initial adhesion through pyocyanin, inducing eDNA release (Zeng et al., 2020).

The consequences of the downregulation of a probable heat shock protein would depend on the specific protein that is affected and its role in the cell. However, in general, a decrease in the level of heat shock proteins could result in a reduced ability of the bacterial cell to respond to environmental stress, such as high temperature or exposure to toxic compounds. This could lead to a decreased survival rate of the bacterium under stress conditions. Additionally, heat shock proteins are known to be involved in bacterial pathogenesis, so the downregulation of these proteins could also impact the ability of the bacterium to cause disease or infect a host. The specific consequences of downregulation would need to be determined by further investigation of the affected protein and its functions within the cell.

Table 2.4. The proteins related to ribosome and protein synthesis in the presence of 4-HBA.

Accession	Protein ID	Protein Name	Localization	q-Value	Downregulation/Upregulation
PA2840	DeaD	ATP-dependent RNA helicase	Cytosol	0.000	Downregulated
PA4403	SecA	Protein translocase subunit	Cytosol	0.000	Downregulated
PA0916	RimO	Ribosomal protein S12 methylthiotransferase	Cytosol	0.000	Downregulated
PA3656	RpsB	30S ribosomal protein S2	Cytosol	0.000	Downregulated
PA5440		Probable peptidase	Cytosol	0.000	Downregulated
PA5049	RpmE	50S ribosomal protein L31	Cytosol	0.000	Downregulated
PA4258	RpIV	50S ribosomal protein L22	Cytosol	0.000	Downregulated
PA4251	RpIE	50S ribosomal protein L5	Cytosol	0.000	Downregulated
PA3162	RpsA	30S ribosomal protein S1	Periplasm	0.000	Downregulated
PA3799	Der	GTPase Der	Cytoplasmic Membrane	0.000	Downregulated
PA1068		Probable heat shock protein (Hsp90 family)	Cytosol	0.000	Downregulated

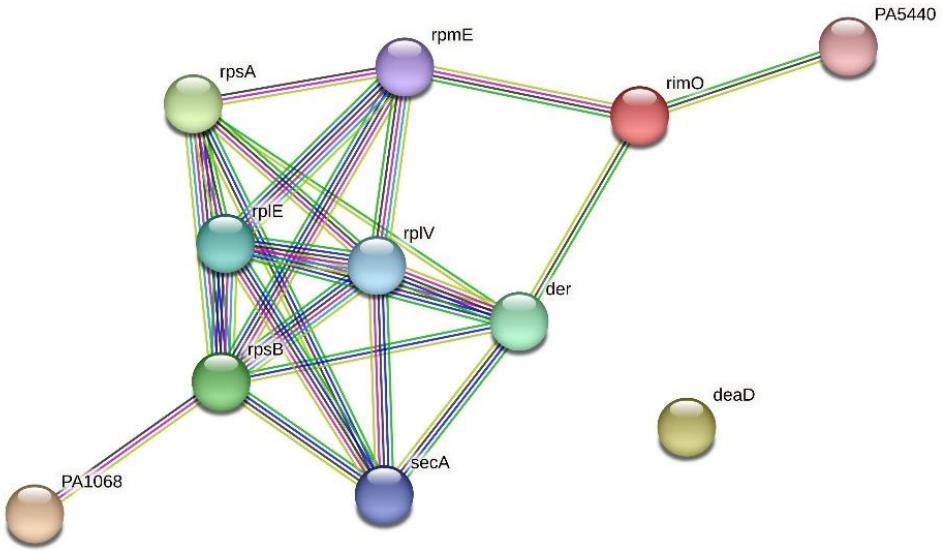

Figure 2.6. String representation of downregulated ribosome and protein-related proteins in the presence of 4-HBA.

2.3.3.4. Cell Wall and Membrane-Related Proteins of 4-HBA Treatment

The interaction network of cell wall and membrane-related proteins was visualized using the STRING database. Figure 2.7.a showcased the network of downregulated proteins, while Figure 2.7.b displayed the network of upregulated proteins. The comprehensive interaction network, encompassing both downregulation and upregulation, was presented in Figure 2.7.c. Moreover, Table 2.5 presents the functions of the downregulated and upregulated proteins.

Throughout the proteomics analysis results, two proteins, PilM and MltD, were found to be downregulated, and one protein, PirA, has been discovered as upregulated.

Type IV pili are important virulence factors in *P. aeruginosa*, contributing to infections in vulnerable individuals. These pili are involved in colonization, and their absence can reduce pathogenicity. The *pilM, N, O, P, and Q* genes are crucial for the assembly of type IV pili and twitching motility. Interactions between PilN/P/O/Q proteins, PilP, and PilA are involved in forming a complex macromolecular machine that spans multiple cellular compartments (Tammam et al., 2013). The downregulation of the PilM protein in *P. aeruginosa* can have significant implications for the expression of type IV pili and twitching motility, which are crucial for virulence and pathogenicity. The PilM protein, along with other encoded proteins from the pilM/N/O/P/Q gene cluster,

forms an inner membrane complex that facilitates the assembly of type IV pili. This complex is essential for the stability and proper functioning of the outer membrane complex involving PilQ. The PilR/S two-component system is also involved in regulating the levels of inner membrane complex components (Ayers et al., 2009). The downregulation of PilM disrupts the formation of the inner membrane complex, leading to a decrease in the stability of the outer membrane complex and PilQ secretion. This disruption can impair pilus assembly and twitching motility, ultimately reducing the virulence and pathogenicity of *P. aeruginosa*. Additionally, targeting type IV pili may offer new therapeutic opportunities by reducing bacterial colonization.

The PirA protein is a receptor for the siderophore ferrienterobactin. Siderophores are molecules that bacteria use to scavenge iron from their environment. The PirA protein is located in the outer membrane of *P. aeruginosa*. When the PirA protein binds to ferrienterobactin, it forms a complex internalized into the cell. The iron is then released from the siderophore and used by the bacterium for growth and development (Ghysels et al., 2005). The PirA protein is also important for the virulence of *P. aeruginosa*. Virulence is the ability of a pathogen to cause disease. *P. aeruginosa* strains lacking the pirA are less virulent than strains with the gene. This is because the *pirA* is required for the bacterium to acquire iron, which is essential for its growth and survival (Moynié et al., 2017). The PirA protein is a potential target for the development of new antibiotics against *P. aeruginosa*. By blocking the ability of the bacterium to acquire iron, it may be possible to prevent it from causing disease (Cornelis and Dingemans, 2013). The upregulation of the PirA protein in *P. aeruginosa* can have a number of effects, for instance, increased iron acquisition, virulence, and resistance to antibiotics. The upregulation of the PirA protein is a major factor in the pathogenesis of *P. aeruginosa* infections. By understanding the mechanisms by which the PirA protein is upregulated, it may be possible to develop new strategies for preventing and treating *P. aeruginosa* infections.

Although the downregulation of the PilM protein reduces the pathogenicity of the bacteria, the upregulation of the PirA protein results in increased pathogenicity and virulence. Therefore, bacteria try to compensate for these aspects by upregulation of PirA protein during the downregulation of PilM protein.

Table 2.5. The proteins related to the cell wall and membrane in the presence of 4-HBA.

Accession	Protein ID	Protein Name	Localization	q-Value	Downregulation/Upregulation
PA5044	PilM	Type IV pilus inner membrane component PilM	Cytoplasmic membrane	0.000	Downregulated
PA1812	MltD	Membrane-bound lytic murein transglycosylase D	Unknown	0.008	Downregulated
PA0931	PirA	Ferric enterobactin receptor PirA	Outer membrane	0.005	Upregulated

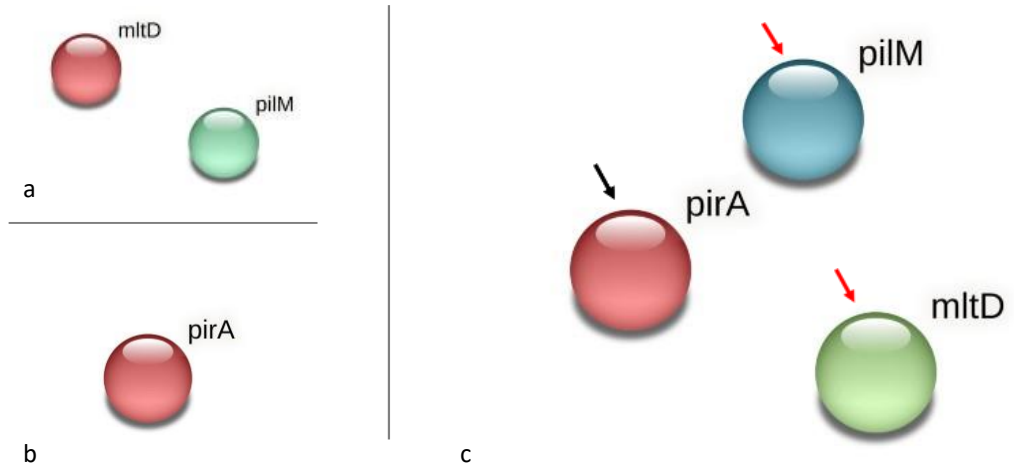

Figure 2.7. String representation of cell wall and membrane-related proteins in the presence of 4-HBA. a) Downregulated proteins, b) Upregulated proteins, c) Both downregulated and upregulated proteins. The red arrows indicate the downregulated proteins, and the black arrows indicate the upregulated proteins.

2.3.3.5. Metabolism-Related Proteins of 4-HBA Treatment

The interaction network of metabolism-related proteins was visualized using the STRING database. Figure 2.8 presents the network of downregulated proteins, while Figure 2.9 presents the network of upregulated proteins. The complete interaction network, encompassing downregulation and upregulation, was demonstrated in Figure

2.10. Furthermore, Table 2.6 showcased the functions of the downregulated and upregulated proteins.

UbiD protein, which is 3-polyprenyl-4-hydroxybenzoate decarboxylase, catalyzes the conversion of 3-polyprenyl-4-hydroxybenzoate to 2-polyprenylphenol in the biosynthesis of ubiquinone has been found as downregulated during the treatment (Jacewicz et al., 2013). Downregulation of the UbiD protein in *P. aeruginosa* would likely result in a decrease in the production of ubiquinone, as UbiD is an essential enzyme in the biosynthesis pathway of ubiquinone. This may have several effects on the cell, as ubiquinone is involved in many important cellular processes, including respiration, oxidative phosphorylation, and electron transport. The specific effects of downregulation of UbiD in *P. aeruginosa* may vary depending on the specific experimental conditions and the extent of downregulation. In our results, it was found to be significantly downregulated with the -6.644 Log2 value. However, in general, a decrease in ubiquinone production could lead to a reduction in energy production and a decrease in the ability of the cell to respond to oxidative stress. In addition to its role in energy production, ubiquinone has also been implicated in other cellular processes, including cell signaling and membrane dynamics. Therefore, the downregulation of UbiD could also have effects on these processes.

Downregulation of the Zwf protein in *P. aeruginosa*, particularly in cystic fibrosis (CF) patients, leads to a decrease in the activity of glucose-6-phosphate dehydrogenase (G6PDH) encoded by the zwf gene. This reduction significantly affects alginate production, a major virulence factor produced by mucoid *P. aeruginosa* strains that contribute to chronic pulmonary disease in CF patients. The regulation of zwf expression differs between CF isolates and other strains, with CF isolates exhibiting relaxed control of zwf expression. This deregulation provides a survival advantage for *P. aeruginosa* in the CF pulmonary environment. Decreased Zwf protein activity not only reduces alginate production but also affects *P. aeruginosa*'s resistance to human sputum, which is dependent on Zwf expression. Additionally, the downregulation of Zwf and subsequent decreased G6PDH activity could impact basic metabolic activities, including carbon catabolism, potentially influencing the bacterium's adaptation to the CF pulmonary environment (Silo-Suh et al., 2005). Understanding the role of Zwf in *P. aeruginosa* infections in CF patients may offer insights into therapeutic strategies targeting alginate production and the pathogenesis of the chronic pulmonary disease.

Downregulation of the HmgA protein in *P. aeruginosa* leads to the hyperproduction of a dark-brown pigment called pyomelanin. This effect is observed in both laboratory and clinical isolates. While the inactivation of hmgA slightly reduces the bacterium's killing ability in acute lung infections, it confers decreased clearance and increased persistence in chronic lung infections. The researchers propose that this increased adaptation to chronicity may be attributed to the pyomelanin pigment's ability to provide higher resistance to oxidative stress in vitro. However, further investigation is required to determine whether pyomelanin production is the cause of the heightened adaptation to chronic infections or simply a side effect of *hmgA* inactivation (Rodríguez-Rojas et al., 2009). These findings suggest that the downregulation of HmgA and subsequent hyperproduction of pyomelanin may play a role in the survival and persistence of *P. aeruginosa* strains in chronic infection settings.

The upregulation of the PchA protein in *P. aeruginosa* leads to enhanced pyochelin formation and increased salicylate production, particularly in cultures grown under iron-limiting conditions. PchA is the first enzyme in the pyochelin biosynthetic pathway and is encoded by the last gene in the *pchDCBA* operon. The study demonstrated that the activity of PchA depends on the presence of Mg(2+) ions and is not influenced by feedback inhibitors or allosteric effectors. The concentration of PchA within the cell was identified as a critical factor in determining the rate of salicylate formation, both in vitro and in vivo. The researchers propose that the PchA concentration plays a crucial role in regulating the flow through the pyochelin biosynthetic pathway, with pyochelin serving as a positive signal and iron acting as a negative signal (Gaille, Reimmann, and Haas, 2003). Consequently, the upregulation of the PchA protein may stimulate pyochelin synthesis in *P. aeruginosa*. These findings provide insights into the molecular mechanisms underlying pyochelin production and its regulation, offering potential avenues for modulating the biosynthesis of this siderophore in the bacterium.

SpeH protein catalyzes the decarboxylation of S-adenosylmethionine to S-adenosylmethioninamine (dcAdoMet), the propylamine donor required for the synthesis of the polyamines spermine and spermidine from the diamine putrescine. SpeE2 protein also has a role in the spermidine biosynthetic process. Since spermidine has a role in growth, biofilm formation, and other surface behaviors, it can be speculated that bacteria’s cell surface integrity is trying to be increased by the upregulated production of these proteins (Piatek et al., 2023).

D-lactate dehydrogenase (LdhA) protein was found to be upregulated during the treatment, as well. A study examines the role and expression patterns of lactate dehydrogenases in *P. aeruginosa*, the most common cause of chronic, biofilm-based lung infections in patients with cystic fibrosis. The study identifies four lactate dehydrogenase enzymes produced by *P. aeruginosa*, three of which contribute to anaerobic or aerobic metabolism in liquid cultures. The fourth enzyme, LldA, performs redundant l-lactate oxidation during growth in aerobic cultures and is explicitly induced by the l-enantiomer of lactate. The study also finds that lactate dehydrogenases play similar roles in colony biofilms as they do in liquid cultures and that enzymes LdhA and LldE have the potential to support metabolic cross-feeding in biofilms. LdhA can catalyze the production of d-lactate in the anaerobic zone, which is then used as a substrate in the aerobic zone (Lin et al., 2018). Upregulation of the LdhA protein in *Pseudomonas aeruginosa* may increase the production of d-lactate during anaerobic survival, as LdhA reduces pyruvate to d-lactate. This could potentially support metabolic cross-feeding in biofilms, as d-lactate produced by LdhA in the anaerobic zone could be used as a substrate in the aerobic zone.

Although downregulation of UbiD, Zwf, and HmgA proteins reduce energy production, virulence, and killing ability of the bacteria, respectively, upregulation of PchA, SpeH, SpeE2, and LdhA proteins results in increased pyochelin biosynthesis, growth, and biofilm formation respectively. Therefore, bacteria try to compensate for growth and survival by upregulation of the PchA, SpeH&SpeE2, and LdhA proteins during the downregulation of UbiD, Zwf, and HmgA proteins.

Table 2.6. The proteins related to metabolism in the presence of 4-HBA.

Accession	Protein ID	Protein Name	Localization	q-Value	Downregulation/Upregulation
PA5252		Probable ATP-binding component of ABC transporter	Cytoplasm	0.000	Downregulated
PA5237	UbiD	3-octaprenyl-4-hydroxybenzoate carboxy-lyase	Cytoplasm	0.000	Downregulated
PA3190		Probable binding protein component of	Periplasm	0.000	Downregulated

		ABC sugar transporter			
PA1818	LdcA	Lysine decarboxylase LdcA	Cytoplasm	0.000	Downregulated
PA4443	CysD	Sulfate adenylyltransferase subunit 2	Cytoplasm	0.000	Downregulated
PA4442	CysNC	Bifunctional enzyme CysN/CysC	Cytoplasm	0.000	Downregulated
PA3183	Zwf	Glucose-6-phosphate 1-dehydrogenase	Cytoplasm	0.000	Downregulated
PA3195	Gap	Glyceraldehyde-3-phosphate dehydrogenase	Cytoplasm	0.000	Downregulated
PA3584	GlpD	Glycerol-3-phosphate dehydrogenase	Cytoplasm	0.007	Downregulated
PA5278	DapF	Diaminopimelate epimerase	Cytoplasm	0.000	Downregulated
PA5209		CYTH domain-containing protein	Unknown	0.000	Downregulated
PA4636		PlsC domain-containing protein	Unknown	0.000	Downregulated
PA3171	UbiG	Ubiquinone biosynthesis O-methyltransferase	Cytoplasm	0.000	Downregulated
PA2903	CobJ	Precorrin-3 methylase CobJ	Cytoplasm	0.000	Downregulated
PA2249	BkdB	Lipoamide acyltransferase component of branched-	Cytoplasm	0.000	Downregulated

		chain alpha-keto acid dehydrogenase complex			
PA2248	BkdA2	2-oxoisovalerate dehydrogenase subunit beta	Cytoplasm	0.002	Downregulated
PA1344		Probable short-chain dehydrogenase	Unknown	0.000	Downregulated
PA0413	ChpA	Histidine kinase	Cytoplasm	0.000	Downregulated
PA0335		Histidinol-phosphatase	Cytoplasm	0.000	Downregulated
PA2009	HmgA	Homogentisate 1,2-dioxygenase	Unknown	0.000	Downregulated
PA4231	PchA	Salicylate biosynthesis isochorismate synthase	Cytoplasm	0.005	Upregulated
PA2531		Probable aminotransferase	Unknown	0.000	Upregulated
PA0231	PcaD	Beta-ketoadipate enol-lactone hydrolase	Unknown	0.000	Upregulated
PA0227		3-oxoadipate CoA-transferase	Cytoplasm	0.000	Upregulated
PA5445		Probable coenzyme A transferase	Cytoplasm	0.002	Upregulated
PA4774	SpeE2	Polyamine aminopropyltransferase 2	Cytoplasm	0.000	Upregulated
PA4773	SpeH	S-adenosylmethionine decarboxylase proenzyme	Unknown	0.001	Upregulated

PA3559		UDP-glucose 6-dehydrogenase	Cytoplasm	0.000	Upregulated
PA3524	GloA1	Lactoylglutathione lyase	Cytoplasm	0.000	Upregulated
PA3277		Probable short-chain dehydrogenase	Cytoplasm	0.000	Upregulated
PA2918		Probable short-chain dehydrogenase	Cytoplasm	0.000	Upregulated
PA2764		AB hydrolase-1 domain-containing protein	Cytoplasm	0.000	Upregulated
PA1830		SCP2 domain-containing protein	Unknown	0.000	Upregulated
PA0927	LdhA	D-lactate dehydrogenase (Fermentative)	Cytoplasm	0.002	Upregulated
PA0835	Pta	Phosphate acetyltransferase	Cytoplasm	0.001	Upregulated
PA0558		Methyltransf_11 domain-containing protein	Cytoplasm	0.000	Upregulated
PA0318		FAA_hydrolase domain-containing protein	Cytoplasm	0.000	Upregulated
PA0245	AroQ2	3-dehydroquinate dehydratase 2	Cytoplasm	0.000	Upregulated

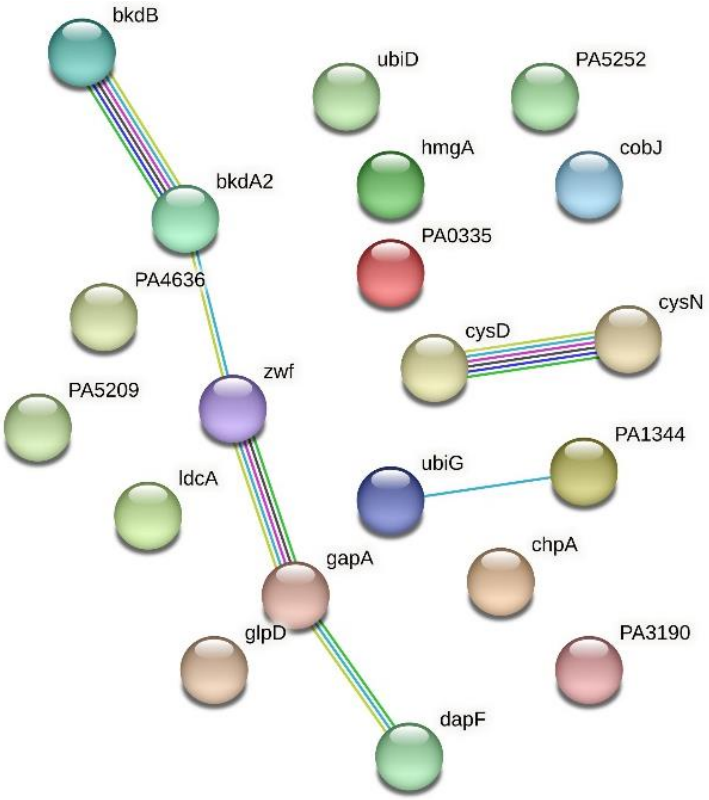

Figure 2.8. String representation of metabolism-related downregulated proteins in the presence of 4-HBA.

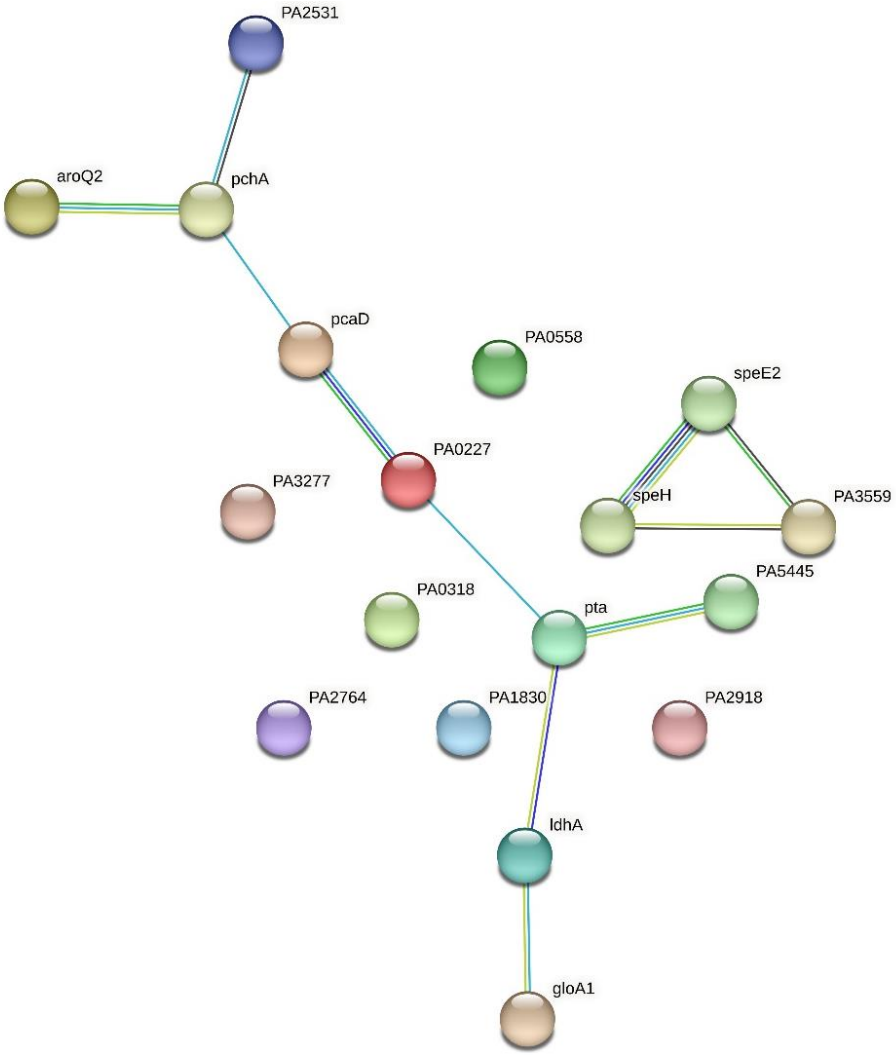

Figure 2.9. String representation of metabolism-related upregulated proteins in the presence of 4-HBA.

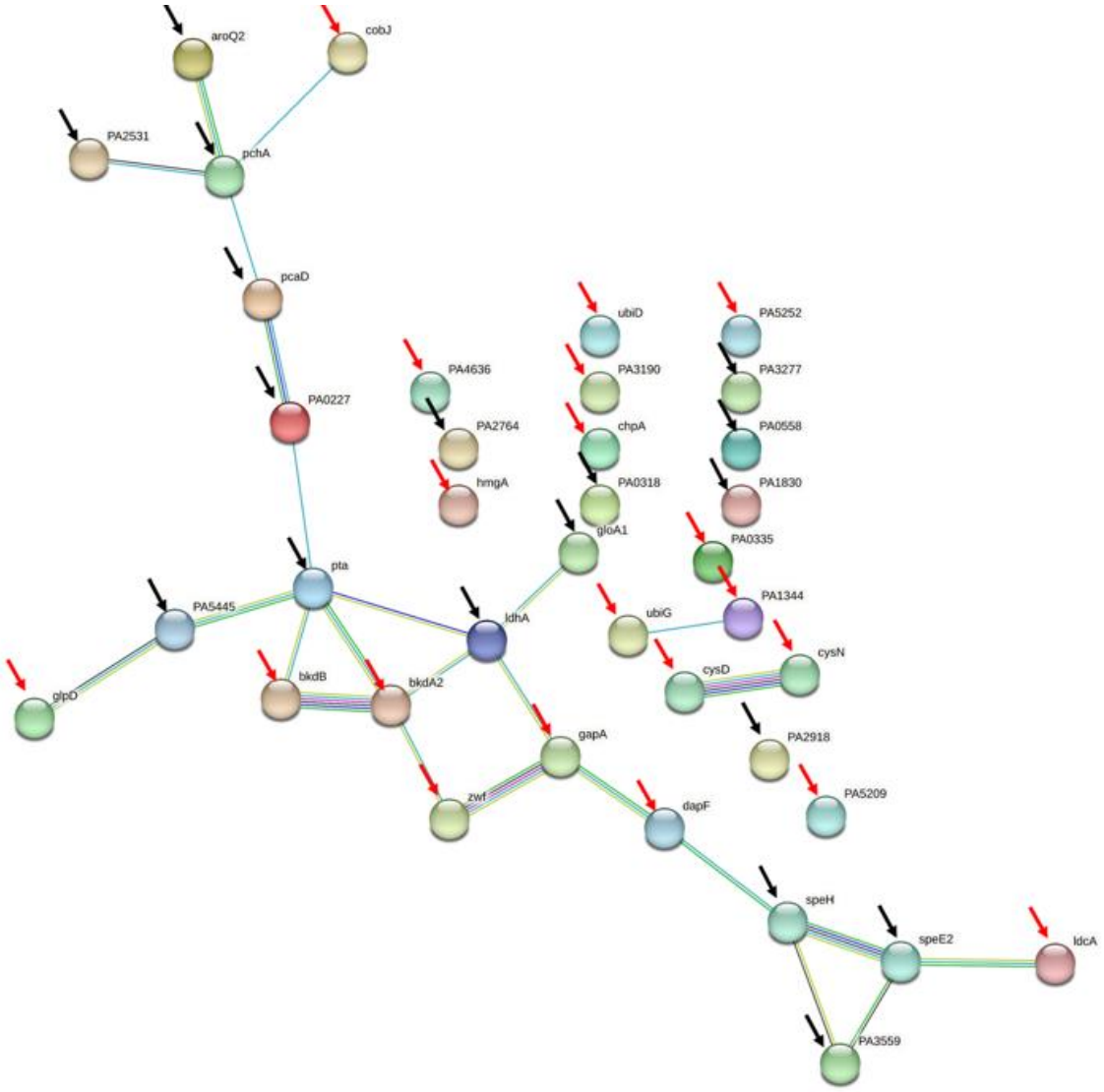

Figure 2.10. String representation of metabolism-related proteins in the presence of 4-HBA. Both downregulated and upregulated proteins. The red arrows indicate the downregulated proteins, and the black arrows indicate the upregulated proteins.

2.3.3.6. Redox and Cell Homeostasis Proteins of 4-HBA Treatment

The interaction network of redox and cell homeostasis-related proteins was visualized using the STRING database. Figure 2.11. a presented the network of downregulated proteins, while Figure 2.11.b depicted the network of upregulated proteins. The complete interaction network, encompassing downregulation and upregulation, was demonstrated in Figure 2.11.c. Furthermore, Table 2.7 showcased the functions of the downregulated and upregulated proteins.

Bacterioferritin protein BfrB was found to be downregulated during the 4-HBA treatment. Iron is a crucial nutrient for bacteria, but it poses difficulties due to the high reactivity of Fe^{2+} and the insolubility of Fe^{3+}. Iron storage proteins play a crucial role in overcoming these challenges by oxidizing Fe^{2+} using O_2 and H_2O_2 as electron acceptors and compartmentalizing Fe^{3+}. Two kinds of iron-storage proteins, ferritins (Ftn) and heme-containing bacterioferritins (Bfr) exist in bacteria, but the reasons for their coexistence are not well understood. *Pseudomonas aeruginosa* cells possess two iron storage proteins, FtnA and BfrB, but their relative contributions to iron homeostasis are currently unknown (Eshelman et al., 2017). Downregulation of the BfrB protein in *Pseudomonas aeruginosa* could potentially lead to disruptions in iron homeostasis and iron metabolism in the bacterium. BfrB is involved in the storage and regulation of iron, and its downregulation could result in reduced capacity for the bacterium to sequester iron ions in the cytoplasm. This could result in an increased susceptibility of the bacterium to oxidative stress and reactive oxygen species. It could also affect the expression of other iron-responsive genes and the ability of the bacterium to acquire iron from its environment. The specific consequences of BfrB downregulation may depend on the availability of other iron storage proteins and the severity of the iron limitation in the environment.

NosZ was found to be downregulated during the treatment, as well. The genes known as *nosZ* encode for an enzyme called nitrous oxide reductase, which contains multiple copper ions. (Zumft et al., 1992). In *P. aeruginosa*, the genes responsible for reducing nitrous oxide (N(2)O), namely *nosRZDFYL*, are grouped together on the chromosome. Promoter assays that fused the lacZ reporter gene to the transcriptional elements found that the *nosZ*, which encodes the nitrous oxide reductase enzyme, is transcribed along with the adjacent *nosR* (Arai et al., 2003). Nitrous oxide (N_2O) reductase is responsible for reducing N_2O in bacteria, and its production is catalyzed by the *nosZ*. If the expression of NosZ is downregulated in *P. aeruginosa*, then the ability of the bacteria to reduce N_2O would be impaired. The downregulation of the NosZ may also impact the bacterial response to environmental stress and the metabolism of other nitrogen-containing compounds. Overall, the downregulation of the NosZ protein could significantly impact the ecological and environmental functions of *P. aeruginosa*.

The upregulation of the SodB protein in *P. aeruginosa* results in increased resistance to oxidative stress caused by reactive oxygen species (ROS) like superoxide,

hydrogen peroxide, and hydroxyl radicals. SodB is an iron-cofactor-associated superoxide dismutase that plays a crucial role in detoxifying superoxide radicals. Its upregulation reduces the levels of ROS, protecting cellular components and promoting survival under stressful conditions. Moreover, elevated SodB levels may lead to higher pyocyanin production in *P. aeruginosa*. Pyocyanin is a blue antibiotic that generates superoxide, similar to paraquat, and its production coincides with increased activities of iron superoxide dismutase and catalase enzymes (Hassett, Schweizer, and Ohman, 1995). The upregulation of SodB could enhance pyocyanin synthesis by stimulating the production of these enzymes, providing *P. aeruginosa* with a competitive advantage against other microorganisms in its environment. In summary, the upregulation of SodB in *P. aeruginosa* confers benefits such as improved resistance to oxidative stress and enhanced pyocyanin production, contributing to the bacterium's overall fitness and survival.

Although downregulation of BfrB results in the susceptibility of the bacterium to oxidative stress and reactive oxygen species, upregulation of the SodB protein increases the organism's resistance to oxidative stress. By these changes, bacteria try to survive during the 4-HBA treatment.

Table 2.7. The proteins related to redox and cell homeostasis in the presence of 4-HBA.

Accession	**Protein ID**	**Protein Name**	**Localization**	**q-Value**	**Downregulation/Upregulation**
PA5421	FdhA	Glutathione-independent formaldehyde dehydrogenase	Cytoplasm	0.000	Downregulated
PA3531	BfrB	Bacterioferritin	Cytoplasm	0.000	Downregulated
PA3392	NosZ	Nitrous-oxide reductase	Periplasm	0.000	Downregulated
PA2630		JmjC domain-containing protein	Cytoplasm	0.000	Downregulated
PA1606		DUF3859 domain-	Unknown	0.000	Downregulated

		containing protein			
P53641	SodB	Superoxide dismutase [Fe]	Periplasm	0.000	Upregulated
PA3795		Probable oxidoreductase	Cytoplasm	0.000	Upregulated
PA3397	Fpr	Ferredoxin--NADP(+) reductase	Cytoplasm	0.000	Upregulated
PA3092	FadH1	2,4-dienoyl-CoA reductase	Cytoplasm	0.000	Upregulated
PA2546		Probable ring-cleaving dioxygenase	Cytoplasm	0.001	Upregulated
PA2482		Probable cytochrome c	Periplasm	0.002	Upregulated
PA1684	MtnD	Acireductone dioxygenase	Cytoplasm	0.000	Upregulated
PA5240	TrxA	Thioredoxin	Cytoplasm	0.000	Upregulated

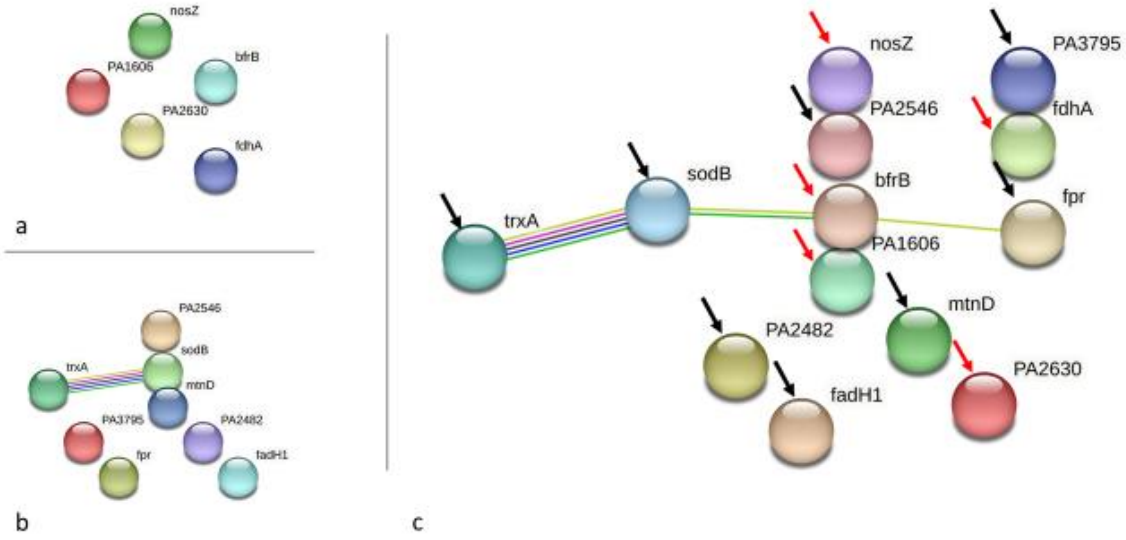

Figure 2.11. String representation of redox and cell homeostasis-related proteins in the presence of 4-HBA. a) Downregulated proteins, b) Upregulated proteins, c) Both downregulated and upregulated proteins. The red arrows indicate the

downregulated proteins, and the black arrows indicate the upregulated proteins.

2.3.3.7. Virulence-Related Proteins of 4-HBA Treatment

The interaction network of virulence-related proteins was visualized using the STRING database. Figure 2.12 presents the network of proteins, and Table 2.8 presents the functions of the downregulated proteins.

The downregulation of VgrG1a protein, a component of the Type VI secretion system (T6SS) in *P. aeruginosa*, can have significant implications for the bacterium's ability to infect host cells. Secretion systems play a crucial role in bacterial pathogenesis by enabling the delivery of proteins that facilitate survival and exploitation of the host environment. Gram-negative bacteria possess various secretion systems, including T6SS, which is involved in delivering effector proteins into host cells. The T6SS system consists of multiple genes and is essential for bacterial pathogenesis and host cell survival. *P. aeruginosa* harbors several gene clusters encoding T6SS components. Mutants lacking T6SS genes were shown to be less virulent in animal infection models. The T6SS system secretes proteins belonging to two families, Hcp and VgrG, with VgrGs forming trimeric complexes resembling bacteriophage tail proteins. VgrG proteins, including VgrG1a, play a crucial role in the T6SS-mediated delivery of effectors. They form trimeric complexes that puncture membranes, facilitating the passage of proteins or macromolecular complexes (Hachani et al., 2011). Therefore, the downregulation of VgrG1a in *P. aeruginosa* would likely impair the bacterium's ability to deliver effector proteins into host cells effectively. This could result in reduced virulence and compromised survival within the host. It is important to note that the specific effects of VgrG downregulation may vary depending on the targeted protein and the context of the infection. However, since the T6SS system is known to be crucial for bacterial pathogenesis and host-cell interactions, the downregulation of VgrG1a would likely have a negative impact on *P. aeruginosa*'s ability to establish infection and manipulate host responses. Further research is needed to fully understand the consequences of VgrG downregulation and its implications for Pseudomonas aeruginosa virulence.

Table 2.8. The proteins related to virulence in the presence of 4-HBA.

Accession	Protein ID	Protein Name	Localization	q-Value	Downregulation/Upregulation
PA0091	VgrG1a	Type VI secretion system spike protein VgrG1a	Extracellular	0.000	Downregulated
PA0082	TssA1	Type VI secretion system component TssA1	Cytoplasm	0.002	Downregulated

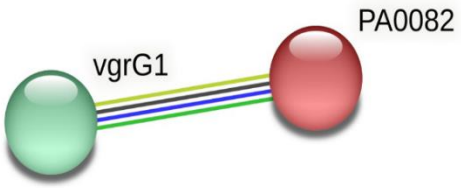

Figure 2.12. String representation of virulence-related downregulated proteins in the presence of 4-HBA.

2.3.3.8. Uncharacterized Proteins of 4-HBA Treatment

Several downregulated and upregulated proteins were shown, uncharacterized in Uniprot. The STRING results demonstrated the interaction network of these downregulated and upregulated proteins (Figure 2.13). Table 2.9 shows the list of the uncharacterized proteins.

The strongly interacted downregulated proteins PA4685, PA4684, and PA0943 are found to play a role in chromosome partitioning, as DUF3375 domain-containing and dehydrogenase, respectively (Klockgether et al., 2010). Similarly, the strongly interacted upregulated protein PA2805, a chromosome partitioning protein (UniProt, 2023). Therefore, bacteria maintain these aspects in the downregulation of PA4685, PA4684, and PA0943 proteins by upregulated production of PA2805 protein.

Table 2.9. The uncharacterized proteins in the presence of 4-HBA.

Accession	Protein ID	Protein Name	Localization	q-Value	Downregulation/Upregulation
PA4684		Uncharacterized protein	Cytoplasm	0.005	Downregulated
PA4685		Uncharacterized protein	Cytoplasm	0.000	Downregulated
PA0943		Uncharacterized protein	Periplasm	0.000	Downregulated
PA4714		Uncharacterized protein	Unknown	0.000	Downregulated
PA3318		Uncharacterized protein	Unknown	0.002	Downregulated
PA0371		Uncharacterized protein	Unknown	0.007	Downregulated
PA2458		Uncharacterized protein	Outer Membrane	0.005	Downregulated
PA2805		Uncharacterized protein	Unknown	0.000	Upregulated
PA4611		Uncharacterized protein	Cytoplasm	0.000	Upregulated
PA2451		Uncharacterized protein	Cytoplasm	0.000	Upregulated
PA3720		Uncharacterized protein	Cytoplasm	0.002	Upregulated
PA4090		Uncharacterized protein	Unknown	0.000	Upregulated
PA3332		Uncharacterized PhzA/B-like protein	Cytoplasm	0.000	Upregulated
PA5339		Uncharacterized protein	Cytoplasm	0.000	Upregulated
PA3123		Uncharacterized protein	Cytoplasm	0.000	Upregulated
PA2770		Uncharacterized isomerase	Cytoplasm	0.000	Upregulated
PA2729		Uncharacterized protein	Cytoplasm	0.000	Upregulated

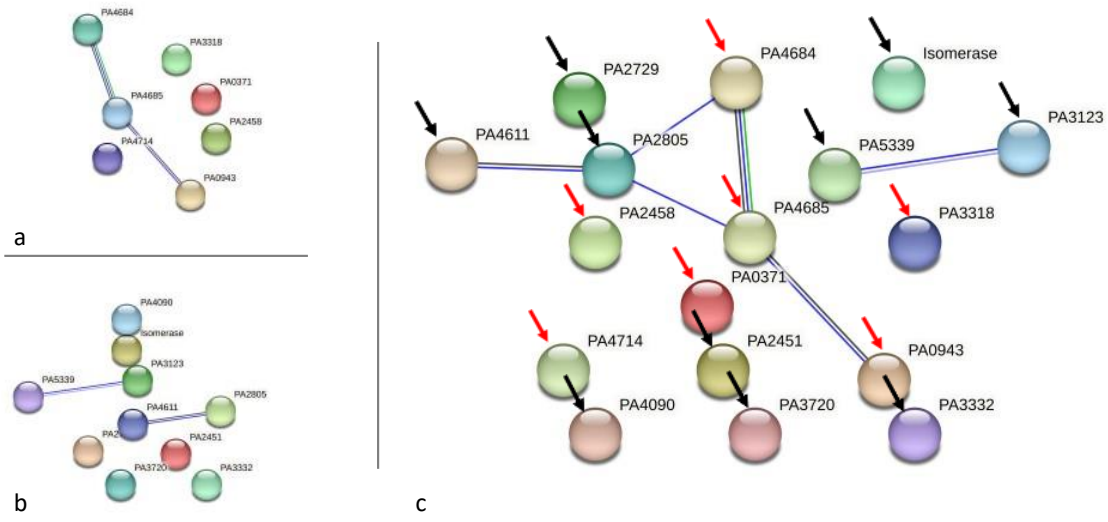

Figure 2.13. String representation of uncharacterized proteins in the presence of 4-HBA. Both downregulated and upregulated proteins. The red arrows indicate the downregulated proteins, and the black arrows indicate the upregulated proteins.

2.3.4. Quantitative Analysis by ddPCR

To validate proteomics data, three genes were selected to conduct ddPCR analysis. Droplet Generation and Droplet digital PCR protocol were used for absolute quantification of *recQ*, *pilM*, and *deaD* gene expression on *P. aeruginosa*. Copies of expressed mRNA levels per µl have been given in the figure below (Figure 2.14).

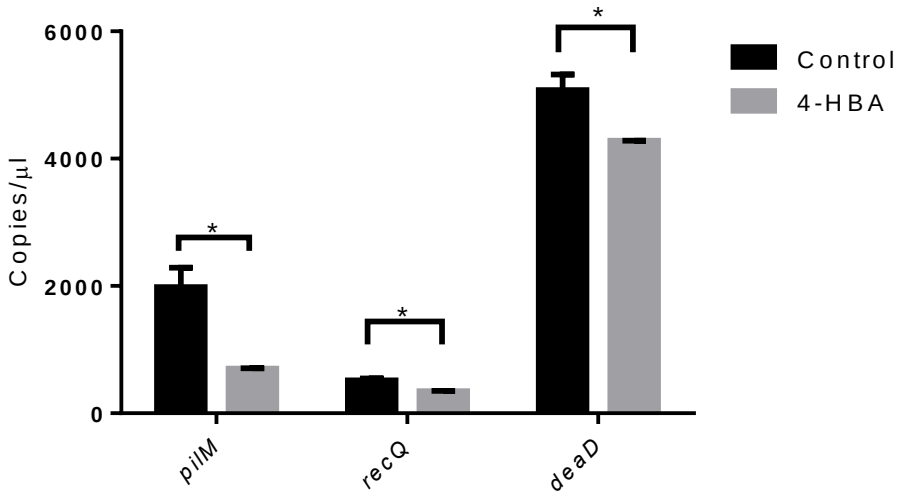

Figure 2.14. Copies/µl results of *recQ*, *pilM*, and *deaD*'s mRNA expression levels on untreated *P. aeruginosa* and 4-HBA treated *P. aeruginosa*. "*" indicates p-value similar or smaller than 0.05.

Three different proteins involved in various functions have been selected to conduct the experiment. RecQ protein is a DNA-related protein; PilM protein is a cell wall and membrane-related protein; and DeaD protein is a ribosome and protein-related protein. According to the results, fewer copies/µl of expressed mRNA levels of the given genes on 4-HBA treated samples were found compared to untreated samples. All genes were significantly changed in the 4-HBA treated sample compared to the untreated sample (Figure 2.14). The PilM/N/O/P/Q gene cluster, particularly PilM, is essential for type IV pili and twitching motility in bacteria. PilM, typically cytoplasmic, colocalizes with other components to form an inner membrane complex. Its loss affects complex stability, impacting outer membrane complex formation (Ayers et al., 2009). RecQ protein in P. aeruginosa also plays pivotal roles in DNA repair, recombination, and replication, ensuring genomic stability. It resolves DNA damage, facilitates genetic recombination, and aids in faithful DNA replication (Berstein, Gangloff, and Rothstein, 2010). In *Pseudomonas aeruginosa*, the DeaD protein (CsdA) functions as an RNA helicase, unwinding RNA structures critical for translation, RNA degradation, and ribosome assembly. It plays a role in ribosome biogenesis, RNA metabolism, and stress responses, aiding the bacterium's adaptability and gene expression regulation in diverse environments (Lv et al., 2022). mRNA expression levels of these two proteins were decreased after 4-HBA treatment.

2.4. Conclusions

In this study, proteomics analysis revealed the functional effects of phenolic acid 4-HBA on opportunistic pathogen *P. aeruginosa*. Differentially expressed proteins were categorized as downregulated and upregulated to interpret their functions using Gene Ontology and STRING. The proteomics data was also validated by ddPCR analysis, and the expected results were reached.

Throughout the results, the most significant changes were observed in the proteins related to protein biosynthesis against 4-HBA treatment. DNA replication was found to be affected as well. Hence, it can be inferred that 4-HBA treatment led to defects in the protein translation machinery, subsequently affecting processes such as DNA repair and transcription.

Another conclusion was reached that the downregulation of certain proteins results in upregulation of some other proteins. For instance, from cell wall and membrane-related proteins, PilM protein, which has a role in pathogenicity and is suggested as a target for new therapeutics, was found to be downregulated. Meanwhile, PirA protein, which is ferrienterobactin that is associated with pathogenicity and virulence, was found to be upregulated. These results suggest that bacteria try to survive by compensating for the downregulation of some proteins by producing new ones.

On the other hand, upregulation of ribosome and protein-related proteins was not observed throughout the treatments. Various proteins that have effects on virulence, protein translocation, and pathogenesis were downregulated, whereas no protein was found to be upregulated. Therefore, it was once again concluded that more significant changes in the result of 4-HBA treatment are related to protein and ribosome.

Lastly, proteomics data validation was conducted by ddPCR analysis by comparing mRNA expression levels of PilM, RecQ, and DeaD proteins in untreated and 4-HBA-treated *P. aeruginosa* samples. According to the results, expression levels decreased after 4-HBA treatment, as was observed in proteomics data.

CHAPTER 3

EFFECT OF 4-HBA-LOADED BONE CEMENTS ON MSSA, MRSA, *S. epidermidis*, *E. faecium* and *P. aeruginosa*

3.1. Introduction

Bone cement, commonly referred to as polymethyl methacrylate (PMMA), finds wide application in orthopedic and trauma surgeries for the fixation of implants. However, the term "cement" can be misleading as it implies a substance that binds two objects together. In reality, PMMA serves as a space-filling material that creates a snug fit, anchoring the implant against the bone and functioning akin to a "glue." Its effectiveness stems from the close mechanical interlock achieved between the irregular surface of the bone and the prosthesis rather than possessing adhesive properties. (Chow et al., 2001). While the utilization and the range of bone cements have advanced significantly over the past century, ongoing research endeavors to explore their clinical applications further and mitigate the associated adverse effects (Vaishya, Chauhan, and Vaish, 2013). The consequences could be allergic reactions, infection, bone cement embolism, heat generation, and mechanical loosening of the implant.

PMMA (polymethyl methacrylate), an acrylic polymer, is produced through the combination of two sterile components: a powdered copolymer composed of MMA (methyl methacrylate) and styrene and a liquid monomer called MMA. When these two elements are mixed, the liquid monomer undergoes polymerization, enveloping the pre-polymerized powder particles and forming solidified PMMA. During this process, an exothermic reaction occurs, generating heat.

PMMA, in combination with different additives, imparts a range of physical and chemical characteristics to the mixture. The liquid component of the mixture can undergo premature polymerization when exposed to light or high temperatures. To prevent this, a stabilizer or inhibitor called hydroquinone is incorporated. The powder component of the PMMA bone cement comprises an initiator, commonly di-benzoyl peroxide (BPO), while the liquid component contains an accelerator, primarily N, N-dimethyl-p-toluidine

(DmpT). These additives function in synergy to promote the polymerization of the monomer at ambient temperature, known as cold curing the cement.

A contrast agent is incorporated to improve the cement's visibility during imaging procedures. Available commercially, bone cement commonly employs either barium sulfate (BaSO4) or zirconium dioxide (ZrO2). Zirconium dioxide is favored due to its notably lower solubility compared to barium sulfate, as well as its minimal influence on the mechanical properties of the cement.

During the exothermic free-radical polymerization process, heating is being endured in the cement. Approximately 82 °C to 86 °C heat within the body is being reached by this polymerization. The relatively thin coating of the cement, not exceeding 5 mm, along with the dissipation of temperature through the large surface area of the prosthesis and the flow of blood, contribute to the lower polymerization temperature observed in the body (Vaishya, Chauhan, and Vaish, 2013).

The number of primary and revision total joint arthroplasty (TJA) surgeries has increased in recent decades. In the United States, it is projected that there will be approximately 635,000 primary total hip arthroplasties (THAs) and 935,000 total knee arthroplasties (TKAs) performed by the year 2030 (Sloan, Premkumar, and Sheth, 2018). Aseptic loosening and infection are the leading causes of arthroplasty revision, particularly in the cases of early failures following total knee arthroplasty (TKA) (Fehring et al., 2001). A recent study conducted in France, which examined a cohort of 1,170 TKA reinterventions, revealed that prosthetic joint infection (PJI) accounted for nearly 50% of all revision procedures (Schmidt et al., 2020). The incidence of PJIs after TJA ranges from 1% to 7% (Sultan et al., 2019)

Bone cement has emerged as a valuable tool due to its ability to incorporate specific active substances, such as antibiotics, into the powder component. This characteristic allows bone cement to serve as a contemporary drug delivery system, delivering necessary medications directly to the surgical site. When bone cement is used, the localized concentration of active substances achieved is significantly lower compared to the standard clinical dosages used for systemic single injections. Studies have shown that incorporating various antibiotics into bone cement, in amounts less than 2 grams per standard packet of bone cement, does not negatively impact the mechanical properties such as compressive or diametrical tensile strengths. However, exceeding the 2-gram threshold can weaken these properties. Antibiotics like gentamicin, tobramycin,

erythromycin, cefuroxime, vancomycin, and colistin have been successfully mixed and applied with bone cement. The crucial requirements for the added antibiotics are having heat resistance and retaining their effectiveness over an extended period.

Specifically, the combination of gentamicin and tobramycin demonstrates a synergistic effect, leading to a significant increase of 68% in the elution of tobramycin ($P = 0.024$) and a substantial increase of 103% in the elution of vancomycin from the bone cement ($P = 0.007$) compared to controls containing a single antibiotic. (Penner, Masri, and Duncan, 1996).

According to Van Staden's research, bacteriocins have emerged as a promising alternative to antibiotics for integration into bone cement. In vitro findings from the study revealed that adding bacteriocins to brushite cement did not result in significant changes to the matrix characteristics, and the peptides were released in an active form. Moreover, the study demonstrated that brushite cement loaded with nisin F effectively managed *S. aureus* infection in mice. (Van Staden et al., 2011).

Silver-containing nanoparticles have demonstrated significant promise as highly effective antibacterial agents that can be incorporated into bone cement (Alt et al., 2004). Additionally, adding 10% vitamin E additives has been observed to positively impact the reduction of free radical oxidation and exothermic activity. This addition also leads to a marginal decrease of less than 5% in tensile strength. (Arora et al., 2013).

Compared to intramuscular administration, the systemic concentration levels of gentamicin when delivered through bone cement are relatively low, typically below 1 μg/mL (less than 10%). After seven days of administration, no detectable systemic levels of gentamicin are present. Following the administration of bone cement, gentamicin levels in urine initially range from 10 μg/mL and, after seven days, decrease to 1-2 μg/mL.

Different bone types of cement exhibit unique chemical compositions, leading to a range of antibiotic-loaded bone cement with varying handling properties. This diversity enables the selection of bone cement tailored to meet diverse clinical needs and surgical techniques. (Vaishya, Chauhan, and Vaish, 2013).

Szymski et al. analyzed 13,612 intracapsular femoral neck fracture cases, comparing hip arthroplasty (HA) with total hip arthroplasty (THA) and assessing infection rates. They found that using antibiotic-loaded cement in HA significantly reduced the infection rate compared to uncemented prostheses. In THA cases, there was no statistical difference between cemented and uncemented prostheses, but after one year,

infections were slightly higher in uncemented THA. Risk factors for periprosthetic joint infection (PJI) were identified as BMI (Body Mass Index) and Elixhauser Comorbidity Index. Cemented THA showed an increased risk within the first 30 days. The study concludes that antibiotic-loaded cement may be a reasonable preventive measure, especially for patients with multiple risk factors for PJI (Szymski et al., 2023).

Although antibiotics play a crucial role in modern medicine by supporting surgical procedures and aiding immunocompromised patients, the rise of antibiotic-resistant bacteria is making existing antibiotics less effective. Antibiotic resistance currently causes around 700,000 deaths annually, and if not addressed through antibiotic stewardship or the continuous development of new antibiotics, it is projected to claim 10 million lives by 2050. Unfortunately, the pipeline for new antibiotics has stagnated due to diminishing commercial incentives for the pharmaceutical industry (Ghosh et al., 2022). To overcome this challenge, we propose a new strategy by loading the bone cement with an antibiotic and another antimicrobial agent: gentamicin and 4-HBA.

Ghosh et al. described the engineering of a drug-device combination for treating orthopedic infections. The demand for orthopedic procedures is increasing due to the aging global population and trauma cases. Despite pre-operative and prophylactic antibiotic therapy, infection related to invasive orthopedic procedures is expected, with rates exceeding 25% in complex open fractures or revision surgeries. Post-operative orthopedic infections, caused not only by *Staphylococcus* but also by *Enterococcus*, *Cutibacterium*, and *Pseudomonas* bacteria, pose significant challenges even in advanced medical settings (Ghosh et al., 2022).

These infections often lead to the formation of dead bone sections, which, along with limited blood supply, create an environment characterized by acidic pH, local immune suppression, and poor penetration of antibiotics. Persistent bacterial biofilms commonly develop, resulting in chronic infections that require surgical interventions. While systemic antibiotics are commonly used, high doses are necessary to achieve efficacy at the necrotic region. However, this systemic antibiotic exposure can disrupt the normal microbiome (Ghosh et al., 2022).

Antibiotic-eluting acrylic bone cement spacers are increasingly being used to address these challenges. The US Food and Drug Administration (FDA) has approved bone cement loaded with vancomycin, gentamicin, tobramycin, or clindamycin. However, these antibiotics were not initially developed for local delivery from bone

cement, and resistance has emerged against them. Therefore, there is a need for next-generation antibiotics that are specifically optimized to meet the emerging demands of localized delivery in orthopedic infections (Ghosh et al., 2022).

Kim et al.'s study focused on the pharmacodynamics of gentamicin (GEN) in relation to Gram-positive organisms, specifically methicillin-resistant *Staphylococcus aureus* (MRSA). Aminoglycosides, including gentamicin, are known for their concentration-dependent killing and post-antibiotic effect in Gram-negative bacteria. The researchers aim to explore the relationship between gentamicin concentration and the rate of bacterial kill or resistance development in *S. aureus* and investigate differences compared to Gram-negative organisms (Kim et al., 2010).

Although comprehensive dose-fractionation studies were not conducted, the bacterial killing rates of once-daily (OD) and traditional twice-daily (TD) dosing regimens of gentamicin were compared. The results suggest that OD dosing over 24-48 hours had superior killing effects compared to TD dosing, similar to observations in Gram-negative organisms. Kim et al. also proposed that the discordance between the regimens may be attributed to the rapid killing of susceptible populations during the early period, followed by the re-growth of resistant subpopulations later. To gain a better understanding of the pharmacodynamics of gentamicin against community-acquired MRSA (CA-MRSA), further studies involving complete dose-fractionation with strains of varying minimum inhibitory concentrations (MICs) are warranted (Kim et al., 2010). Their vitro studies have shown that exposure of CA-MRSA to gentamicin for 24 hours at concentrations ranging from 1 to 64 times the MIC resulted in the development of high-level resistance. This suggests that gentamicin monotherapy may not be an effective treatment choice despite most CA-MRSAs being initially susceptible to gentamicin. Optimal bactericidal activity of aminoglycosides, including gentamicin, is achieved when the exposure concentration is approximately 8-10 times the MIC against Gram-negative organisms. Moreover, maintaining a peak/MIC ratio of 8:1 was correlated with a decrease in the selection and re-growth of resistant subpopulations during treatment with netilmicin. The study simulated human pharmacokinetics over a 48-hour evaluation period and identified the development of resistance in TD dosing with individualized pharmacokinetics/pharmacodynamics modeling. Resistance and re-growth of colonies were observed after 24 hours of TD dosing. In contrast, OD dosing with a peak/MIC ratio over 8.6 in both CA-MRSA strains exhibited more profound bactericidal activity with no

resistance or re-growth. This could be attributed to the longer duration of the post-antibiotic effect (PAE) in the OD regimen against CA-MRSA. The findings provide clues for maximizing the efficacy of aminoglycosides against CA-MRSA, even though most peak/MIC data are associated with Gram-negative bacteria. The authors plan to extend their experiments to include combination therapies and the development of resistance against MRSA (Kim et al., 2010).

Regarding small colony variants (SCVs) of *S. aureus*, optimal therapy has yet to be defined. Gentamicin is generally not recommended for SCVs due to reduced uptake caused by the interruption of electron transport, resulting in decreased susceptibility compared to the parent strain. However, recent studies have shown that OD dosing of arbekacin could reduce or eliminate the appearance of SCVs in individualized pharmacokinetic/pharmacodynamics modeling. In the current study, SCVs appeared after 30 hours of TD treatment with gentamicin but were not observed with OD dosing. Combination therapies such as ceftriaxone plus gentamicin, administered once daily, have shown potential for selected cases of staphylococcal endocarditis. Further investigations are needed to determine whether OD or higher dose treatment can overcome the reduced uptake of gentamicin in *S. aureus* SCVs and to identify the beneficial period (Kim et al., 2010). Since gentamicin's required dosage is still an unknown subject and monotherapy with it is not suggested for treating MRSA strains, there is a specific need for new antimicrobial agents to combat these infections (Kim et al., 2010).

The effectiveness of antibiotics being released from bone cement plays a crucial role in determining the antibacterial capabilities of antibiotic-loaded bone cement (ALBC). A higher porosity level is anticipated to enhance the discharge of antibiotics from ALBC, leading to increased antibacterial activity. Powder antibiotics have shown effective release from bone cement, according to studies. On the other hand, using a liquid form of antibiotics in bone cement was limited due to a significant decrease in the cement's mechanical strengths, up to 50%. However, it was reported that liquid gentamicin in bone cement can be effectively released while maintaining its ability to kill bacteria. Furthermore, when liquid gentamicin is combined with vancomycin in the same cement, a mutually beneficial effect was observed on the release of both antibiotics (Chang et al., 2014).

The enhanced release of antibiotics with the addition of liquid gentamicin to bone cement is attributed to the increased porosity of the cement. Consequently, incorporating

a liquid antibiotic can increase the porosity of antibiotic-loaded bone cements (ALBCs). Dissolving antibiotic powder in distilled water before mixing it with bone cement could provide a more efficient release of antibiotics compared to loading the same amount of antibiotic powder directly (Chang et al., 2014). In this study, the antimicrobial properties of bone cement loaded with 4-HBA (4-hydroxybenzoic acid) were investigated in comparison to gentamicin-loaded bone cement as an antimicrobial agent. Our findings revealed that 4-HBA-liquid-loaded bone cement exhibited superior antibacterial activity compared to their powder-loaded counterparts, primarily due to increased porosity, as extensively discussed in the literature.

An ideal antibiotic is characterized by its ability to eliminate infections while preserving the healthy microbiome and minimizing the development of antibiotic resistance. Numerous studies indicate that alternative broad-spectrum antibacterial compounds (ABLCs) have demonstrated effectiveness in combating infections. However, an unresolved issue remains regarding the optimal dosage and duration required to achieve satisfactory outcomes (Mensah and Love, 2021). As a concern, a natural product showing antimicrobial, anticancer, and anti-inflammatory features has been tested in the study.

Herein, we describe a novel study investigating the effects of a natural antimicrobial agent, 4-HBA, and gentamicin-loaded bone cement on several pathogens. The primary objective of this chapter is to assess the impact of 4-HBA-loaded bone cement on various bacterial strains, including MSSA (methicillin-sensitive *Staphylococcus aureus*), MRSA (methicillin-resistant *Staphylococcus aureus*), *S. epidermidis*, *E. faecium*, and *P. aeruginosa*. Furthermore, our study aims to elucidate the contrasting effects of bone cements loaded with 4-HBA in powder and liquid forms. Additionally, the potential synergistic effects of 4-HBA when combined with gentamicin, an aminoglycoside-type antibiotic, in loaded bone cement was explored and discussed.

3.2. Materials and Methods

In this study, methicillin-susceptible *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA), *Staphylococcus epidermidis*, *Enterococcus faecium,* and *Pseudomonas aeruginosa* were utilized to examine the antimicrobial activity of phenolic acid 4-hydroxybenzoic acid-loaded bone cement.

3.2.1. Preparation of Bone Cement Samples

The antimicrobial agents utilized in bone cement preparation were i) 1 g gentamicin powder, ii) 2 g gentamicin powder, iii) 10 mL gentamicin solution that contains 2 g gentamicin powder, iv) 2 g 4-HBA powder, v) 10 mL 4-HBA solution that contains 2 g 4- HBA powder, vi) 1 g of gentamicin powder with 1 g of 4-HBA powder and, vii) 1 g gentamicin powder with 2 g 4-HBA powder. 10 ml of sterile ddH_2O was used to dissolve antimicrobial agents in the solutions. The bone cement samples containing gentamicin were used as a positive control. The bone cement samples without adding antimicrobial agents were used as negative control and indicated as "null" during the experimental procedure.

All steps for bone cement sample preparations were conducted under aseptic conditions. The size of the bone cement samples is 8 mm in diameter and 3 mm in height.

Radiopaque Cement Oliga 1 and Radiopaque Cement Oliga 1 Genta were purchased commercially. Both contained 40 g of PMMA radiopaque powder and 20 mL of liquid methyl methacrylate (MMA). Radiopaque Cement Oliga 1 Genta also contained 1 g of gentamicin. The 20 mL of liquid MMA was combined in a fast way with the 40 g of powder of PMMA. Prepared bone cement discs were stored at -20 °C and in the dark to prevent light exposure (Dokumacı et al., 2022).

3.2.2. Antimicrobial Effects of Gentamicin and 4-Hydroxybenzoic Acid Loaded Bone Cements on MSSA, MRSA, *S. epidermidis*, *E. faecium* and *P. aeruginosa*

PMMA bone cement samples, including null, 2 g of Gentamicin powder, 2 g of 4-HBA, 2 g of Gentamicin liquid, 2 g of 4-HBA liquid, 1 g of Gentamicin, and 1 g of 4-HBA and 1g of Gentamicin and 2 g of 4-HBA concentrations were weighted, and 1 g of bone cement (approximately 6 or 7 discs) were added to test tubes including 5 ml of TSB. The test tubes were pre-incubated for 7 days at 37 °C at 75 rpm. The experiment was conducted for each bacterium individually for three replicates. Bacteria were inoculated from a single colony to 4 ml of TSB and incubated for 18 hours at 37 °C without shaking. Then, the bacteria were diluted to 10^6 CFU/ml, and 1 ml was inoculated in pre-incubated

cement-containing tubes. The samples were subjected to OD measurements at 600 nm at time zero. Parallel to this, 100 µl from each tube was spread onto TSA plates for viable count. After the inoculation, tubes were incubated for 24 hours at 37 °C with 75 rpm to provide kinetically co-incubation effects of the bone cement. Then, the OD measurements and viable count were conducted to observe the growth inhibition of the bacteria for 24-hour and 48-hour time points.

3.2.3. Statistical Analysis

Three technical replicates were used with two sets to conduct experiments. GraphPad Prism 6 was used to interpret the data. Statistical significance was found by unpaired t-test.

3.3. Results and Discussions

In this study, seven different types of PMMA bone cement samples containing a) Null (no antimicrobial agent), b) 2 g of gentamicin powder (Genta powder), c) 10 mL of gentamicin solution that includes 2 g of gentamicin powder (Genta liquid), d) 2 g of 4-HBA powder, e) 10 mL of 4-HBA solution that includes 2 g of 4-HBA powder (4-HBA liquid), f) 1 g of gentamicin powder with 1 g of 4-HBA powder and, g) 1 g of gentamicin powder with 2 g of 4-HBA powder were examined for antimicrobial activity against MSSA, MRSA, *S. epidermidis*, *E. faecium*, *P. aeruginosa*.

"4-HBA-liquid" or "Gentamicin-liquid" loaded bone cement samples were prepared by dissolving 2 g of the antimicrobial agent in 10 mL of the sterile ddH_2O. The bone cements with the addition of gentamicin were used as a positive control for 4-HBA-containing samples. "Null" labeled bone cement didn't include any antimicrobial agent and was the negative control of the study.

3.3.1. Antimicrobial Effects of Gentamicin and 4-Hydroxybenzoic Acid-Loaded Bone Cements on MSSA

The study aimed to assess the antimicrobial efficacy of bone cement loaded with antimicrobial agents against Methicillin-Susceptible *Staphylococcus aureus* (MSSA). Different combinations of gentamicin and 4-HBA were compared alongside control groups to evaluate their effectiveness in inhibiting bacterial growth. The antimicrobial effects of 7 days of preincubated 1 g bone cement samples were shown in Figure 3.1.

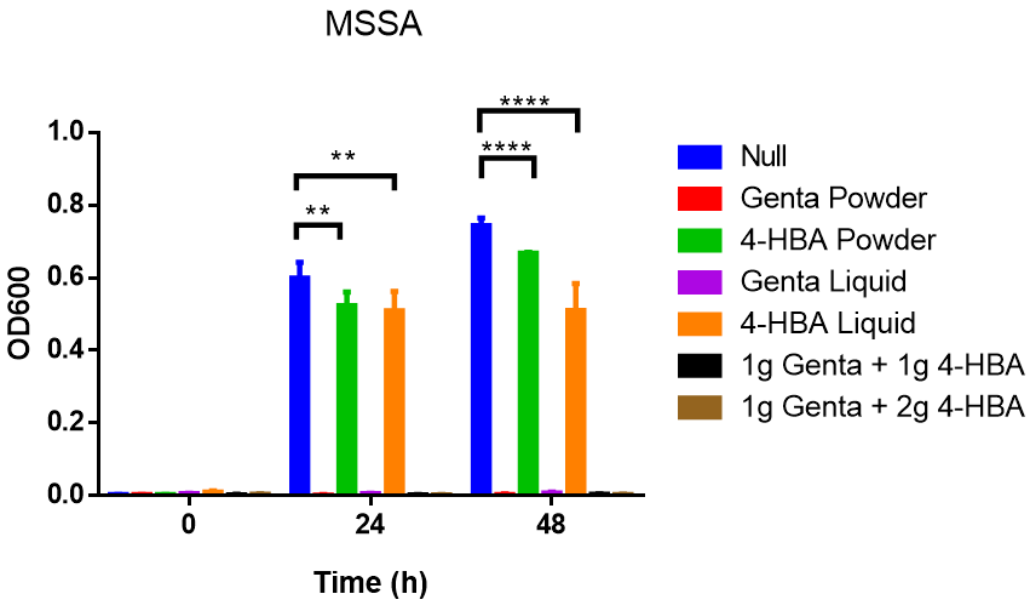

Figure 3.1. Antimicrobial effect of bone cement samples on MSSA (10^6 CFU/ml). The optical densities (OD_{600nm}) were measured at 600 nm at the 0^{th}, 24^{th}, and 48^{th} hours of MSSA inoculation. "**" indicates a p-value equal to or smaller than 0.01, and "****" indicates a p-value equal to or smaller than 0.0001, found by unpaired t-test.

Although all OD values were similar at the 0^{th} hour, the null displayed the highest at the 24^{th} and 48^{th} hour time points (Figure 3.1). It was an expected result since no antimicrobial agents were loaded to null samples. 4-HBA liquid and 4-HBA powder containing bone cement samples were found below the null significantly. All gentamicin-loaded cement samples displayed the lowest OD values, indicating bacterial growth inhibition. At the end of the 48^{th} hour, the result indicated that 4-HBA liquid inhibited the

growth of MSSA more than the 4-HBA powder containing cement samples because of its better release properties. It was distinctly visible that all gentamicin-loaded cement samples inhibited the growth of MSSA at the 24^{th} and 48^{th} hours.

In a study involving patients with infected total hip replacements, where gentamicin-loaded bone cement was primarily utilized, it was found that 88% of these patients carried at least one strain of *Staphylococcus* bacteria that showed resistance to gentamicin (Hilbrand van Belt et al., 2000). This indicates a potential risk for developing antibiotic-resistant strains due to prolonged exposure to low concentrations of gentamicin in the vicinity of the implant. Hence, it is imperative to utilize a specific subtype of phenolic acid known as 4-HBA, which, according to unpublished data, does not exhibit resistance in MSSA and MRSA. This particular subtype is deemed necessary for the purposes of this study. Furthermore, published evidence supports the anti-resistance properties of another subtype of phenolic acid called 3-HPAA, thereby substantiating the data (Keman and Soyer 2019).

The strength of the bone cement is a significant aspect since the increased roughness increases the porosity and thus causes inclined antimicrobial agent release. A recent study showed that caffeic acid phenethyl ester (CAPE) increased the strength of the cement, which was found in more than 130 MPa, which is higher than the strength of gentamicin-loaded bone cement. Therefore, the strength of the 4-HBA-loaded bone cement and *in vivo* implantation test could be done further to observe the cement's release rate and inflammatory response, respectively (Lee and Chang, 2015). A similar result was reached by our unpublished data (Soyer Lab) about increasing the strength of the cement by 4-HBA. Higher doses of 4-HBA would be loaded into the bone cement; for instance, up to eight times higher concentrations than gentamicin concentration would be tried to observe the antimicrobial effect of the bone cement as it is researched in the literature (Lee and Chang, 2015).

An ideal antibiotic is characterized by its ability to eliminate infections while preserving the healthy microbiome as much as possible and minimizing the development of antibiotic resistance. Numerous studies indicate that alternative broad-spectrum antibacterial compounds (ABLCs) have demonstrated effectiveness in combating infections. However, an unresolved issue remains regarding the optimal dosage and duration required to achieve satisfactory outcomes (Mensah and Love, 2021). As a

concern, a natural product, 4-HBA, that showed antimicrobial, anticancer, and anti-inflammatory features was tested in our study.

Furthermore, there is an associated mechanical drawback when incorporating high doses of antibiotics into bone cement. The strength of the cement is negatively impacted, leading to a reduction in structural stability. A study by Singh et al. demonstrated that adding more than 3 g of vancomycin decreased the bending strength and stiffness of ALBCs (Singh et al., 2019). Therefore, up to 2 g of gentamicin was utilized in our study to load the cements. Thus, a maximum of 2 g 4-HBA was loaded into the cements and was investigated in our study.

Antibiotic-loaded bone cements could be utilized in patients with diabetic foot osteomyelitis for wound healing, as well. Although the usage should be researched more, it was found that pathogens eradication was increased and hospital stays decreased (Ehya et al., 2021). Therefore, The study serves for joint replacements and other attention-required wounds.

The use of gentamicin, an aminoglycoside antibiotic, has declined significantly in orthopedic infections over the past three decades due to its associated toxicity. However, the experimental utilization of gentamicin during this period has provided valuable insights into microbial pathogenesis and antibiotic resistance, presenting opportunities for drug development. In 1973, it was discovered that gentamicin does not enter mammalian cells, leading to the development of the "Gentamicin Protection Assay" in the early 1980s. This assay involves infecting a layer of murine primary bone marrow macrophages with *S. aureus* expressing green fluorescent protein (GFP) and observing the clearance of extracellular GFP-positive bacteria upon gentamicin treatment. This allows for the study of *S. aureus* within macrophages, which is important as intracellular persistence is a characteristic of chronic infections. Researchers have also developed methods to investigate antibiotic resistance to generate virulent gentamicin-induced small colony variants (SCVs) of *Staphylococcus aureus*. These nonhemolytic SCVs have become a well-established experimental model for studying antibiotic resistance. Therefore, while gentamicin's use has declined due to its toxicity, the insights gained from studying its properties have opened up new avenues for developing an ideal antibiotic treatment for musculoskeletal and orthopedic infections that effectively targets intracellular pathogens (Schwarz et al., 2020).

The use of low-dose (with a median exposure of only 4 days at a daily dose of 2–3 mg/kg) gentamicin in combination with either vancomycin or antistaphylococcal penicillins for the treatment of *S. aureus* bacteremia and native valve endocarditis was associated with notable renal dysfunction (Cosgrove et al., 2009). Low-dose gentamicin usage, therefore, should be increased; however, the increased gentamicin usage causes strength values that are under standardized level in the bone cement. In our study, an additional antimicrobial agent is used to eliminate issues related to drug resistance, inadequate gentamicin doses, and strength of the bone cements.

In Table 3.1. percent inhibitions values were presented according to the OD values. All gentamicin-loaded cement samples killed all the bacteria, according to the table. It is observable that 2 g of 4-HBA liquid killed more bacteria than 2 g of 4-HBA powder. It was measured that the release of 4-HBA liquid-loaded cement samples was more efficient than the 4-HBA powder-loaded samples (Dokumaci, 2022). Chang et al. (2014) also had similar findings with the release of liquid antibiotics from bone cement samples.

Table 3.1. Percent inhibition values of MSSA after 24 and 48 hours of incubation.

Bacteria	**Bone Cement Sample**	**% Inhibition**	
		24th hour	**48th hour**
MSSA	2 g of Gentamicin Powder	99.7%	99.5%
	2 g of 4-HBA Powder	12.7%	10.2%
	2 g of Gentamicin Liquid	99.0%	98.9%
	2 g of 4-HBA Liquid	15.1%	31.4%
	1 g of Gentamicin + 1 g of 4-HBA	99.5%	99.4%
	1 g of Gentamicin + 2 g of 4-HBA	99.5%	99.5%

The viable cell counts of the MSSA at the initial inoculum, after 24 hours and 48 hours of incubation, were presented in Table 3.2. The bacterial growth in the presence of null samples was the negative control. The viable count indicated that the initial inoculum of MSSA was 10^6 CFU/mL. No growth was observed in the group that contained all gentamicin-loaded cement samples from the time zero of the experiment. This showed that gentamicin has an immediate bacteriocidal effect on MSSA. After 24 hours of

incubation, the growth of MSSA was increased by 3 logs than the initial values at the tubes that contained null, 4-HBA powder, and 4-HBA liquid bone cement samples. Then, after 48 hours, the bacterial count was increased by 1 log in null; however, it almost did not change for 4-HBA powder and slightly decreased in 4-HBA liquid samples.

Table 3.2. Viable cell counts of MSSA after incubation with bone cement samples.

Bacteria	**Bone Cement Sample**	**Viable cell counts (CFU/mL)**		
		0^{th} Hour	**24^{th} Hour**	**48^{th} Hour**
MSSA	Null	2.16×10^6	9.75×10^9	5.05×10^{10}
	2 g of Gentamicin Powder	0	0	0
	2 g of 4-HBA Powder	1.76×10^6	5.50×10^9	5.72×10^9
	2 g of Gentamicin Liquid	0	0	0
	2 g of 4-HBA Liquid	1.66×10^6	6.97×10^9	3.64×10^9
	1 g of Gentamicin and 1 g of 4-HBA	0	0	0
	1 g of Gentamicin and 2 g of 4-HBA	0	0	0

3.3.2. Antimicrobial Effects of Gentamicin and 4-Hydroxybenzoic Acid Loaded Bone Cements on MRSA

The study aimed to evaluate the antimicrobial effect of bone cement loaded with antimicrobial agents against Methicillin-Resistant *Staphylococcus aureus* (MRSA). Various combinations of gentamicin and 4-HBA were assessed in comparison to control groups to determine their capacity to inhibit bacterial growth. The antimicrobial effects of 7 days of preincubated 1 g bone cement samples were shown in Figure 3.2.

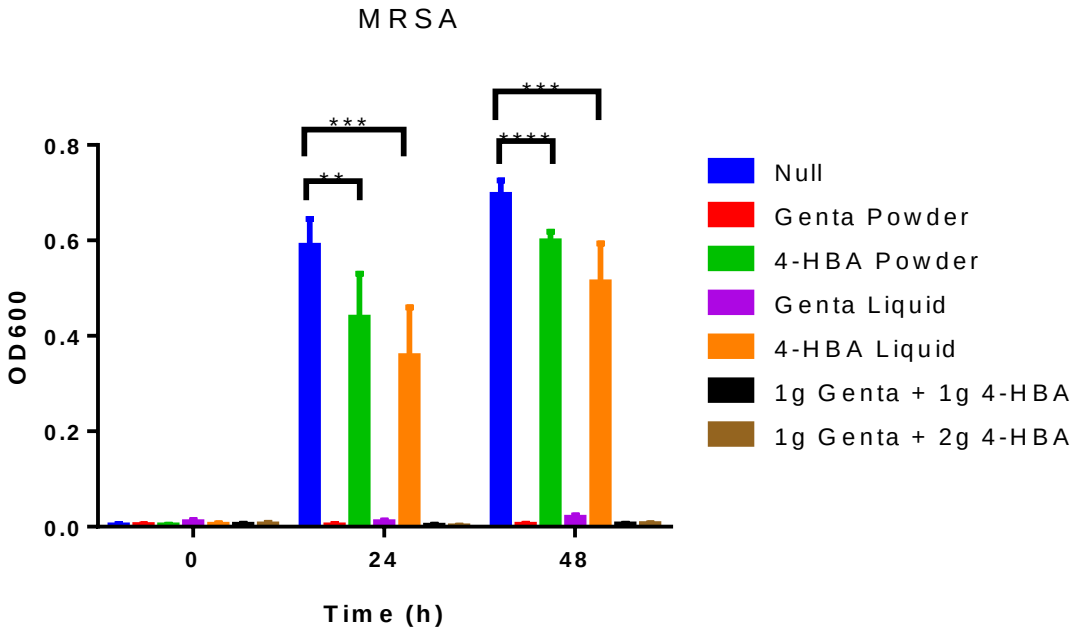

Figure 3.2. Antimicrobial effect of bone cement samples on MRSA growth. The optical densities (OD600nm) were measured at 600 nm at the 0th, 24th, and 48th hours of MRSA inoculation. "**" indicates a p-value that is equal to or smaller than 0.01, "***" indicates a p-value that is equal to or smaller than 0.001, and "****" indicates a p-value that is equal to or smaller than 0.0001, found by unpaired t-test.

In orthopedic surgeries, antibiotic-loaded PMMA bone cement is commonly used to address implant-related infections associated with bacterial biofilm formation. However, due to the development of antibiotic resistance in pathogens, Melicherčík et al. investigated the effectiveness of an antimicrobial peptide (AMP) mixed in bone cement to inhibit bacterial adhesion and biofilm formation. The study focused on methicillin-resistant MRSA biofilms on PMMA implants loaded with a 12-amino acid residue AMP. Results showed that the AMP prevented MRSA adhesion and biofilm formation on over 80% of the implants, while control implants made from plain cement did form biofilms. The study suggests that this murine osteomyelitis model has the potential to simulate actual orthopedic operations (Melicherčík et al., 2022). In our study, the same approach has been tested using 4-HBA on MRSA, and a statistically significant decrease has been reached.

The use of gentamicin-loaded bone cement for infected total hip replacements has shown a potential risk of antibiotic resistance development, with 88% of patients carrying gentamicin-resistant strains of *Staphylococcus* bacteria. Thus, a need for a product that

does not induce antibiotic resistance is crucial. The strength of bone cement must be considered when an antimicrobial agent is incorporated into the cement. A recent study demonstrated that incorporating caffeic acid phenethyl ester (CAPE) increases the cement's strength, surpassing that of gentamicin-loaded bone cement (Lee and Chang, 2015). Therefore, further research is recommended to observe the release rate and inflammatory response of 4-HBA-loaded bone cement through in *vivo* implantation tests. Additionally, *in vivo* experiments should be conducted to assess the anti-inflammatory properties of phenolic acids, which can reduce inflammation in implanted tissues by decreasing TNF-α production.

An ideal antibiotic should effectively eliminate infections while preserving the healthy microbiome and minimizing the development of antibiotic resistance. Alternative broad-spectrum antibacterial compounds (ABLCs) have shown promise in combating infections, but the optimal dosage and duration for satisfactory outcomes remain unresolved. Incorporating high doses of antibiotics into bone cement can negatively impact its strength, leading to reduced structural stability (Singh et al., 2019). Furthermore, antibiotic-loaded bone cements may also be utilized for wound healing in patients with diabetic foot osteomyelitis, as it was demonstrated the increased pathogen eradication and decreased hospital stays (Ehya et al., 2021).

Despite the declining use of gentamicin due to its associated toxicity, experimental utilization has provided valuable insights into microbial pathogenesis and antibiotic resistance. The study of gentamicin's properties has led to the development of assays and models for investigating antibiotic resistance and targeting intracellular pathogens. Low-dose gentamicin usage, in combination with other antibiotics, was associated with renal dysfunction. Therefore, incorporating an additional antimicrobial agent like 4-HBA into the bone cement can address drug resistance issues, the need for less effective gentamicin doses, and the strength of the cements.

In Figure 3.2. at the 24^{th} and 48^{th} hours, null was observed as the highest OD level. It was an expected result since null did not contain any antimicrobial agents. At the 24^{th} hour, 4-HBA powder was found to be lower than the null, and 4-HBA-liquid with a 0.358 OD600 value was found at almost half of the null with an OD value of 0.589. As in the MSSA, MRSA also showed a higher antibacterial effect in 4-HBA-liquid bone cement samples compared to 4-HBA-powder bone cement samples. This result is expected since 4-HBA-liquid-loaded bone cement samples had a higher antimicrobial agent release rate.

In Table 3.3. percent inhibition values of MRSA after 48 hours of incubation with bone cements were presented. All gentamicin-loaded cement samples showed a bacteriocidal effect on MRSA (Table 3.4). Both at 24 and 48 hours of incubation, 4-HBA-liquid-loaded bone cement samples showed higher percent inhibition than 4-HBA-powder samples. However, the viable count results displayed similar CFU/ml for both samples.

Table 3.3. Percent inhibition values of MRSA after 24 and 48 hours of incubation.

Bacteria	**Bone Cement Sample**	**% Inhibition**	
		24th hour	**48th hour**
MRSA	2 g of Gentamicin Powder	99.5%	99.4%
	2 g of 4-HBA Powder	25.6%	14.0%
	2 g of Gentamicin Liquid	98.3%	97.3%
	2 g of 4-HBA Liquid	39.2%	26.4%
	1 g of Gentamicin + 1 g of 4-HBA	99.6%	99.3%
	1 g of Gentamicin + 2 g of 4-HBA	99.8%	99.2%

Viable cell counts of all cement samples against MRSA were presented in Table 3.4. The initial inoculum was 10^5 CFU/mL in null and 4-HBA-liquid and powder-loaded bone cement samples. Both 2 g of gentamicin-powder-loaded and 2 g of gentamicin-liquid-loaded bone cement samples displayed bacteriocidal effect starting from the 0th time point. At the 24th hour null, 2 g of 4-HBA-powder and 2 g of 4-HBA-liquid bone cement samples showed approximately 3 log higher viable cell counts, similar to those of MSSA counts for the same bone cement sample groups.

Table 3.4. Viable cell counts of MRSA after incubation with bone cement samples.

Bacteria	Bone Cement Sample	Viable cell counts (CFU/mL)		
		0th Hour	**24th Hour**	**48th Hour**
MRSA	Null	1.18×10^5	4.55×10^8	1.64×10^8
	2 g of Gentamicin Powder	0	0	0
	2 g of 4-HBA Powder	4.38×10^5	5.70×10^8	1.56×10^8
	2 g of Gentamicin Liquid	0	0	0
	2 g of 4-HBA Liquid	1.36×10^5	3.47×10^8	1.55×10^8
	1 g of Gentamicin and 1 g of 4-HBA	1.17×10^1	0	0
	1 g of Gentamicin and 2 g of 4-HBA	1×10^1	0	0

3.3.3. Antimicrobial Effects of Gentamicin and 4-Hydroxybenzoic Acid Loaded Bone Cements on *S. epidermidis*

The study aimed to assess the antimicrobial efficacy of bone cement loaded with antimicrobial agents against *S. epidermidis*. Different combinations of gentamicin and 4-HBA were compared alongside control groups to evaluate their effectiveness in inhibiting bacterial growth. The antimicrobial effects of 7 days of preincubated 1 g bone cement samples were shown in Figure 3.3.

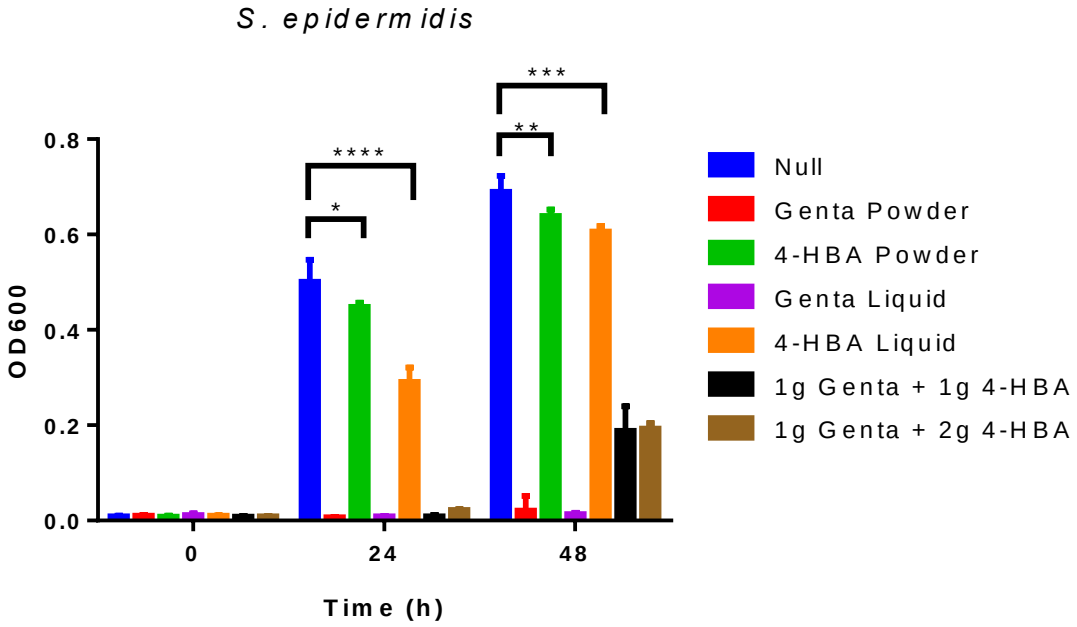

Figure 3.3. Antimicrobial effect of bone cement samples on *S. epidermidis* growth. The optical densities (OD600nm) were measured at 600 nm at the 0th, 24th, and 48th hours of *S. epidermidis* inoculation. "*" indicates a p-value equal to or smaller than 0.05, "**" indicates a p-value equal to or smaller than 0.01, "***" indicates a p-value equal to or smaller than 0.001, and "****" indicates a p-value equal to or smaller than 0.0001, found by unpaired t-test.

Periprosthetic joint infections (PJI) are challenging to treat due to biofilm formation on implant surfaces. This in vitro study investigated the efficacy of methylene blue photodynamic therapy (MB-PDT) on PJI-causing biofilms on various implant materials. The study tested different strains of bacteria in planktonic form and with early and mature biofilms on prosthetic materials such as polyethylene, titanium alloys, cobalt-chrome-based alloys, and bone cement. MB-PDT effectively eliminated common PJI pathogens in both the planktonic bacteria and the biofilms, regardless of the strain, orthopedic material, or biofilm maturity. The treatment did not cause relevant surface modifications to the implants. These findings suggest that MB-PDT could be a promising treatment approach for PJI, potentially replacing invasive methods and reducing the duration of antibiotic treatment, thereby improving patient quality of life and reducing associated healthcare costs (Prinz et al., 2023). Since implant-related infections are an overwhelming issue, new treatment approaches are gaining valuable insights into this field. Combining these approaches with natural products would promise a great strategy against biofilm formation on implant surfaces.

A recent study investigated the pharmacological properties of *Solenostemma argel*, a desert medicinal plant native to African countries. The research focuses on analyzing the antibacterial, antioxidant, anticancer, and anti-inflammatory activities of the aerial parts (leaves and flowers) of *Solenostemma argel* (Delile) Hayane. Additionally, the study characterized the phenolic and flavonoid contents of the plant. The antioxidant activity of *Solenostemma argel* extract shows an increase in effectiveness, ranging from 12.16% to 94.37%, as the concentration of the extract is raised from 10 μg/mL to 1280 μg/mL. Among the tested organisms, *S. epidermidis* demonstrates the highest sensitivity to chloroform extract (Elsanhoty et al., 2022). As it is parallel with the literature, in this study, 4-HBA-loaded bone cement samples showed a significant decline in the growth of the bacteria with a 1 log difference, as shown in Table 3.6.

The current treatment approach for traumatic wounds, like those caused by gunshot injuries, involves using gauze and tourniquets. However, these dressings are inadequate for wounds that cannot be compressed and do not provide sufficient protection against infections, leading to high morbidity and mortality rates. To address this critical clinical need, a recent study developed a foam made of shape memory polymers (SMP) with antimicrobial and antioxidant properties. The shape memory properties of the foam allow for easy application onto deep and irregularly shaped wounds. Moreover, the presence of antimicrobial polyphenols, specifically 4-HBA, within the foam provides effective defense against common wound pathogens such as *E. coli*, *S. aureus*, and *S. epidermidis* (Du et al., 2022). The findings of this recent study align with the conclusions reached in this chapter.

Gentamicin, an aminoglycoside antibiotic, is commonly employed in orthopedic surgery due to its favorable bacteriological and physico-chemical properties for local infection treatment. In a study, the growth of *S. epidermidis* biofilm was observed to increase slime production on the biomaterials after 3 hours of exposure to gentamicin (as shown in Figure 5). This aligns with previous findings indicating that antibiotics can induce slime production. Slime production serves as a protective response by bacteria, enabling them to endure unfavorable conditions, including the presence of antibiotics. Furthermore, embedding biofilms within slime is recognized as a significant cause of failure in treating or preventing biofilm-associated infections (BAI) with antibiotics. Evidence suggests that the binding of positively charged aminoglycosides to slime hampers the penetration of these agents (Nuryastuti et al., 2010). This study explains why

the immediate effect of gentamicin-loaded bone cements was not observable in the *S. epidermidis* cultures as it was found in MSSA and MRSA. Additionally, the study provides information about the inadequate effect of gentamicin on the dispersal of biofilm of the bacteria, which suggests the urgent need for the usage of new antimicrobial agents.

In the Figure 3.3. null was found with the highest OD value at both the 24th hour and the 48th hour. 4-HBA powder follows null and can be seen lower than the null at both the 24th hour and 48th hour in the graph. 4-HBA-liquid was significantly lower than the null, according to the graph. Combinations, which include 1 g of gentamicin other than 2 g of gentamicin powder and liquid, did not kill all the bacteria. This suggests that gentamicin does not affect *S. epidermidis* as much as it does MSSA and MRSA. An interesting result was found also in the combined effects of 4-HBA and gentamicin. 1 g of gentamicin and 1 g of 4-HBA-loaded cement samples (0.188 OD600 value) resulted in almost the same killing rate as 1 g of gentamicin and 2 g of 4-HBA-loaded cement samples (0.193 OD600 value). This was not an expected result since 4-HBA was found to be effective on MSSA and MRSA; an increased amount of it should have led to the increased killing rate. Since *S. epidermidis* is a biofilm-producing bacteria, 4-HBA and gentamicin could not affect the bacteria as they happen in MSSA and MRSA (Ammann et al., 2017). Finally, gentamicin-powder-loaded bone cement samples and gentamicin-liquid-loaded bone cement samples containing 2 grams killed most of the bacteria at both time points.

Table 3.5. shows percent inhibition values of *S. epidermidis* after 48 hours of bone cement treatment; 2 g of gentamicin powder killed almost all the bacteria as indicated as 98.6% and 97.0% of inhibition values at the 24th and 48th hours, respectively. A similar result is observable in 2 g of gentamicin-liquid-loaded bone cements since 98.3% and 98.0% of inhibitions were found at the 24th and 48th hour time points, respectively. 2 g of 4-HBA powder-loaded bone cements show a significant change in the term of inhibition. However, 2 g of 4-HBA-liquid-loaded bone cement killed 41.9% of the bacteria at the 24th hour. Since the result hasn't been reached at the 48th hour, where 12.1% inhibition was observed, it cannot be said that 4-HBA leads to a very significant inhibition rate on *S. epidermidis*.

Table 3.5. Percent inhibition values of *S. epidermidis* after 24 and 48 hours of incubation.

Bacteria	**Bone Cement Sample**	**% Inhibition**	
		24th hour	**48th hour**
S. epidermidis	2 g of Gentamicin Powder	98.6%	97.0%
	2 g of 4-HBA Powder	10.5%	7.36%
	2 g of Gentamicin Liquid	98.3%	98.0%
	2 g of 4-HBA Liquid	41.9%	12.1%
	1 g of Gentamicin + 1 g of 4-HBA	98.2%	72.7%
	1 g of Gentamicin + 2 g of 4-HBA	95.5%	72.0%

Viable cell counts of *S. epidermidis* were indicated in the table after the bacterial load at the initial time point and after the 24th and 48th hours in Table 3.6. The bacterial growth in the presence of null bone cement discs was negative control. The initial inoculum of *S. epidermidis* was 10^5 CFU/mL, and the initial spread was performed after inoculation. At the initial (0th) time point, no immediate effect of gentamicin was observed when starting with a bacterial concentration of 10^5 CFU/mL in all the bone cement samples. However, the immediate effect of gentamicin had been observed on MSSA and MRSA in the previous results. Growth of null, 2 g of 4-HBA powder, and 2 g of 4-HBA liquid increased 1000 times at the 24th hour. An increase is observable at the 48th hour; however, 4-HBA exhibits lower growth compared to the control (null). Additionally, 4-HBA liquid shows less viable cell counts than 4-HBA powder because of the release level explained before. 2 g of gentamicin-loaded bone cements resulted in a higher killing rate than the combinations loaded ones. The reason for the result could be less gentamicin amount (1 g) in combinations than gentamicin alone loaded bone cements (2g). Additionally, combinations showed less growth than only 4-HBA-loaded cements; this indicated that 4-HBA decreased the growth slightly.

Table 3.6. Viable cell counts of *S. epidermidis* after incubation with bone cement samples.

Bacteria	Bone Cement Sample	Viable cell counts (CFU/mL)		
		0th Hour	**24th Hour**	**48th Hour**
S. epidermidis	Null	5.9×10^5	8.27×10^8	1.81×10^9
	2 g of Gentamicin Powder	7.45×10^5	1.3×10^3	1.50×10^1
	2 g of 4-HBA Powder	7.07×10^5	1.83×10^8	9.05×10^8
	2 g of Gentamicin Liquid	7.38×10^5	8.80×10^2	2.05×10^2
	2 g of 4-HBA Liquid	9.43×10^5	1.61×10^8	2.63×10^8
	1 g of Gentamicin and 1 g of 4-HBA	8.46×10^5	9.1×10^6	2.26×10^8
	1 g of Gentamicin and 2 g of 4-HBA	9.20×10^5	1.10×10^7	2.00×10^8

3.3.4. Antimicrobial Effects of Gentamicin and 4-Hydroxybenzoic Acid Loaded Bone Cements on *E. faecium*

The study aimed to assess the antimicrobial efficacy of bone cement loaded with antimicrobial agents against *E. faecium*. Different combinations of gentamicin and 4-HBA were compared alongside control groups to evaluate their effectiveness in inhibiting bacterial growth. The antimicrobial effects of 7 days of preincubated 1 g bone cement samples were shown in Figure 3.4.

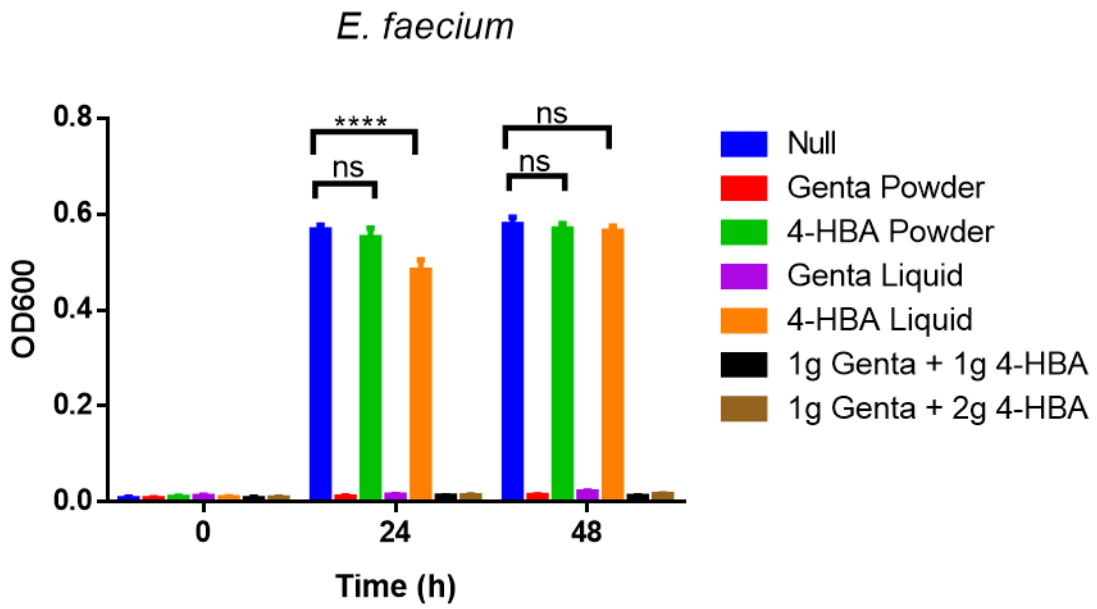

Figure 3.4. Antimicrobial effect of bone cement samples on *E. faecium* growth. The optical densities (OD600nm) were measured at 600 nm at the 0th, 24th, and 48th hours of *E. faecium* inoculation. "ns" indicates a p-value higher than 0.05, and "****" indicates a p-value equal to or smaller than 0.0001, found by unpaired t-test.

In the management of Enterococcal infections, the administration of both gentamicin and either a beta-lactam antibiotic or a glycopeptide is employed to achieve a synergistic bactericidal impact. Nevertheless, strains that display significant resistance to gentamicin have lost their susceptibility to this combined therapeutic approach (Kaçmaz and Aksoy, 2005). Therefore, generating new strategies to combat *E. faecium*-derived infections is urgent. However, no significant decrease in the bacterial growth rate was found after 4-HBA-loaded bone cement treatment. However, as mentioned before, higher doses of 4-HBA would be investigated further.

In a recent study on *Enterococcus faecium*, a common cause of hospital-acquired infections, researchers analyzed antibiotic-resistant genomes, identifying potential drug targets from core genes. Functional analysis revealed metabolic pathways, and subtractive genomics identified 26 unique therapeutic targets. 3-dehydroquinate dehydrogenase was proposed as a promising target. Virtual screening with natural compounds found potential inhibitors. Experimental validation is required for these novel therapeutics (Khan and Uddin, 2023). For our study, 4-HBA's higher doses would be further tested on the bacteria, as mentioned above.

Null was found with the highest OD values at the 24th and 48th hours. All the gentamicin-loaded bone cements, including combinations, killed most of the bacteria at the 24th and 48th hours (Figure 3.4). A significant decrease was found in using 4-HBA liquid and powder-loaded bone cements in the 24th hour. However, compared to null, slightly decreased bacterial growth in 4-HBA powder and liquid-loaded bone cement samples was observed at the 48th hour. This data indicates no significant changes occurred because of 4-HBA addition in *E. faecium* growth.

In the table 3.7. indicating percent inhibition values of *E. faecium*, 2 g of gentamicin powder-loaded bone cements show more than 90% inhibition rate as found in the other bacteria (MSSA, MRSA, *S. epidermidis*) in all time points. 4-HBA liquid-loaded bone cements showed more inhibition percentage than 4-HBA powder-loaded bone cements in both the 24th and 48th hours of time points. However, no significant inhibition rate was reached overall by 4-HBA on *E. faecium*.

Table 3.7. Percent inhibition values of *E. faecium* after 24 and 48 hours of incubation.

Bacteria	**Bone Cement Sample**	**% Inhibition**	
		24th hour	**48th hour**
E. faecium	2 g of Gentamicin Powder	98.2%	99.6%
	2 g of 4-HBA Powder	2.79%	1.60%
	2 g of Gentamicin Liquid	97.4%	96.3%
	2 g of 4-HBA Liquid	14.8%	2.47%
	1 g of Gentamicin + 1 g of 4-HBA	97.8%	97.9%
	1 g of Gentamicin + 2 g of 4-HBA	97.7%	97.4%

Viable cell counts of *E. faecium* were indicated in the table after the bacterial load at the initial time point and after the 24th and 48th hours in the Table... The bacterial growth in the presence of null bone cement discs was negative control. The initial inoculum of E. faecium was 10^6 CFU/mL, and the initial spread was performed after inoculation. Growth of null, 2 g of 4-HBA powder, and 2 g of 4-HBA liquid increased 100 times at the 24th hour. Additionally, 4-HBA liquid shows less viable cell counts than 4-HBA powder because of the release level explained before. 2 g of gentamicin-loaded

bone cements resulted in a higher cell death rate than the combinations loaded ones. The reason for the result could be less gentamicin amount (1 g) in combinations than gentamicin alone loaded cements (2g). Additionally, cement samples containing combinations with gentamicin showed less growth than only 4-HBA-loaded cements; this indicated that 4-HBA decreases the growth slightly.

Table 3.8. Viable cell counts of *E. faecium* after incubation with bone cement samples.

Bacteria	**Bone Cement Sample**	**Viable cell counts (CFU/mL)**		
		0th Hour	**24th Hour**	**48th Hour**
E. faecium	Null	1.55×10^6	2.38×10^8	2.34×10^8
	2 g of Gentamicin Powder	1.01×10^4	0	0
	2 g of 4-HBA Powder	1.70×10^6	1.15×10^8	5.17×10^8
	2 g of Gentamicin Liquid	1.55×10^4	0	0
	2 g of 4-HBA Liquid	1.66×10^6	2.55×10^8	5.00×10^8
	1 g of Gentamicin and 1 g of 4-HBA	7.53×10^4	0	0
	1 g of Gentamicin and 2 g of 4-HBA	9.67×10^4	0	0

3.3.5. Antimicrobial Effects of Gentamicin and 4-Hydroxybenzoic Acid Loaded Bone Cements on *P. aeruginosa*

The study aimed to assess the antimicrobial efficacy of bone cement loaded with antimicrobial agents against *P. aeruginosa*. Different combinations of gentamicin and 4-HBA were compared alongside control groups to evaluate their effectiveness in inhibiting bacterial growth (Figure 3.5).

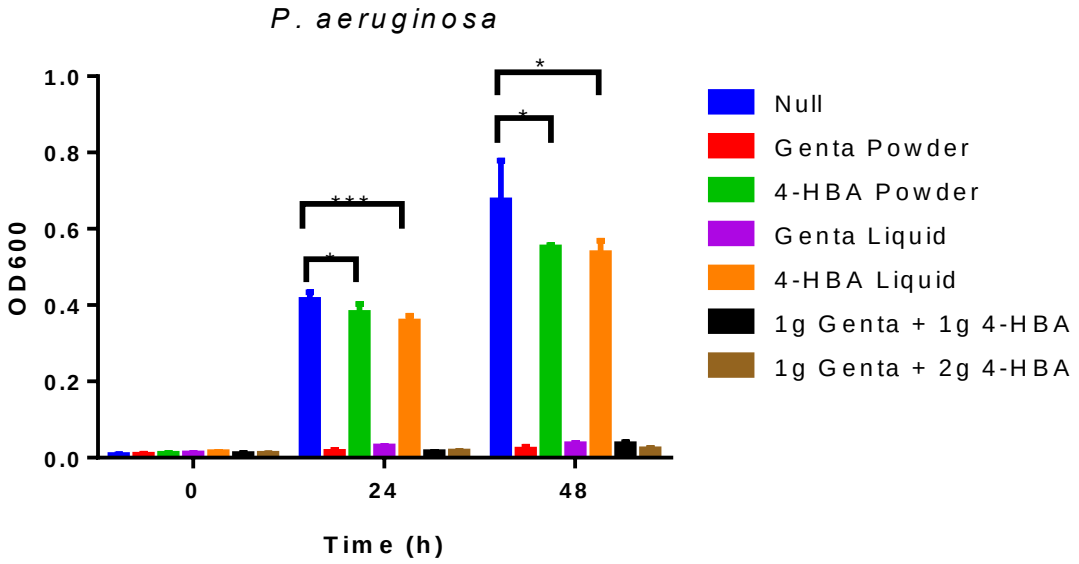

Figure 3.5. Antimicrobial effect of bone cement samples on *P. aeruginosa* growth. The optical densities (OD600nm) were measured at 600 nm at the 0th, 24th, and 48th hours of *P. aeruginosa* inoculation. "*" indicates a p-value equal to or smaller than 0.05, and "***" indicates a p-value equal to or smaller than 0.001, found by unpaired t-test.

A recent study evaluated the addition of various antibiotics (tobramycin, meropenem, piperacillin, ceftazidime, ciprofloxacin, and aztreonam) to acrylic cement for preventing skeletal infections caused by gram-negative bacteria. The researchers conducted both in vitro and animal studies. They found that meropenem-loaded cement had the most stable and effective antimicrobial properties against gram-negative bacteria in vitro. In the animal study using a rat model, meropenem-loaded cement demonstrated improved body weight, reduced inflammation, and better radiological and histopathological outcomes compared to control unloaded cement. The study suggests that meropenem-loaded acrylic cement could be a potentially effective preventive measure against skeletal infections caused by gram-negative bacteria, but further clinical research is required to assess its safety and efficacy in humans (Wei et al., 2022)

Amikacin, gentamicin, and tobramycin are commonly used aminoglycosides for the treatment of *Pseudomonas aeruginosa* infections. The primary resistance mechanism involves the inactivation of these antibiotics by aminoglycoside-modifying enzymes. These enzymes reduce the binding affinity of aminoglycosides to the target subunit 30S of the bacterial ribosome, thereby impairing their effectiveness. Amikacin is relatively

less susceptible to this resistance mechanism among the three aminoglycosides. However, it is important to note that these antibiotics should not be used alone as monotherapy for infections beyond the urinary tract. In addition to the intravenous formulation, alternative forms of administration have demonstrated efficacy, such as inhaled tobramycin, which was effective in managing acute exacerbations of cystic fibrosis. Plazomicin, a novel aminoglycoside agent, is ineffective against resistance mechanisms such as altered membrane permeability and other aminoglycoside resistance mechanisms. Therefore, its usage is limited to treating *P. aeruginosa* urinary tract infections (Karruli et al., 2023).

Microbial biofilms are communities of cells that adhere to surfaces and form a protective matrix of extracellular polymeric substances. The increased use of antibiotics to treat infections associated with biofilms has led to the emergence of multiple drug-resistant strains. *Pseudomonas aeruginosa* is a well-known pathogenic bacterium that forms biofilms and is a model for studying biofilm-related infections. Vitexin, a polyphenolic phytochemical with antimicrobial properties, was investigated for its potential as an antibiofilm agent against *Pseudomonas aeruginosa* when combined with azithromycin and gentamicin. The minimum inhibitory concentration (MIC) of vitexin was found to be 260 μg/ml. Its antibiofilm activity was assessed through safranin staining, protein extraction, microscopy techniques, extracellular polymeric substances (EPS) quantification, and in vivo models using sub-MIC doses. The results demonstrated a significant reduction in biofilm formation and QS-mediated phenotypes of *Pseudomonas aeruginosa* in 110 μg/ml vitexin combined with azithromycin and gentamicin separately. This study highlights the potential of vitexin as a novel antibiofilm agent against microbial biofilm-associated pathogenicity, specifically targeting *Pseudomonas aeruginosa* (Das et al., 2016). A Similar study was conducted in this thesis; firstly, a combination of gentamicin and 4-HBA-loaded bone cements was tested. Afterward, the effects of 4-HBA on the biofilm formation of *P. aeruginosa* were examined.

A recent study revealed that the trans-translation system is crucial for *Pseudomonas aeruginosa*'s ability to tolerate both azithromycin and various aminoglycoside antibiotics. Additionally, they demonstrated that gentamicin can inhibit the trans-translation activation induced by azithromycin. When combined, gentamicin and azithromycin exhibited increased efficacy in killing both planktonic and biofilm-associated *P. aeruginosa* cells. This effect was observed in different strains, including the reference strain PA14, its carbapenem-resistant oprD mutant, the mucoid strain FRD1,

and multiple clinical isolates. Furthermore, combining gentamicin and azithromycin improved bacterial clearance in murine models of acute pneumonia, biofilm implant, and cutaneous abscess infection (Ren et al., 2019). In the previous chapter, the effect of 4-HBA on *P. aeruginosa* was described by mostly leading defects in protein translation machinery. Therefore, using 4-HBA with gentamicin could potentially decrease bacteria's tolerance to it. Although the mode of action of 4-HBA was not explicitly identified, it can be seen in Figure 3.5 that in combination with bone cements, increasing the 4-HBA amount in bone cement increases the elimination of the bacteria at the 48th hour.

In Figure 3.5. null was found with the highest OD value at the 24th and 48th hours. All the gentamicin-loaded, including combinations and bone cements, killed all the bacteria at the 24th and 48th hours. Significantly decreased bacterial growth provided by 4-HBA powder and liquid-loaded bone cements was observed at the 24th hour, and 4-HBA liquid cements caused a significantly decreased growth rate than 4-HBA powder-loaded cements. At the 48th hour, both 4-HBA liquid and powder-loaded bone cements significantly decreased when compared with the null. Additionally, at both the 24th and 48th hours, 4-HBA liquid shows the highest decrease in the growth of *P. aeruginosa*. According to the data, it was concluded that 4-HBA is significantly effective on the bacterium.

In Table 3.9., it was shown that 2 g of gentamicin liquid bone cements has a higher inhibition percentage than 2 g of gentamicin powder bone cements at all time points. It is observable that 2 g of 4-HBA liquid-loaded bone cements indicated higher inhibition rates than 2 g of 4-HBA powder-loaded cements at all time points. The same result was also repeated by the data of a combination of 1 g of gentamicin and 2 g of 4-HBA-loaded cements having a higher inhibition rate than 1 g of gentamicin and 1 g of 4-HBA-loaded bone cements.

Table 3.9. Percent inhibition values of *P. aeruginosa* after 24 and 48 hours of incubation.

Bacteria	**Bone Cement Sample**	**% Inhibition**	
		24th hour	**48th hour**
P. aeruginosa	2 g of Gentamicin Powder	96.2%	96.8%
	2 g of 4-HBA Powder	8.28%	18.9%
	2 g of Gentamicin Liquid	92.9%	94.7%
	2 g of 4-HBA Liquid	13.8%	20.4%
	1 g of Gentamicin + 1 g of 4-HBA	96.5%	94.7%
	1 g of Gentamicin + 2 g of 4-HBA	96.1%	96.7%

Viable cell counts of *P. aeruginosa* have been indicated after the bacterial load at the initial point and after the 24th and 48th hours in Table 3.10. The bacterial growth in the presence of null bone cement discs was negative control. The initial inoculum of *P. aeruginosa* was 10^7 CFU/mL, and the initial spread was performed after inoculation. Growth of null, 2 g of 4-HBA powder, and 2 g of 4-HBA liquid increased 100 times at the 24th hour. At the 48th hour, 4-HBA powder-loaded bone cements caused 1 log(10) reduction, and 4-HBA liquid-loaded bone cements caused 2 log (10) reduction. Additionally, 4-HBA liquid cements showed less viable cell counts than 4-HBA powder at the 48th hour because of the release level explained before.

Table 3.10. Viable cell counts of *P. aeruginosa* after incubation with bone cement samples.

Bacteria	**Bone Cement Sample**	**Viable cell counts (CFU/mL)**		
		0th Hour	**24th Hour**	**48th Hour**
P. aeruginosa	Null	1.17×10^7	1.33×10^8	4.90×10^{10}
	2 g of Gentamicin Powder	1.67×10^1	0	0
	2 g of 4-HBA Powder	1.82×10^7	1.12×10^8	3.70×10^9
	2 g of Gentamicin Liquid	1.67×10^1	0	0
	2 g of 4-HBA Liquid	1.44×10^7	2.63×10^8	5.80×10^8
	1 g of Gentamicin and 1 g of 4-HBA	3.33×10^1	0	0
	1 g of Gentamicin and 2 g of 4-HBA	6.66×10^1	0	0

3.4. Conclusions

The aim of this chapter was to observe the effect of 4-HBA-loaded bone cements on MSSA, MRSA, *S. epidermidis*, *E. faecium,* and *P. aeruginosa* for the first time. Another aim was to compare the effect of powder and liquid-loaded bone cements on the bacteria. Additionally, gentamicin was used as both a control and to observe a synergistic effect with 4-HBA.

Polymethyl methacrylate (PMMA) is a bone cement widely used in orthopedic surgeries. It acts as a space-filling material, securing implants through mechanical interlock. The incidence of complications, including infection and implant loosening, emphasizes the need for ongoing research. Antibiotic-loaded bone cement has shown efficacy in inhibition of infections. Statistics project around 635,000 primary total hip arthroplasties (THAs) and 935,000 total knee arthroplasties (TKAs) in the US by 2030. Nearly 50% of revision procedures were accounted for in prosthetic joint infection (PJI). In summary, PMMA bone cement plays a crucial role in orthopedics, with ongoing research addressing complications and antibiotic-loaded cement providing localized drug delivery for infection prevention. Our study found that after 48 hours of incubation, 4-HBA liquid-loaded bone cements delivered more than 20% inhibition rate on MSSA, MRSA, and *P. aeruginosa*. Although gentamicin provided a much higher killing rate of more than 90%, synergistic effects of 4-HBA and gentamicin evaluated that increasing 4-HBA amount caused more killing rate on *P. aeruginosa*. Based on these results, it can be concluded that where antimicrobial resistance occurs, phenolic acids could be the second line of defense. A recent study stated that experimental models and clinical data indicate that achieving high local concentrations of antimicrobials is crucial for maximizing prophylactic and therapeutic efficacy. It is essential to assess novel carriers and diverse antimicrobial agents to enhance clinical outcomes (Sabater-Martos et al., 2023). Since antimicrobial resistance is a growing problem worldwide, the usage of phenolic acids has become a need. 4-hydroxybenzoic acid is a phenolic acid used for the first time for loading bone cements to observe the synergistic effect of gentamicin on several different bacteria. According to our results, a significant decline in the growth of three of the bacteria was observed. Therefore, it can be concluded that the phenolic acid 4-HBA is a promising molecule that can be used to eliminate bacterial growth using gentamicin.

CHAPTER 4

EFFECTS OF 4-HYDROXYBENZOIC ACID ON BIOFILM OF *Pseudomonas aeruginosa*

4.1. Introduction

A biofilm can be described as a well-organized population of microorganisms, predominantly bacteria, which adhere to a particular surface and are encompassed by a matrix of substances they produce externally. Biofilms are found in various environments, including medical devices, natural habitats, and industrial systems (Muhammad et al., 2020).

The formation of biofilms is a multifaceted procedure comprising the initial adherence of individual bacteria to a surface and subsequent synthesis of extracellular polymeric substances (EPS) that construct the matrix. This EPS matrix grants the bacteria both physical and metabolic shelter, rendering them more resilient to antibiotics, immune responses, and environmental pressures when compared to bacteria existing in a planktonic state (freely floating). (Hall-Stoodley et al., 2004).

Biofilms play a significant role in many important biological processes, including nutrient cycling and energy transfer in ecosystems, and they can cause significant problems in various industrial, medical, and environmental applications. For example, biofilms can cause corrosion and fouling of pipes and heat exchangers in industrial systems, infections associated with medical devices such as catheters, and persistent infections in individuals with weakened immune systems (Sharma et al., 2019).

Understanding the mechanisms of biofilm formation and developing strategies to prevent and control biofilm growth are important in various fields, including medicine, environmental science, and engineering.

4.1.1. Biofilm of *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is an opportunistic bacterium commonly found in the environment, including soil and water. One of its unique characteristics is its ability to form biofilms, which are communities of microorganisms attached to a surface and surrounded by a self-produced extracellular matrix (Tuon et al., 2022).

In the case of *P. aeruginosa*, biofilm formation is a common mode of growth, and it can play a significant role in the persistence and resistance of this bacterium to antibiotics and host defenses. *P. aeruginosa* biofilms are frequently associated with chronic infections, particularly in individuals with weakened immune systems, and are often difficult to treat. The biofilm structure provides physical and metabolic protection for the bacteria, making it more resistant to antibiotics and host defenses compared to planktonic (free-floating) bacteria (Tuon et al., 2022).

Biofilm formation by *P. aeruginosa* can occur in various environments, including medical devices such as catheters, artificial joints, and contact lenses, as well as in natural environments like water and soil. Understanding the mechanisms of biofilm formation by *P. aeruginosa* is important in developing strategies to prevent and treat infections associated with this bacterium.

4.1.1.1. *Pseudomonas aeruginosa* biofilms and resistance

Pseudomonas aeruginosa, a well-established model organism in biofilm research, exhibits distinct attributes compared to its planktonic counterparts. These characteristics include heightened resistance to antimicrobial agents and the ability to evade the host's immune system. (Costerton et al., 1995). The bacteria can be found in both planktonic and biofilm forms. Cells surrounded by an extracellular polymeric (EPS) matrix are within the biofilm of *P. aeruginosa*. A biofilm matrix consists of proteins, polysaccharides, lipids, as well as nucleic acids (specifically extracellular DNA or eDNA and RNA), and biosurfactants. These components play crucial roles in facilitating the initial adhesion of cells and establishing the structural framework of the biofilm. In the case of *P. aeruginosa*, the production of the matrix occurs during the later stages of biofilm development, leading to a significant degree of autoaggregation among the cells.

(Friedman and Kolter et al., 2004). *P. aeruginosa* produces Psl (polysaccharide), Pel (polysaccharide), alginate, and exopolysaccharides that are involved in biofilm formation (Ryder, Byrd, and Wozniak et al., 2007). Cyclic di-GMP, a specific nucleotide signaling molecule known as a bacterial second messenger, serves as the primary regulator of extracellular polymeric substance (EPS) production. Moreover, it enables bacterial cells to transition from a motile state to a non-motile (sessile) state and acts as a regulator for the adherence of cells within biofilms. (Jenal and Malone, 2006). *P. aeruginosa* biofilm formation involves four primary stages: initial attachment to a surface (either living or non-living), subsequent proliferation, formation of microcolonies, and maturation into a well-organized and resilient microbial community. (Whiteley et al., 2001). Biofilms offer an alternative mechanism for evading antimicrobial agents, thus rendering the resulting infections more challenging to treat. (Ghadaksaz et al., 2015).

4.1.1.2. *Pseudomonas aeruginosa* biofilms confer resistance to antimicrobials

The exopolysaccharide matrix of the biofilm functions as a barrier that hinders the diffusion of antimicrobial agents, effectively immobilizing antibiotics. (Drenkard et al., 2003). Moreover, the diffusion barriers within the biofilm also affect nutrient production. This reduces the growth rate and metabolic activity, subsequently promoting the accumulation of more persistent cells. (Sultana, Call and Beyenal, 2016). The growth patterns of bacterial cells within the biofilm vary depending on the varying levels of oxygen and nutrient availability across different regions. Surface-dwelling bacterial cells within the biofilm display higher metabolic activity, while cells in the inner layers grow at a slower pace. Antibiotics exhibit differential efficacy in distinct biofilm regions, with certain drugs like polymyxins targeting cells under unfavorable growth conditions, whereas beta-lactams and aminoglycosides tend to eliminate actively growing cells. (Drenkard et al., 2003). When antibiotics are administered at low concentrations, they effectively eliminate a majority of the *P. aeruginosa* biofilm cells. However, increasing the antibiotic concentration does not eradicate the persistent cells within the biofilm. Consequently, researchers have determined that only a small portion of biofilm cells are accountable for the heightened antibiotic resistance observed. Similar to planktonic cells, the majority of biofilm cells are susceptible to the effects of antibiotics. (Brooun, Liu, and

Lewis, 2000). Since the presence of LPS prevents penetration of antibiotics, Gram-negative bacteria show more resistance to antibiotics (Sultana, Call, and Beyenal, 2016).

Bacterial cells within the *P. aeruginosa* biofilm exhibit a range of genetic, metabolic, physiological, and phenotypic alterations. These changes manifest in the form of diverse colony characteristics, including dwarfed size, mucoid appearance, increased pilus formation (hyperpiliation), deficiency in lipopolysaccharides (LPS), rough texture, wrinkled morphology, and antibiotic resistance. (Webb, Lau and Kjelleberg, 2004). Overproduction of Exopolysaccharide Pel ends up with wrinkled colonies; meanwhile, alginate overproduction leads to finger-like colonies. Additionally, the generation of alginate causes an increase in biofilm resistance to the human defense system and antibiotics (Ghafoor, Hay, and Rehm, 2011). Cystic fibrosis (CF) is an autosomal recessive disorder primarily attributed to cystic fibrosis transmembrane conductance regulator (CFTR) gene mutations, resulting in impaired chloride ion channel function. In CF patients, *P. aeruginosa* biofilms tend to develop in the lungs, and these biofilms exhibit resistance to antibiotic therapies. They are not effectively targeted by the host's inflammatory response involving polymorphonuclear leukocytes (PMNLs). CF can lead to severe consequences, including mortality, the necessity for lung transplantation, or respiratory failure. (Høiby, Ciofu and Bjarnsholt, 2010). During the progression of infection, a specific subset of phenotypes is favored in *P. aeruginosa* strains that invade the lungs of cystic fibrosis (CF) patients. These selected phenotypic variants exhibit characteristics such as being nonmotile and mucoid. They possess a rough lipopolysaccharide (LPS) texture and produce excessive amounts of alginate, resulting in a mucoid appearance. (Garrett, Perlegas and Wozniak, 1999).

In this chapter, the antibiofilm effect of 4-HBA was evaluated in the biofilm of *P. aeruginosa*. The aim of the chapter is to understand the impact of 4-HBA on the inhibition of biofilm formation in *P. aeruginosa*. The hypothesis was that since 4-HBA could inhibit *P. aeruginosa* growth, the biofilm of the bacteria could be inhibited by the phenolic acid 4-HBA, as well.

4.2. Materials and Methods

In this study, *Pseudomonas aeruginosa* (ATCC 27853) was utilized to examine the antibiofilm activity of phenolic acid 4-hydroxybenzoic acid by culture colors, crystal

violet assay, and scanning electron microscopy (SEM). Culture conditions and bacteria maintenance were conducted, as mentioned in Chapter 2.

4.2.1. Culture Colors

Subinhibitory 4-HBA concentrations that were investigated, as mentioned previously in Chapter 2 were applied to observe the difference in culture color in the presence of 4-HBA.

Overnight *P. aeruginosa* culture was inoculated to a total volume of 4 ml TSB culture, including 1.6 mg/mL of 4-HBA as 10^6 CFU/mL. The same number of bacteria were inoculated in the total volume of TSB culture as control. Then, the images of the cultures in 0, 1, 2, 4, 6, 12, and 24 hours of intervals were taken.

4.2.2. Crystal Violet (CV) Assay

Overnight *P. aeruginosa* culture was diluted to 10^6 CFU/ml in TSB medium. CV assay was then carried out according to O'Toole et al., 2011 with minor modifications for both control and 1.6 mg/ml 4-HBA added samples. 100 µl of the standardized inoculum was added to each well of 96-well plates. Then, incubation was conducted for 0, 4, 8, 12, 24, and 36 hours at 37 °C, individually. At each incubation time point, the culture media was removed, and wells were washed with distilled water twice to remove planktonic cells. Biofilms were air-dried for 15 minutes at 37 °C. Then, 125 µl of 0.1% (w/v) crystal violet solution was added to the wells. After 15 minutes, the excess stain was removed by extensive washing with water. Then, de-staining was conducted by adding 150 µl of 30% (v/v) acetic acid to the wells, and the plate was then incubated for 15 minutes at room temperature. Finally, 125 µl of 30% (v/v) acetic acid was gently pipetted for 1 minute to release bound crystal violet completely. The acetic acid was transferred into a new 96 well-plate, and the absorbance was quantified at 570 nm at spectrophotometry. 125 µl of 30% of acetic acid was used as blank control. The quantity of biofilm biomass is proportional to absorbance values.

4.2.3. Scanning Electron Microscopy

For scanning electron microscopy (SEM) imaging, a single colony of the bacteria was incubated for 18 hours at 4 mL of TSB. The next day, 1.6 mg/mL of 4-HBA treatment was conducted for one group, and the other group remained as control. The treatment was applied for 10^1, $10^{3,}$ and 10^6 CFU/mL of bacteria. Biofilms were grown for 18 hours on half of the autoclave-sterilized, 0.2-µm type 25 mm membrane filters (Gelman Sciences) in glass petri plates. After 18 hours of biofilm formation, membrane filters containing biofilms from treated and untreated groups were collected under aseptic conditions. The collected membrane filters were then fixed overnight using a solution containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Subsequently, the filters were rinsed three times for 10 minutes each with 0.1 M phosphate buffer. To gradually dehydrate the membranes, they were washed sequentially with alcohol solutions of increasing concentrations: 10%, 30%, 50%, 70%, and 95% for 10 minutes each, followed by three sets of 100% alcohol for 10 minutes each. The membranes were then subjected to overnight drying using hexamethyldisilizane (HMDS). Before visualization, the membrane filters were sputter-coated with gold for 90 seconds. Microscopic images were captured using a Phillips XL-30S FEG SEM and FEI Quanta 250 FEG. The SEM scanning was performed at an accelerating voltage of 10 kV.

4.3. Results and Discussion

Biofilm of *P. aeruginosa* was observed phenotypically using photos of culture colors, by measuring quantitatively using crystal violet assay, and morphologically by SEM. In each experiment, a single colony was incubated, and the following subinhibitory concentration of 4-HBA (1.6 mg/mL) treatment was applied.

4.3.1. Phenotypic Effects of 4-HBA on *Pseudomonas aeruginosa*

To observe the phenotypic effects of 4-HBA treatment on *P. aeruginosa*, color differences of control and 1.6 mg/mL of 4-HBA treated *P. aeruginosa* cultures were compared (Figure 4.1).

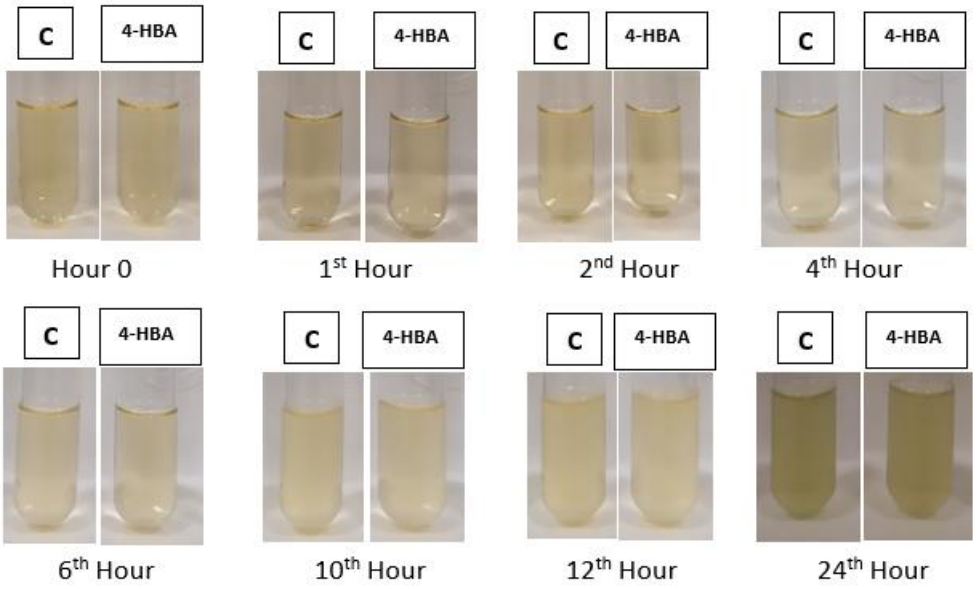

Figure 4.1. 4-HBA treatment against *P. aeruginosa* liquid cultures. The subinhibitory concentration (1.6 mg/mL of 4-HBA) was used. C refers to control samples. The experiment was carried out at different time intervals in the range between 0 to 24 hours.

In each time point, more opaque culture colors were observed in every control sample with respect to 1.6 mg/mL of 4-HBA treated samples. A greenish color was shown at the 24th hour of the control sample, whereas a yellow color was found at the 24th hour of the 4-HBA treated sample. Since the greenish color depends on the pyoverdine presence and thus colonization and biofilm formation related, it was assumed that biofilm formation of the *P. aeruginosa* cultures was disrupted.

4.3.2. Quantitative Analysis on *Pseudomonas aeruginosa* after 4-HBA Treatment

To assess whether 4-HBA treatment inhibits biofilm formation by *P. aeruginosa* by using crystal violet biofilm assay, a time kinetic experiment was performed to observe the effects of the 4-HBA on biofilm biomass (Figure 4.2).

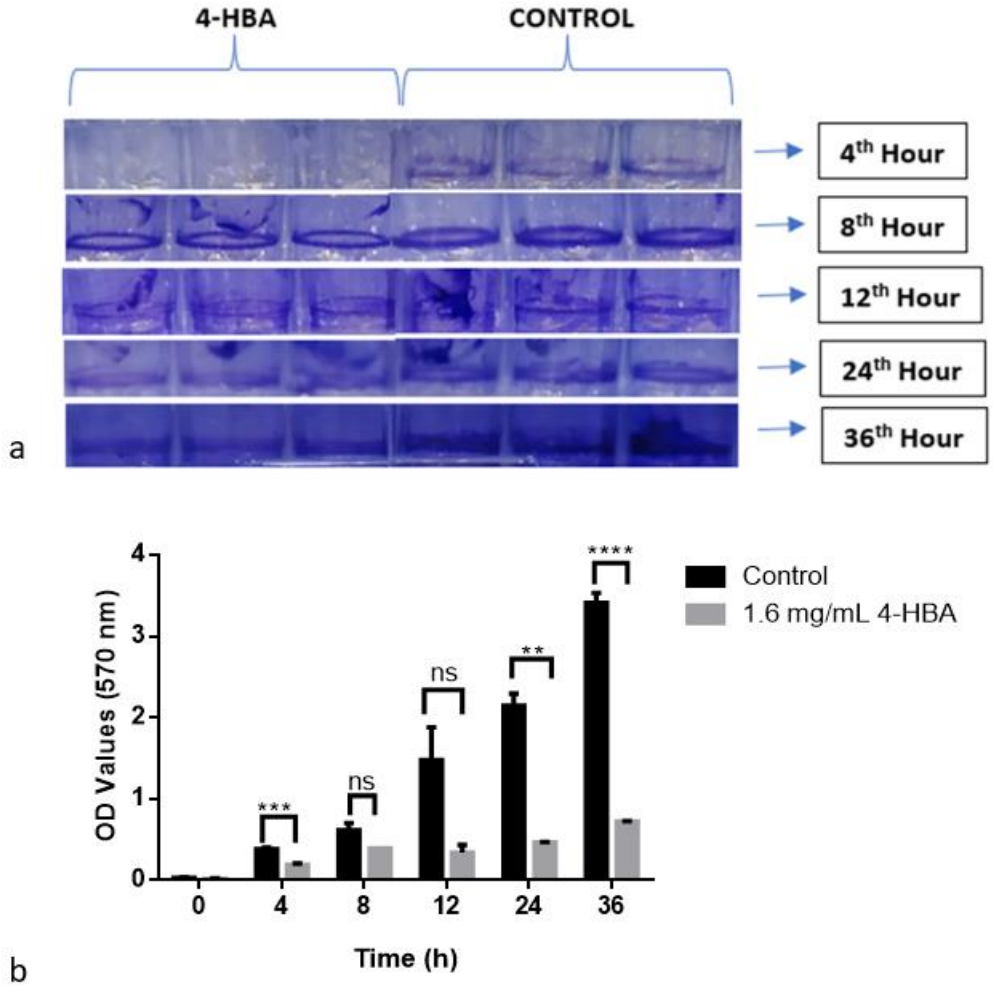

Figure 4.2. Crystal violet assay for biofilm biomass of *P. aeruginosa* treated with 4-HBA at a subinhibitory concentration of 1.6 mg/mL. OD measurements were taken at 570 nm using a spectrophotometer. Error bars represent standard deviations. "ns" indicates a p-value higher than 0.05, "**" indicates a p-value less than or equal to 0.01, "***" indicates a p-value less than or equal to 0.001, and "****" indicates a p-value less than or equal to 0.0001, as determined by unpaired t-tests.

At all time periods, biofilm biomass of 4-HBA treated *P. aeruginosa* has decreased compared with control of *P. aeruginosa*. They showed that a subinhibitory (1.6 mg/mL) concentration of 4-HBA inhibits the formation of *P. aeruginosa* biofilms. The significant difference is visible in the thicker bands in the control group compared to the 4-HBA treated group in the 4th hour (Figure 4.2.a). As time points increase, although the biofilm amount of 4-HBA treated bacteria slightly increases, there is a significant observable decrease compared to the control biofilm amount (Figure 4.2.b). Thus, since the molecular effects of 4-HBA on *P. aeruginosa* were discussed in previous chapters, the phenotypic effects of the phenolic acid also desired to be investigated.

Crystal violet assay has been conducted for bone cement experiments, as well. It has been previously found by HPLC analysis that liquid 4-HBA loaded bone cement releases 0.250 mg/mL of 4-HBA, whereas powder 4-HBA loaded bone cement releases 0.213 mg/mL of 4-HBA (Dokumacı, 2022). Therefore, the effects of the different releasing amounts of 4-HBA on the biofilm of *P. aeruginosa* were examined (Figure 4.3).

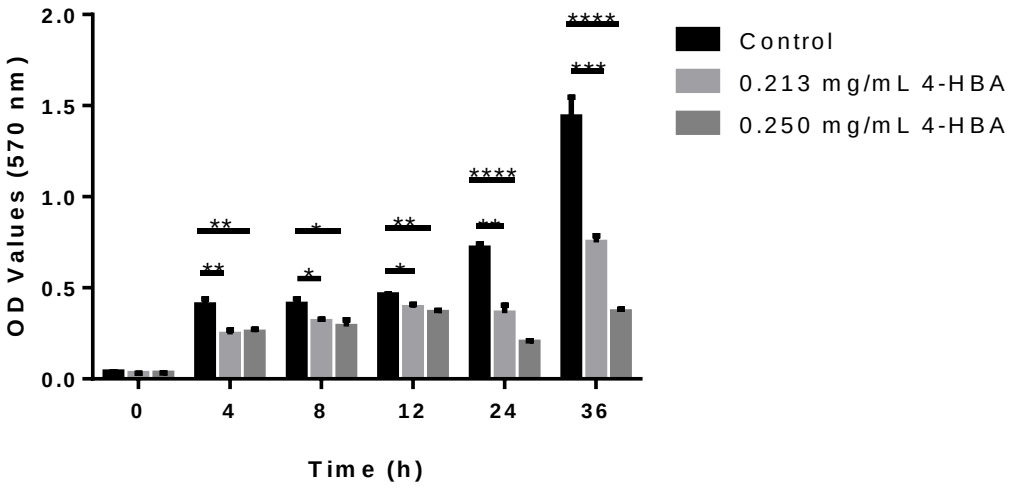

Figure 4.3. Crystal violet assay for biofilm biomass of *P. aeruginosa* treated with 4-HBA at two different release rates of liquid and powder 4-HBA loaded bone cement, 0.250 mg/mL and 0.213 mg/mL, respectively. OD measurements were taken at 570 nm using a spectrophotometer. Error bars represent standard deviations. "*" indicates a p-value less than or equal to 0.05, "**" indicates a p-value less than or equal to 0.01, "***" indicates a p-value less than or equal to 0.001, and "****" indicates a p-value less than or equal to 0.0001, as determined by unpaired t-tests.

Since it was found in the experiment where a subinhibitory concentration of 4-HBA was applied to the biofilm of *P. aeruginosa*, in all time points, 4-HBA treated samples produced less biofilm than the control sample (Figure 4.3). The most significant

differences were observed in the 24th and 36th hours, where biofilm biomass increase was expected to reach the highest level (Žiemytė et al., 2021).

4.3.3. Morphological Effects of 4-HBA on Biofilm of *Pseudomonas aeruginosa*

To observe the morphological effects of 4-HBA treatment on *P. aeruginosa*, SEM images of overnight grown 10^3 CFU/mL of control and 1.6 mg/mL of 4-HBA treated *P. aeruginosa* cultures were compared.

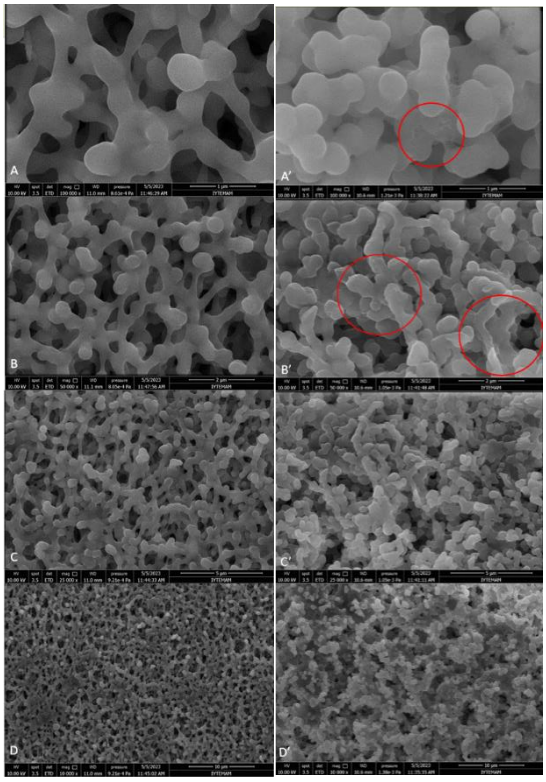

Figure 4.4. Scanning electron microscopy (SEM) images showing untreated (A, B, C, and D) and 4-HBA (A', B,' C' and D') treated *P. aeruginosa* biofilm cells. A and A' were taken at 100000X magnification. B and B' were taken at 50000X magnification. C and C' were taken as 25000X magnification. D and D' were taken at 10000X magnification. Red circles indicate the ruptured biofilm structure.

As presented in Chapter 2, proteomic analysis of 4-HBA treated *P. aeruginosa* cells showed that the biofilm of *P. aeruginosa* was affected by the 4-HBA treatment. The ruptured biofilm structure can also be seen in the cells in Figure 4.4. The figure also shows the decreased number of cells and dispersed cell shapes throughout the images (A', B,' C,' and D'). Ruptured biofilm structure is observable in red circled images (Figure 4. A'

and B'). On the other hand, the smooth and standard distribution of cells and biofilm structure can be examined in untreated cells (Figure 4. A, B, C, and D). Raptured and irregularly distributed biofilm formation is also observable in Figures B1.B' (Appendix B) and C1.B' and C' (Appendix C).

4.4. Conclusions

Since biofilm provides both metabolic and physical protection of the bacteria against antibiotics, eradicating them is an emerging and significant need for dealing with antibiotic-resistant bacteria. 4-HBA effect on Candida albicans biofilm has been studied previously by crystal violet biofilm assay (Ottaviano et al., 2021). In this study, it was shown that 4-HBA resulted as an effective compound in inhibiting biofilm biomass production. In our research, color differences between 4-HBA treated and no treated cultures have been observed in a time-dependent manner. The greenish color of *P. aeruginosa* culture is provided by fluorescent siderophore pyoverdine, which has a role in biofilm formation. Therefore, it has been hypothesized that the biofilm of the bacteria is being seriously affected by the 4-HBA treatment. Therefore, a crystal violet biofilm assay has been conducted to analyze biofilm amount in a time-kinetic experiment, and it has been found that biofilm biomass remained the same from the 8^{th} hour; meanwhile, non-treated bacteria showed exponential growth. According to the SEM images, it has been found that dispersed accumulation of cells, less amount of cells, and scattered cell shape of the biofilm cells are observable in the 4-HBA treated group. Since proteomic analysis and crystal violet assay gave similar results, it was an expected result to examine a ruptured biofilm structure on the biofilm cells of *P. aeruginosa*. Overall, according to proteomics data, virulence-related proteins were found to be only downregulated. Secondly, virulence-related, a component of the T4P *pilM* gene's mRNA expression level, was seen as significantly downregulated. Thirdly, the yellowish color, mainly associated with pyoverdine expression level, has been found in 4-HBA treated *P. aeruginosa* liquid cultures since pyoverdine is directly related to biofilm formation and it was a morphological hallmark of the study. Then, biofilm biomass was calculated by crystal violet assay, and 4-HBA treatment affected it negatively. Lastly, SEM micrographs showed less cell quantity, dispersed biofilm formation, and raptured biofilm structure occurred in the 4-HBA-treated *P. aeruginosa* biofilms.

REFERENCES

"4-Hydroxybenzoic Acid." National Center for Biotechnology Information. PubChem Compound Database. U.S. National Library of Medicine. Accessed January 29, 2023. https://pubchem.ncbi.nlm.nih.gov/compound/4-Hydroxybenzoic-acid.

"Centers for Disease Control and Prevention." Accessed February 15, 2023. https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf.

"New Report Calls for Urgent Action to Avert Antimicrobial Resistance Crisis." World Health Organization. World Health Organization. Accessed February 15, 2023. https://www.who.int/news/item/29-04-2019-new-report-calls-for-urgent-action-to-avert-antimicrobial-resistance-crisis.

"Uniprot Website Q9I038 · Q9I038_PSEAE." UniProt. Accessed June 7, 2023. https://www.uniprot.org/uniprotkb/Q9I038/entry.

Abreu, Ana Cristina, Andrew J. McBain, and Manuel Simões. 2012. "Plants as Sources of New Antimicrobials and Resistance-Modifying Agents." *Natural Product Reports* 29, no. 9: 1007. https://doi.org/10.1039/c2np20035j.

Ajit Singh, Vivek, Bong Chun Haw, Amber Haseeb, and Cindy Shuan Ju Teh. 2019. "Hand-Mixed Vancomycin versus Commercial Tobramycin Cement Revisited: A Study on Mechanical and Antibacterial Properties." *Journal of Orthopaedic Surgery* 27, no. 2: 230949901983961. https://doi.org/10.1177/2309499019839616.

Alberts, Bruce, A. Johnson, and J. Lewis. 2002. "Introduction to Pathogens." Essay. In *Molecular Biology of the Cell*. New York: Garland Science.

Allesen-Holm, Marie, Kim Bundvig Barken, Liang Yang, Mikkel Klausen, Jeremy S. Webb, Staffan Kjelleberg, Soren Molin, Michael Givskov, and Tim Tolker-Nielsen. 2006. "A Characterization of DNA Release in *Pseudomonas* Aeruginosa Cultures

and Biofilms." *Molecular Microbiology* 59, no. 4: 1114–28. https://doi.org/10.1111/j.1365-2958.2005.05008.x.

Almajano, M.P., R. Carbó, M.E. Delgado, and M.H. Gordon. 2007. "Effect of Ph on the Antimicrobial Activity and Oxidative Stability of Oil-in-Water Emulsions Containing Caffeic Acid." *Journal of Food Science* 72, no. 5. https://doi.org/10.1111/j.1750-3841.2007.00387.x.

Alonso, Beatriz, Laia Fernández-Barat, Enea Gino Di Domenico, Mercedes Marín, Emilia Cercenado, Irene Merino, Manuela de Pablos, Patricia Muñoz, and María Guembe. 2020. "Characterization of the Virulence of *Pseudomonas* Aeruginosa Strains Causing Ventilator-Associated Pneumonia." *BMC Infectious Diseases* 20, no. 1. https://doi.org/10.1186/s12879-020-05534-1.

Alt, Volker, Thorsten Bechert, Peter Steinrücke, Michael Wagener, Peter Seidel, Elvira Dingeldein, Eugen Domann, and Reinhard Schnettler. 2004. "An in Vitro Assessment of the Antibacterial Properties and Cytotoxicity of Nanoparticulate Silver Bone Cement." *Biomaterials* 25, no. 18: 4383–91. https://doi.org/10.1016/j.biomaterials.2003.10.078.

Ammann, Christoph G., David Neuhauser, Claudia Eberl, Michael Nogler, and Débora Coraça-Huber. 2017. "Tolerance towards Gentamicin Is a Function of Nutrient Concentration in Biofilms of Patient-Isolated Staphylococcus Epidermidis." *Folia Microbiologica* 63, no. 3: 299–305. https://doi.org/10.1007/s12223-017-0568-x.

Anand, Uttpal, Anukool Vaishnav, Sushil K. Sharma, Jagajjit Sahu, Sarfaraz Ahmad, Kumari Sunita, S. Suresh, et al. 2022. "Current Advances and Research Prospects for Agricultural and Industrial Uses of Microbial Strains Available in World Collections." *Science of The Total Environment* 842: 156641. https://doi.org/10.1016/j.scitotenv.2022.156641.

Anantharaman, Vivek, Eugene V. Koonin, and L. Aravind. 2001. "Tram, a Predicted RNA-Binding Domain, Common to Trna Uracil Methylation and Adenine Thiolation Enzymes." *FEMS Microbiology Letters* 197, no. 2: 215–21. https://doi.org/10.1111/j.1574-6968.2001.tb10606.x.

Antibiotic resistance threats in the United States 2019. [accessed on 10 June 2020]; Available online: https://www.cdc.gov/drugresistance/pdf/threats-report/2019-ar-threats-report-508.pdf

Arai, H. 2003. "Transcriptional Regulation of the NOS Genes for Nitrous Oxide Reductase in *Pseudomonas* Aeruginosa." *Microbiology* 149, no. 1: 29–36. https://doi.org/10.1099/mic.0.25936-0.

Aravind, L., and Eugene V. Koonin. 2000. "The STAS Domain — a Link between Anion Transporters and Antisigma-Factor Antagonists." *Current Biology* 10, no. 2. https://doi.org/10.1016/s0960-98220000335-3.

Arias, Cesar A., and Barbara E. Murray. 2012. "The Rise of the Enterococcus: Beyond Vancomycin Resistance." *Nature Reviews Microbiology* 10, no. 4: 266–78. https://doi.org/10.1038/nrmicro2761.

Arora, Manit. 20139. "Polymethylmethacrylate Bone Cements and Additives: A Review of the Literature." *World Journal of Orthopedics* 4, no. 2: 67. https://doi.org/10.5312/wjo.v4.i2.67.

Arragain, Simon, Samuel K. Handelman, Farhad Forouhar, Fan-Yan Wei, Kazuhito Tomizawa, John F. Hunt, Thierry Douki, Marc Fontecave, Etienne Mulliez, and Mohamed Atta. 2010. "Identification of Eukaryotic and Prokaryotic Methylthiotransferase for Biosynthesis of 2-Methylthio-N6-Threonylcarbamoyladenosine in Trna." *Journal of Biological Chemistry* 285, no. 37: 28425–33. https://doi.org/10.1074/jbc.m110.106831.

Ayers, M., L.M. Sampaleanu, S. Tammam, J. Koo, H. Harvey, P.L. Howell, and L.L. Burrows. 2009. "PilM/n/o/P Proteins Form an Inner Membrane Complex That Affects the Stability of the *Pseudomonas* Aeruginosa Type IV Pilus Secretin." *Journal of Molecular Biology* 394, no. 1: 128–42. https://doi.org/10.1016/j.jmb.2009.09.034.

Azam, Mohd W., and Asad U. Khan. 2019. “Updates on the Pathogenicity Status of *Pseudomonas* Aeruginosa.” *Drug Discovery Today* 24, no. 1: 350–59. https://doi.org/10.1016/j.drudis.2018.07.003.

Baker, Perrin, Preston J. Hill, Brendan D. Snarr, Noor Alnabelseya, Matthew J. Pestrak, Mark J. Lee, Laura K. Jennings, et al. 2016. “Exopolysaccharide Biosynthetic Glycoside Hydrolases Can Be Utilized to Disrupt and Prevent*Pseudomonas Aeruginosa*Biofilms.” *Science Advances* 2, no. 5. https://doi.org/10.1126/sciadv.1501632.

Baldassarri, L. *et al.* 2005 “Pathogenesis of implant infections by Enterococci,” *The International Journal of Artificial Organs*, 2811, pp. 1101–1109. Available at: https://doi.org/10.1177/039139880502801107.

Balloux, Francois, and Lucy van Dorp. 2017. “Q&A: What Are Pathogens, and What Have They Done to and for Us?” *BMC Biology* 15, no. 1. https://doi.org/10.1186/s12915-017-0433-z.

Balouiri, Mounyr, Moulay Sadiki, and Saad Koraichi Ibnsouda. 2016. “Methods for in Vitro Evaluating Antimicrobial Activity: A Review.” *Journal of Pharmaceutical Analysis* 6, no. 2: 71–79. https://doi.org/10.1016/j.jpha.2015.11.005.

Baron, Samuel, and Barbara H Iglewski. 1996. “*Pseudomonas*.” Essay. In *Medical Microbiology*. Galveston, TX: University of Texas Medical Branch at Galveston.

Baron, Samuel, Charles P. Davis, Gail Woods, and David Niesel. 1996. “Introduction to Bacteriology.” Essay. In *Medical Microbiology*, 4th ed. Galveston, TX: University of Texas Medical Branch at Galveston.

Belt, Hilbrand van, Daniëlle Neut, Willem Schenk, Jim R Horn, Henny C Mei, and Henk J Busscher. 2000. “Gentamicin Release from Polymethylmethacrylate Bone Cements and Staphylococcus Aureus Biofilm Formation.” *Acta Orthopaedica Scandinavica* 71, no. 6: 625–29. https://doi.org/10.1080/000164700317362280.

Bernstein, Kara A., Serge Gangloff, and Rodney Rothstein. 2010. "The RecQ DNA Helicases in DNA Repair." *Annual Review of Genetics* 44, no. 1: 393–417. https://doi.org/10.1146/annurev-genet-102209-163602.

Bhuwan, Manish, Hui-Ju Lee, Hwei-Ling Peng, and Hwan-You Chang. 2012. "Histidine-Containing Phosphotransfer Protein-B HptB Regulates Swarming Motility through Partner-Switching System in *Pseudomonas* Aeruginosa PAO1 Strain." *Journal of Biological Chemistry* 287, no. 3: 1903–14. https://doi.org/10.1074/jbc.m111.256586.

Billings, Nicole, Maria Ramirez Millan, Marina Caldara, Roberto Rusconi, Yekaterina Tarasova, Roman Stocker, and Katharina Ribbeck. 2013. "The Extracellular Matrix Component PSL Provides Fast-Acting Antibiotic Defense in *Pseudomonas* Aeruginosa Biofilms." *PLoS Pathogens* 9, no. 8. https://doi.org/10.1371/journal.ppat.1003526.

Bistolfi, Alessandro, Giuseppe Massazza, Enrica Verné, Alessandro Massè, Davide Deledda, Sara Ferraris, Marta Miola, Fabrizio Galetto, and Maurizio Crova. 2011. "Antibiotic-Loaded Cement in Orthopedic Surgery: A Review." *ISRN Orthopedics* 2011: 1–8. https://doi.org/10.5402/2011/290851.

Bjarnsholt, Thomas, Maria Alhede, Morten Alhede, Steffen R. Eickhardt-Sørensen, Claus Moser, Michael Kühl, Peter Østrup Jensen, and Niels Høiby. 2013. "The in Vivo Biofilm." *Trends in Microbiology* 21, no. 9: 466–74. https://doi.org/10.1016/j.tim.2013.06.002.

Bonomo, Robert A. 2016. "B-Lactamases: A Focus on Current Challenges." *Cold Spring Harbor Perspectives in Medicine* 7, no. 1. https://doi.org/10.1101/cshperspect.a025239.

Bonten, Marc JM, Rob Willems, and Robert A Weinstein. 2001. "Vancomycin-Resistant Enterococci: Why Are They Here, and Where Do They Come from?" *The Lancet Infectious Diseases* 1, no. 5: 314–25. https://doi.org/10.1016/s1473-30990100145-1.

Boucher, Helen W., and G. Ralph Corey. 2008. “Epidemiology of Methicillin-Resistant Staphylococcus *Aureus*.” *Clinical Infectious Diseases* 46, no. S5. https://doi.org/10.1086/533590.

Brooun, Alexei, Songhua Liu, and Kim Lewis. 2000 “A Dose-Response Study of Antibiotic Resistance in *Pseudomonas Aeruginosa* Biofilms.” *Antimicrobial Agents and Chemotherapy* 44, no. 3: 640–46. https://doi.org/10.1128/aac.44.3.640-646.2000.

Brown, Sam P., Daniel M. Cornforth, and Nicole Mideo. 2012. “Evolution of Virulence in Opportunistic Pathogens: Generalism, Plasticity, and Control.” *Trends in Microbiology* 20, no. 7: 336–42. https://doi.org/10.1016/j.tim.2012.04.005.

Büyükköroğlu, Gülay, Devrim Demir Dora, Filiz Özdemir, and Candan Hızel. 2018. “Techniques for Protein Analysis.” *Omics Technologies and Bio-Engineering*, 317–51. https://doi.org/10.1016/b978-0-12-804659-3.00015-4.

Byrd, Matthew S., Irina Sadovskaya, Evgueny Vinogradov, Haiping Lu, April B. Sprinkle, Stephen H. Richardson, Luyan Ma, et al. 2009. “Genetic and Biochemical Analyses of the*Pseudomonas Aeruginosa*PSL Exopolysaccharide Reveal Overlapping Roles for Polysaccharide Synthesis Enzymes in PSL and LPS Production.” *Molecular Microbiology* 73, no. 4: 622–38. https://doi.org/10.1111/j.1365-2958.2009.06795.x.

Campbell, Elizabeth A, Lars F Westblade, and Seth A Darst. 2008. “Regulation of Bacterial RNA Polymerase σ Factor Activity: A Structural Perspective.” *Current Opinion in Microbiology* 11, no. 2: 121–27. https://doi.org/10.1016/j.mib.2008.02.016.

Campos, F.M., J.A. Couto, A.R. Figueiredo, I.V. Tóth, A.O.S.S. Rangel, and T.A. Hogg. 2009. “Cell Membrane Damage Induced by Phenolic Acids on Wine Lactic Acid Bacteria.” *International Journal of Food Microbiology* 135, no. 2: 144–51. https://doi.org/10.1016/j.ijfoodmicro.2009.07.031.

Cara, A. *et al.* 2021 “Antibiotics in bone cements used for prosthesis fixation: An efficient way to prevent Staphylococcus aureus and Staphylococcus epidermidis prosthetic joint infection,” *Frontiers in Medicine*, 7. Available at: https://doi.org/10.3389/fmed.2020.576231.

Cassini, Alessandro, Liselotte Diaz Högberg, Diamantis Plachouras, Annalisa Quattrocchi, Ana Hoxha, Gunnar Skov Simonsen, Mélanie Colomb-Cotinat, et al. 2019. “Attributable Deaths and Disability-Adjusted Life-Years Caused by Infections with Antibiotic-Resistant Bacteria in the EU and the European Economic Area in 2015: A Population-Level Modelling Analysis.” *The Lancet Infectious Diseases* 19, no. 1: 56–66. https://doi.org/10.1016/s1473-30991830605-4.

Centers for Disease Control and Prevention CDC. 2003 “Public Health Dispatch: Outbreaks of Community-Associated Methicillin-Resistant Staphylococcus Aureus Skin Infections—Los Angeles County, California, 2002-2003.” *Archives of Dermatology* 139, no. 4: 550. https://doi.org/10.1001/archderm.139.4.550.

Chambers, Henry F. 2005. “Community-Associated MRSA — Resistance and Virulence Converge.” *New England Journal of Medicine* 352, no. 14: 1485–87. https://doi.org/10.1056/nejme058023.

Chang, Y. H., C. L. Tai, H. Y. Hsu, P. H. Hsieh, M. S. Lee, and S. W. Ueng. 2014. “Liquid Antibiotics in Bone Cement.” *Bone & Joint Research* 3, no. 8: 246–51. https://doi.org/10.1302/2046-3758.38.2000305.

Charollais, J. 2004. “CSDA, a Cold-Shock RNA Helicase from Escherichia Coli, Is Involved in the Biogenesis of 50S Ribosomal Subunit.” *Nucleic Acids Research* 32, no. 9: 2751–59. https://doi.org/10.1093/nar/gkh603.

Chen, Jinxiang, Jing Yang, Lanlan Ma, Jun Li, Nasir Shahzad, and Chan Kyung Kim. 2020. “Structure-Antioxidant Activity Relationship of Methoxy, Phenolic Hydroxyl, and Carboxylic Acid Groups of Phenolic Acids.” *Scientific Reports* 10, no. 1. https://doi.org/10.1038/s41598-020-59451-z.

Cherny, Kathryn E., and Karin Sauer. 2019. "*Pseudomonas* Aeruginosa Requires the DNA-Specific Endonuclease Enda to Degrade Extracellular Genomic DNA to Disperse from the Biofilm." *Journal of Bacteriology* 201, no. 18. https://doi.org/10.1128/jb.00059-19.

Cho, William C.S. 2007. "Proteomics Technologies and Challenges." *Genomics, Proteomics & Bioinformatics* 5, no. 2: 77–85. https://doi.org/10.1016/s1672-02290760018-7.

Chow, L.C. 2001. "Calcium Phosphate Cements." *Monographs in Oral Science*, 148–63. https://doi.org/10.1159/000061653.

Chu, V H, J M Miro, B Hoen, C H Cabell, P A Pappas, P Jones, M E Stryjewski, et al. 2008. "Coagulase-Negative Staphylococcal Prosthetic Valve Endocarditis--a Contemporary Update Based on the International Collaboration on Endocarditis: Prospective Cohort Study." *Heart* 95, no. 7: 570–76. https://doi.org/10.1136/hrt.2008.152975.

Chua, Song Lin, Louise D Hultqvist, Mingjun Yuan, Morten Rybtke, Thomas E Nielsen, Michael Givskov, Tim Tolker-Nielsen, and Liang Yang. 2015. "In Vitro and in Vivo Generation and Characterization of *Pseudomonas* Aeruginosa Biofilm–Dispersed Cells via C-Di-GMP Manipulation." *Nature Protocols* 10, no. 8: 1165–80. https://doi.org/10.1038/nprot.2015.067.

Chua, Song Lin, Sean Yang-Yi Tan, Morten Theil Rybtke, Yicai Chen, Scott A. Rice, Staffan Kjelleberg, Tim Tolker-Nielsen, Liang Yang, and Michael Givskov. 2013. "Bis-3′-5′-Cyclic Dimeric GMP Regulates Antimicrobial Peptide Resistance in *Pseudomonas* Aeruginosa." *Antimicrobial Agents and Chemotherapy* 57, no. 5: 2066–75. https://doi.org/10.1128/aac.02499-12.

Chua, Song Lin, Yang Liu, Joey Kuok Yam, Yicai Chen, Rebecca Munk Vejborg, Bryan Giin Tan, Staffan Kjelleberg, Tim Tolker-Nielsen, Michael Givskov, and Liang Yang. 2014. "Dispersed Cells Represent a Distinct Stage in the Transition from Bacterial Biofilm to Planktonic Lifestyles." *Nature Communications* 5, no. 1. https://doi.org/10.1038/ncomms5462.

Ciofu, Oana, Tim Tolker-Nielsen, Peter Østrup Jensen, Hengzhuang Wang, and Niels Høiby. 2015. "Antimicrobial Resistance, Respiratory Tract Infections and Role of Biofilms in Lung Infections in Cystic Fibrosis Patients." *Advanced Drug Delivery Reviews* 85: 7–23. https://doi.org/10.1016/j.addr.2014.11.017.

Coates, Anthony RM, Gerry Halls, and Yanmin Hu. 2011. "Novel Classes of Antibiotics or More of the Same?" *British Journal of Pharmacology* 163, no. 1: 184–94. https://doi.org/10.1111/j.1476-5381.2011.01250.x.

Colvin, Kelly M., Noor Alnabelseya, Perrin Baker, John C. Whitney, P. Lynne Howell, and Matthew R. Parsek. 2013. "Pela Deacetylase Activity Is Required for Pel Polysaccharide Synthesis in *Pseudomonas* Aeruginosa." *Journal of Bacteriology* 195, no. 10: 2329–39. https://doi.org/10.1128/jb.02150-12.

Colvin, Kelly M., Yasuhiko Irie, Catherine S. Tart, Rodolfo Urbano, John C. Whitney, Cynthia Ryder, P. Lynne Howell, Daniel J. Wozniak, and Matthew R. Parsek. 2011. "The Pel and PSL Polysaccharides Provide *Pseudomonas* Aeruginosa Structural Redundancy within the Biofilm Matrix." *Environmental Microbiology* 14, no. 8: 1913–28. https://doi.org/10.1111/j.1462-2920.2011.02657.x.

Cornelis, Pierre, and Jozef Dingemans. 2013. "*Pseudomonas* Aeruginosa Adapts Its Iron Uptake Strategies in Function of the Type of Infections." *Frontiers in Cellular and Infection Microbiology* 3. https://doi.org/10.3389/fcimb.2013.00075.

Cosgrove, Sara E., Gloria A. Vigliani, Marilyn Campion, Vance G. Fowler, Jr., Elias Abrutyn, G. Ralph Corey, Donald P. Levine, et al. 2009. "Initial Low-dose Gentamicin for *Staphylococcus aureus* Bacteremia and Endocarditis Is Nephrotoxic." *Clinical Infectious Diseases* 48, no. 6: 713–21. https://doi.org/10.1086/597031.

Costerton, J. William, Zbigniew Lewandowski, Douglas E. Caldwell, Darren R. Korber, and Hilary M. Lappin-Scott. 1995. "Microbial Biofilms." *Annual Review of Microbiology* 49, no. 1: 711–45. https://doi.org/10.1146/annurev.mi.49.100195.003431.

Cranford-Smith, Tamar, and Damon Huber. 2018. “The Way Is the Goal: How Seca Transports Proteins across the Cytoplasmic Membrane in Bacteria.” *FEMS Microbiology Letters* 365, no. 11. https://doi.org/10.1093/femsle/fny093.

Crespo, Anna, Núria Blanco-Cabra, and Eduard Torrents. 2018. “Aerobic Vitamin B12 Biosynthesis Is Essential for *Pseudomonas* Aeruginosa Class II Ribonucleotide Reductase Activity During Planktonic and Biofilm Growth.” *Frontiers in Microbiology* 9. https://doi.org/10.3389/fmicb.2018.00986.

Croft, Kevin D. 1998. “The Chemistry and Biological Effects of Flavonoids and Phenolic Acids.” *Annals of the New York Academy of Sciences* 854, no. 1 TOWARDS PROLO: 435–42. https://doi.org/10.1111/j.1749-6632.1998.tb09922.x.

Cueva, Carolina, M. Victoria Moreno-Arribas, Pedro J. Martín-Álvarez, Gerald Bills, M. Francisca Vicente, Angela Basilio, Concepción López Rivas, Teresa Requena, Juan M. Rodríguez, and Begoña Bartolomé. 2010. “Antimicrobial Activity of Phenolic Acids against Commensal, Probiotic and Pathogenic Bacteria.” *Research in Microbiology* 161, no. 5: 372–82. https://doi.org/10.1016/j.resmic.2010.04.006.

Daddaoua, A., A. Corral-Lugo, J.-L. Ramos, and Tino Krell. 2017. “Identification of GNTR as Regulator of the Glucose Metabolism in *Pseudomonas aeruginosa*.” *Environmental Microbiology* 19, no. 9: 3721–33. https://doi.org/10.1111/1462-2920.13871.

Darouiche, Rabih O. 2004. “Treatment of Infections Associated with Surgical Implants.” *New England Journal of Medicine* 350, no. 14: 1422–29. https://doi.org/10.1056/nejmra035415.

Das, Manash C., Padmani Sandhu, Priya Gupta, Prasenjit Rudrapaul, Utpal C. De, Prosun Tribedi, Yusuf Akhter, and Surajit Bhattacharjee. 2016. “Attenuation of *Pseudomonas* Aeruginosa Biofilm Formation by Vitexin: A Combinatorial Study with Azithromycin and Gentamicin.” *Scientific Reports* 6, no. 1. https://doi.org/10.1038/srep23347.

Davies, Bryan W., Caroline Köhrer, Asha I. Jacob, Lyle A. Simmons, Jianyu Zhu, Lourdes M. Aleman, Uttam L. RajBhandary, and Graham C. Walker. 2010. "Role of Escherichia Coli YbeY, a Highly Conserved Protein, in Rrna Processing." *Molecular Microbiology* 78, no. 2: 506–18. https://doi.org/10.1111/j.1365-2958.2010.07351.x.

Del Pozo, Jose L., and Robin Patel. 2009. "Infection Associated with Prosthetic Joints." *New England Journal of Medicine* 361, no. 8: 787–94. https://doi.org/10.1056/nejmcp0905029.

DeLeo, Frank R., Binh An Diep, and Michael Otto. 2009. "Host Defense and Pathogenesis in Staphylococcus Aureus Infections." *Infectious Disease Clinics of North America* 23, no. 1: 17–34. https://doi.org/10.1016/j.idc.2008.10.003.

Dokumacı, Ecem. 2022. "Investigation of Antimicrobial Properties of 4-Hydroxybenzoic Acid and Its Application in Bone Cement MSc Thesis, Izmir Institute of Technology, 2022".

Donlan, Rodney M. 2002. "Biofilms: Microbial Life on Surfaces." *Emerging Infectious Diseases* 8, no. 9: 881–90. https://doi.org/10.3201/eid0809.020063.

Drenkard, Eliana. 2003. "Antimicrobial Resistance of *Pseudomonas* Aeruginosa Biofilms." *Microbes and Infection* 5, no. 13: 1213–19. https://doi.org/10.1016/j.micinf.2003.08.009.

Du, Changling, Jingyi Liu, David Anthony Fikhman, Katheryn Shi Dong, and Mary Beth Monroe. 2022. "Shape Memory Polymer Foams with Phenolic Acid-Based Antioxidant and Antimicrobial Properties for Traumatic Wound Healing." *Frontiers in Bioengineering and Biotechnology* 10. https://doi.org/10.3389/fbioe.2022.809361.

Durand, J M, G R Björk, A Kuwae, M Yoshikawa, and C Sasakawa. 1997. "The Modified Nucleoside 2-Methylthio-N6-Isopentenyladenosine in Trna of Shigella Flexneri Is Required for Expression of Virulence Genes." *Journal of Bacteriology* 179, no. 18: 5777–82. https://doi.org/10.1128/jb.179.18.5777-5782.1997.

Elegir, G., A. Kindl, P. Sadocco, and M. Orlandi. 2008. “Development of Antimicrobial Cellulose Packaging through Laccase-Mediated Grafting of Phenolic Compounds.” *Enzyme and Microbial Technology* 43, no. 2: 84–92. https://doi.org/10.1016/j.enzmictec.2007.10.003.

Elsanhoty, Rafaat M., Mohamed S. Soliman, Yehia A. Khidr, Gamal O. Hassan, Ahmed R. Hassan, Mohammed Aladhadh, and Asmaa Abdella. 2022. “Pharmacological Activities and Characterization of Phenolic and Flavonoid Compounds in Solenostemma Argel Extract.” *Molecules* 27, no. 23: 8118. https://doi.org/10.3390/molecules27238118.

Eshelman, Kate, Huili Yao, Achala N. Punchi Hewage, Jacqueline J. Deay, Josephine R. Chandler, and Mario Rivera. 2017. “Inhibiting the Bfrb: BFD Interaction in *Pseudomonas* Aeruginosa Causes Irreversible Iron Accumulation in Bacterioferritin and Iron Deficiency in the Bacterial Cytosol.” *Metallomics* 9, no. 6: 646–59. https://doi.org/10.1039/c7mt00042a.

Evans, Leigh R., and Alfred Linker. 1973. “Production and Characterization of the Slime Polysaccharide of *Pseudomonas Aeruginosa*.” *Journal of Bacteriology* 116, no. 2: 915–24. https://doi.org/10.1128/jb.116.2.915-924.1973.

Fehring, Thomas K., Susan Odum, William L. Griffin, J. Bohannon Mason, and Matthew Nadaud. 2001. “Early Failures in Total Knee Arthroplasty.” *Clinical Orthopaedics and Related Research* 392: 315–18. https://doi.org/10.1097/00003086-200111000-00041.

Fleming, Derek, and Kendra Rumbaugh. 2018. “The Consequences of Biofilm Dispersal on the Host.” *Scientific Reports* 8, no. 1. https://doi.org/10.1038/s41598-018-29121-2.

Folkesson, Anders, Lars Jelsbak, Lei Yang, Helle Krogh Johansen, Oana Ciofu, Niels Høiby, and Søren Molin. 2012. “Adaptation of *Pseudomonas* Aeruginosa to the Cystic Fibrosis Airway: An Evolutionary Perspective.” *Nature Reviews Microbiology* 10, no. 12: 841–51. https://doi.org/10.1038/nrmicro2907.

Foster, Timothy J. 2005. “Immune Evasion by Staphylococci.” *Nature Reviews Microbiology* 3, no. 12: 948–58. https://doi.org/10.1038/nrmicro1289.

Friedman, Lisa, and Roberto Kolter. 2003. “Genes Involved in Matrix Formation in *Pseudomonas* Aeruginosa PA14 Biofilms.” *Molecular Microbiology* 51, no. 3: 675–90. https://doi.org/10.1046/j.1365-2958.2003.03877.x.

Friedman, Lisa, and Roberto Kolter. 2004. “Two Genetic Loci Produce Distinct Carbohydrate-Rich Structural Components of the *Pseudomonas Aeruginosa* Biofilm Matrix.” *Journal of Bacteriology* 186, no. 14: 4457–65. https://doi.org/10.1128/jb.186.14.4457-4465.2004.

Fuxman Bass, Juan I., Daniela M. Russo, Maria L. Gabelloni, Jorge R. Geffner, Mirta Giordano, Mariana Catalano, Ángeles Zorreguieta, and Analía S. Trevani. 2010. “Extracellular DNA: A Major Proinflammatory Component of *Pseudomonas Aeruginosa* Biofilms.” *The Journal of Immunology* 184, no. 11: 6386–95. https://doi.org/10.4049/jimmunol.0901640.

Gaille, Catherine, Cornelia Reimmann, and Dieter Haas. 2003. “Isochorismate Synthase PCHA, the First and Rate-Limiting Enzyme in Salicylate Biosynthesis of *Pseudomonas* Aeruginosa.” *Journal of Biological Chemistry* 278, no. 19: 16893–98. https://doi.org/10.1074/jbc.m212324200.

Garrett, Edward S., Demetra Perlegas, and Daniel J. Wozniak. 1999. “Negative Control of Flagellum Synthesis in *Pseudomonas Aeruginosa* Is Modulated by the Alternative Sigma Factor Algt Algu.” *Journal of Bacteriology* 181, no. 23 1999: 7401–4. https://doi.org/10.1128/jb.181.23.7401-7404.1999.

Ghadaksaz, Abdolamir, Abbas Ali Fooladi, Hamideh Mahmoodzadeh Hosseini, and Mohsen Amin. 2015. “The Prevalence of Some *Pseudomonas* Virulence Genes Related to Biofilm Formation and Alginate Production among Clinical Isolates.” *Journal of Applied Biomedicine* 13, no. 1: 61–68. https://doi.org/10.1016/j.jab.2014.05.002.

Ghafoor, Aamir, Iain D. Hay, and Bernd H. Rehm. 2011. “Role of Exopolysaccharides in *Pseudomonas* Aeruginosa Biofilm Formation and Architecture.” *Applied and Environmental Microbiology* 77, no. 15: 5238–46. https://doi.org/10.1128/aem.00637-11.

Ghosh, Sumana, Mau Sinha, Ritwik Samanta, Suresh Sadhasivam, Anamika Bhattacharyya, Ashis Nandy, Swamini Saini, et al. 2022. “A Potent Antibiotic-Loaded Bone-Cement Implant against Staphylococcal Bone Infections.” *Nature Biomedical Engineering* 6, no. 10: 1180–95. https://doi.org/10.1038/s41551-022-00950-x.

Ghysels, Bart, Urs Ochsner, Lothar Heinisch, Michael Vasil, Pierre Cornelis, and Sandra Matthijs. 2005. “The*Pseudomonas Aeruginosa Pira*Gene Encodes a Second Receptor for Ferrienterobactin and Synthetic Catecholate Analogues.” *FEMS Microbiology Letters* 246, no. 2: 167–74. https://doi.org/10.1016/j.femsle.2005.04.010.

Gilmore, Michael S, Francois Lebreton, and Willem van Schaik. 2013. “Genomic Transition of Enterococci from Gut Commensals to Leading Causes of Multidrug-Resistant Hospital Infection in the Antibiotic Era.” *Current Opinion in Microbiology* 16, no. 1: 10–16. https://doi.org/10.1016/j.mib.2013.01.006.

Gloag, Erin S., Guy K. German, Paul Stoodley, and Daniel J. Wozniak. 2018. “Viscoelastic Properties of *Pseudomonas* Aeruginosa Variant Biofilms.” *Scientific Reports* 8, no. 1. https://doi.org/10.1038/s41598-018-28009-5.

Gloag, Erin S., Lynne Turnbull, Alan Huang, Pascal Vallotton, Huabin Wang, Laura M. Nolan, Lisa Mililli, et al. 2013. “Self-Organization of Bacterial Biofilms Is Facilitated by Extracellular DNA.” *Proceedings of the National Academy of Sciences* 110, no. 28: 11541–46. https://doi.org/10.1073/pnas.1218898110.

Griffiths, E, J Humphreys, A Leach, and L Scanlon. 1978. “Alterations in the Trna's of Escherichia Coli Recovered from Lethally Infected Animals.” *Infection and Immunity* 22, no. 2: 312–17. https://doi.org/10.1128/iai.22.2.312-317.1978.

Griffiths, W. 1996. “Food Phenolics: Sources, Chemistry, Effects, Applications.” *Trends in Food Science & Technology* 7, no. 7: 243. https://doi.org/10.1016/0924-22449681226-x.

Guilbaud, Morgan, Jérôme Bruzaud, Emeline Bouffartigues, Nicole Orange, Alain Guillot, Anne Aubert-Frambourg, Véronique Monnet, Jean-Marie Herry, Sylvie Chevalier, and Marie-Noëlle Bellon-Fontaine. 2017. “Proteomic Response of *Pseudomonas* Aeruginosa pao1 Adhering to Solid Surfaces.” *Frontiers in Microbiology* 8. https://doi.org/10.3389/fmicb.2017.01465.

Gupta, Riti, Dmitri Toptygin, and Christian M. Kaiser. 2020. “The Seca Motor Generates Mechanical Force during Protein Translocation.” *Nature Communications* 11, no. 1. https://doi.org/10.1038/s41467-020-17561-2.

Ha, Jun Yong, Hye Kyong Kim, Do Jin Kim, Kyoung Hoon Kim, Sung Jin Oh, Hyung Ho Lee, Hye Jin Yoon, Hyun Kyu Song, and Se Won Suh. 2007. “The Recombination-Associated Protein RdgC Adopts a Novel Toroidal Architecture for DNA Binding.” *Nucleic Acids Research* 35, no. 8: 2671–81. https://doi.org/10.1093/nar/gkm144.

Hachani, Abderrahman, Nadine S. Lossi, Alexander Hamilton, Cerith Jones, Sophie Bleves, David Albesa-Jové, and Alain Filloux. 2011. “Type VI Secretion System in *Pseudomonas* Aeruginosa.” *Journal of Biological Chemistry* 286, no. 14: 12317–27. https://doi.org/10.1074/jbc.m110.193045.

Hall-Stoodley, L., Costerton, J.W. and Stoodley, P. 2004 “Bacterial biofilms: From the natural environment to infectious diseases,” *Nature Reviews Microbiology*, 22, pp. 95–108. Available at: https://doi.org/10.1038/nrmicro821.

Hassett, D J, H P Schweizer, and D E Ohman. 1995. “*Pseudomonas* Aeruginosa SodA and SodB Mutants Defective in Manganese- and Iron-Cofactored Superoxide Dismutase Activity Demonstrate the Importance of the Iron-Cofactored Form in Aerobic Metabolism.” *Journal of Bacteriology* 177, no. 22: 6330–37. https://doi.org/10.1128/jb.177.22.6330-6337.1995.

Hay, Iain D., Zahid Ur Rehman, M. Fata Moradali, Yajie Wang, and Bernd H. Rehm. 2013. “Microbial Alginate Production, Modification and Its Applications.” *Microbial Biotechnology* 6, no. 6: 637–50. https://doi.org/10.1111/1751-7915.12076.

Hickman, Jason W., and Caroline S. Harwood. 2008. “Identification of Fleq from*Pseudomonas Aeruginosa*as a c-Di-GMP-Responsive Transcription Factor.” *Molecular Microbiology* 69, no. 2: 376–89. https://doi.org/10.1111/j.1365-2958.2008.06281.x.

Hishida, Takashi, Yong-Woon Han, Tatsuya Shibata, Yoshino Kubota, Yoshizumi Ishino, Hiroshi Iwasaki, and Hideo Shinagawa. 2004. “Role of the *Escherichia Coli* Recq DNA Helicase in SOS Signaling and Genome Stabilization at Stalled Replication Forks.” *Genes & Development* 18, no. 15: 1886–97. https://doi.org/10.1101/gad.1223804.

Høiby, Niels, Oana Ciofu, and Thomas Bjarnsholt. 2010. “*Pseudomonas Aeruginosa* Biofilms in Cystic Fibrosis.” *Future Microbiology* 5, no. 11: 1663–74. https://doi.org/10.2217/fmb.10.125.

Hsu, Jye-Lin, Hsuan-Cheng Chen, Hwei-Ling Peng, and Hwan-You Chang. 2008. “Characterization of the Histidine-Containing Phosphotransfer Protein B-Mediated Multistep Phosphorelay System in *Pseudomonas* Aeruginosa PAO1.” *Journal of Biological Chemistry* 283, no. 15: 9933–44. https://doi.org/10.1074/jbc.m708836200.

Intile, Peter J., Grant J. Balzer, Matthew C. Wolfgang, and Timothy L. Yahr. 2015. “The RNA Helicase Dead Stimulates EXSA Translation to Promote Expression of the *Pseudomonas* Aeruginosa Type III Secretion System.” *Journal of Bacteriology* 197, no. 16: 2664–74. https://doi.org/10.1128/jb.00231-15.

Irie, Yasuhiko, Aled E. Roberts, Kasper N. Kragh, Vernita D. Gordon, Jaime Hutchison, Rosalind J. Allen, Gavin Melaugh, Thomas Bjarnsholt, Stuart A. West, and Stephen P. Diggle. 2017. “The *Pseudomonas Aeruginosa* PSL Polysaccharide Is a Social

but Noncheatable Trait in Biofilms." *mBio* 8, no. 3. https://doi.org/10.1128/mbio.00374-17.

Irie, Yasuhiko, Bradley R. Borlee, Jennifer R. O'Connor, Preston J. Hill, Caroline S. Harwood, Daniel J. Wozniak, and Matthew R. Parsek. 2012. "Self-Produced Exopolysaccharide Is a Signal That Stimulates Biofilm Formation in *Pseudomonas Aeruginosa*." *Proceedings of the National Academy of Sciences* 109, no. 50: 20632–36. https://doi.org/10.1073/pnas.1217993109.

Jacewicz, Agata, Atsushi Izumi, Katharina Brunner, Robert Schnell, and Gunter Schneider. 2013. "Structural Insights into the UbiD Protein Family from the Crystal Structure of PA0254 from *Pseudomonas* Aeruginosa." *PLoS ONE* 8, no. 5. https://doi.org/10.1371/journal.pone.0063161.

Jackson, Kara D., Melissa Starkey, Stefanie Kremer, Matthew R. Parsek, and Daniel J. Wozniak. 2004. "Identification of *Psl*, a Locus Encoding a Potential Exopolysaccharide That Is Essential for *Pseudomonas Aeruginosa* pao1 Biofilm Formation." *Journal of Bacteriology* 186, no. 14 2004: 4466–75. https://doi.org/10.1128/jb.186.14.4466-4475.

Jenal, Urs, and Jacob Malone. 2006. "Mechanisms of Cyclic-Di-GMP Signaling in Bacteria." *Annual Review of Genetics* 40, no. 1: 385–407. https://doi.org/10.1146/annurev.genet.40.110405.090423.

Jennings, Laura K., Kelly M. Storek, Hannah E. Ledvina, Charlène Coulon, Lindsey S. Marmont, Irina Sadovskaya, Patrick R. Secor, et al. 2015. "Pel Is a Cationic Exopolysaccharide That Cross-Links Extracellular DNA in the *Pseudomonas Aeruginosa* Biofilm Matrix." *Proceedings of the National Academy of Sciences* 112, no. 36: 11353–58. https://doi.org/10.1073/pnas.1503058112.

Jones, Christopher J., and Daniel J. Wozniak. 2017. "PSL Produced by Mucoid *Pseudomonas Aeruginosa* Contributes to the Establishment of Biofilms and Immune Evasion." *mBio* 8, no. 3. https://doi.org/10.1128/mbio.00864-17.

Kaçmaz, Birgül, and Altan Aksoy. 2005. "Antimicrobial Resistance of Enterococci in Turkey." *International Journal of Antimicrobial Agents* 25, no. 6: 535–38. https://doi.org/10.1016/j.ijantimicag.2005.02.020.

Kang, C M, M S Brody, S Akbar, X Yang, and C W Price. 1996. "Homologous Pairs of Regulatory Proteins Control Activity of Bacillus Subtilis Transcription Factor Sigmab in Response to Environmental Stress." *Journal of Bacteriology* 178, no. 13: 3846–53. https://doi.org/10.1128/jb.178.13.3846-3853.1996.

Kapadia, Bhaveen H, Richard A Berg, Jacqueline A Daley, Jan Fritz, Anil Bhave, and Michael A Mont. 2016. "Periprosthetic Joint Infection." *The Lancet* 387, no. 10016: 386–94. https://doi.org/10.1016/s0140-67361461798-0.

Karruli, Arta, Christian Catalini, Chiara D'Amore, Francesco Foglia, Fabio Mari, Arjan Harxhi, Massimiliano Galdiero, and Emanuele Durante-Mangoni. 2023. "Evidence-Based Treatment of *Pseudomonas* Aeruginosa Infections: A Critical Reappraisal." *Antibiotics* 12, no. 2: 399. https://doi.org/10.3390/antibiotics12020399.

Keffer-Wilkes, Laura Carole, Govardhan Reddy Veerareddygari, and Ute Kothe. 2016. "RNA Modification Enzyme TruB Is a Trna Chaperone." *Proceedings of the National Academy of Sciences* 113, no. 50: 14306–11. https://doi.org/10.1073/pnas.1607512113.

Keman, Deniz, and Ferda Soyer. 2019. "Antibiotic-Resistant *Staphylococcus Aureus* Does Not Develop Resistance to Vanillic Acid and 2-Hydroxycinnamic Acid after Continuous Exposure in Vitro." *ACS Omega* 4, no. 13: 15393–400. https://doi.org/10.1021/acsomega.9b01336.

Khan, Kanwal, Khurshid Jalal, and Reaz Uddin. 2023. "Pangenome Profiling of Novel Drug Target against Vancomycin-Resistant *Enterococcus Faecium*." *Journal of Biomolecular Structure and Dynamics*, 1–14. https://doi.org/10.1080/07391102.2023.2191134.

Khodadadi, Ehsaneh, Elham Zeinalzadeh, Sepehr Taghizadeh, Bahareh Mehramouz, Fadhil S Kamounah, Ehsan Khodadadi, Khudaverdi Ganbarov, Bahman Yousefi, Milad Bastami, and Hossein Samadi Kafil. 2020. "Proteomic Applications in Antimicrobial Resistance and Clinical Microbiology Studies." *Infection and Drug Resistance* Volume 13: 1785–1806. https://doi.org/10.2147/idr.s238446.

Kim, Han, Song Yi Kim, Geun Young Sim, and Joong-Hoon Ahn. 2020. "Synthesis of 4-Hydroxybenzoic Acid Derivatives in *Escherichia Coli*." *Journal of Agricultural and Food Chemistry* 68, no. 36: 9743–49. https://doi.org/10.1021/acs.jafc.0c03149.

Kim, Sun Woo, Dong-Gun Lee, Su-Mi Choi, Chulmin Park, Jae-Cheol Kwon, Si-Hyun Kim, Sun Hee Park, Jung-Hyun Choi, Jin-Hong Yoo, and Wan-Shik Shin. 2010 "Once-Daily Gentamicin Administration for Community-Associated Methicillin Resistant *Staphylococcus aureus* in an *in Vitro* Pharmacodynamic Model: Preliminary Reports for the Advantages for Optimizing Pharmacodynamic Index." *Yonsei Medical Journal* 51, no. 5: 722. https://doi.org/10.3349/ymj.2010.51.5.722.

Klausen, Mikkel, Anders Aaes-Jørgensen, Søren Molin, and Tim Tolker-Nielsen. 2003. "Involvement of Bacterial Migration in the Development of Complex Multicellular Structures in *Pseudomonas* Aeruginosa Biofilms." *Molecular Microbiology* 50, no. 1: 61–68. https://doi.org/10.1046/j.1365-2958.2003.03677.x.

Klockgether, Jens, Antje Munder, Jens Neugebauer, Colin F. Davenport, Frauke Stanke, Karen D. Larbig, Stephan Heeb, et al. 2010. "Genome Diversity of*Pseudomonas Aeruginosa*PAO1 Laboratory Strains." *Journal of Bacteriology* 192, no. 4.

Kloos, Wesley E., and Margaret S. Musselwhite. 1975. "Distribution and Persistence of *Staphylococcus* and *Micrococcus* Species and Other Aerobic Bacteria on Human Skin." *Applied Microbiology* 30, no. 3 1975: 381–95. https://doi.org/10.1128/am.30.3.381-395.1975.

Koshla, Oksana, Oleksandr Yushchuk, Iryna Ostash, Yuriy Dacyuk, Maksym Myronovskyi, Gunilla Jäger, Roderich D. Süssmuth, et al. 2019. "Gene*Miaa*for Post-Transcriptional Modification of TrnaXxaIs Important for Morphological and

Metabolic Differentiation in *Streptomyces*." *Molecular Microbiology* 112, no. 1: 249–65. https://doi.org/10.1111/mmi.14266.

Kumar, Naresh, and Nidhi Goel. 2019. "Phenolic Acids: Natural Versatile Molecules with Promising Therapeutic Applications." *Biotechnology Reports* 24. https://doi.org/10.1016/j.btre.2019.e00370.

Lamichhane, Tek N., Nathan H. Blewett, Amanda K. Crawford, Vera A. Cherkasova, James R. Iben, Thomas J. Begley, Philip J. Farabaugh, and Richard J. Maraia. 2013. "Lack of Trna Modification Isopentenyl-A37 Alters Mrna Decoding and Causes Metabolic Deficiencies in Fission Yeast." *Molecular and Cellular Biology* 33, no. 15: 2918–29. https://doi.org/10.1128/mcb.00278-13.

Le, Katherine Y., and Michael Otto. 2015. "Quorum-Sensing Regulation in Staphylococci—an Overview." *Frontiers in Microbiology* 6. https://doi.org/10.3389/fmicb.2015.01174.

Lee, Hye Sun, and Jeong Ho Chang. 2015. "Antimicrobial spine-bone cement with caffeic acid phenethyl ester for controlled release formulation and in vivo biological assessments" *MedChemComm* 6, no. 2: 327–33. https://doi.org/10.1039/c4md00272e.

Lewis, Kim. 2001. "Riddle of Biofilm Resistance." *Antimicrobial Agents and Chemotherapy* 45, no. 4: 999–1007. https://doi.org/10.1128/aac.45.4.999-1007.2001.

Lewis, Kim. 2020. "The Science of Antibiotic Discovery." *Cell* 181, no. 1: 29–45. https://doi.org/10.1016/j.cell.2020.02.056.

Lin, Qiqi, Jiahui Huang, Zhiqing Liu, Qunyi Chen, Xinbo Wang, Guohui Yu, Ping Cheng, Lian-Hui Zhang, and Zeling Xu. 2022. "TRNA Modification Enzyme Miab Connects Environmental Cues to Activation of *Pseudomonas* Aeruginosa Type III Secretion System." *PLOS Pathogens* 18, no. 12. https://doi.org/10.1371/journal.ppat.1011027.

Lin, Yu-Cheng, William Cole Cornell, Jeanyoung Jo, Alexa Price-Whelan, and Lars E. Dietrich. 2018. “The *Pseudomonas* Aeruginosa Complement of Lactate Dehydrogenases Enables Use of d - and l -Lactate and Metabolic Cross-Feeding.” *mBio* 9, no. 5. https://doi.org/10.1128/mbio.00961-18.

Liu, Yi-Yun, Yang Wang, Timothy R Walsh, Ling-Xian Yi, Rong Zhang, James Spencer, Yohei Doi, et al. 2016. “Emergence of Plasmid-Mediated Colistin Resistance Mechanism MCR-1 in Animals and Human Beings in China: A Microbiological and Molecular Biological Study.” *The Lancet Infectious Diseases* 16, no. 2: 161–68. https://doi.org/10.1016/s1473-30991500424-7.

Lladó, Salvador, Rubén López-Mondéjar, and Petr Baldrian. 2017. “Forest Soil Bacteria: Diversity, Involvement in Ecosystem Processes, and Response to Global Change.” *Microbiology and Molecular Biology Reviews* 81, no. 2. https://doi.org/10.1128/mmbr.00063-16.

Lowy, Franklin D. 1998 “*Staphylococcus Aureus*Infections.” *New England Journal of Medicine* 339, no. 8: 520–32. https://doi.org/10.1056/nejm199808203390806.

Lundquist, Karl, Evan Billings, Maxine Bi, James Wellnitz, and Nicholas Noinaj. 2020. “The Assembly of β-Barrel Membrane Proteins by Bam and Sam.” *Molecular Microbiology* 115, no. 3: 425–35. https://doi.org/10.1111/mmi.14666.

Lv, Xiaoli, Ran Zhang, Jing Wang, and Morigen. 2022. “The Absence of CSDA in Escherichia Coli Increases DNA Replication and Cell Size but Decreases Growth Rate at Low Temperature.” *Biochemical and Biophysical Research Communications* 631: 41–47. https://doi.org/10.1016/j.bbrc.2022.09.005.

Lynch, S. V., L. Dixon, M. R. Benoit, E. L. Brodie, M. Keyhan, P. Hu, D. F. Ackerley, G. L. Andersen, and A. Matin. 2007. “Role of the *Rapa* Gene in Controlling Antibiotic Resistance of *Escherichia Coli* Biofilms.” *Antimicrobial Agents and Chemotherapy* 51, no. 10: 3650–58. https://doi.org/10.1128/aac.00601-07.

Ma, Luyan, Matthew Conover, Haiping Lu, Matthew R. Parsek, Kenneth Bayles, and Daniel J. Wozniak. 2009. “Assembly and Development of the *Pseudomonas*

Aeruginosa Biofilm Matrix." *PLoS Pathogens* 5, no. 3. https://doi.org/10.1371/journal.ppat.1000354.

Ma, Luyan, Shiwei Wang, Di Wang, Matthew R. Parsek, and Daniel J. Wozniak. 2012. "The Roles of Biofilm Matrix Polysaccharide PSL in Mucoid*Pseudomonas Aeruginosa*Biofilms." *FEMS Immunology & Medical Microbiology* 65, no. 2: 377–80. https://doi.org/10.1111/j.1574-695x.2012.00934.x.

Mandal, Santi M., Dipjyoti Chakraborty, and Satyahari Dey. 2010. "Phenolic Acids Act as Signaling Molecules in Plant-Microbe Symbioses." *Plant Signaling & Behavior* 5, no. 4: 359–68. https://doi.org/10.4161/psb.5.4.10871.

Martínez, José Luis, and Fernando Baquero. 2014. "Emergence and Spread of Antibiotic Resistance: Setting a Parameter Space." *Upsala Journal of Medical Sciences* 119, no. 2: 68–77. https://doi.org/10.3109/03009734.2014.901444.

Martinez-Gomez, N. Cecilia, Lauren D. Palmer, Eugenio Vivas, Peter L. Roach, and Diana M. Downs. 2011. "The Rhodanese Domain of Thii Is Both Necessary and Sufficient for Synthesis of the Thiazole Moiety of Thiamine in Salmonella Enterica." *Journal of Bacteriology* 193, no. 18 2011: 4582–87. https://doi.org/10.1128/jb.05325-11.

Melicherčík, Pavel, Karel Kotaška, David Jahoda, Ivan Landor, and Václav Čeřovský. 2022. "Antimicrobial Peptide in Polymethylmethacrylate Bone Cement as a Prophylaxis of Infectious Complications in Orthopedics–an Experiment in a Murine Model." *Folia Microbiologica* 67, no. 5: 785–91. https://doi.org/10.1007/s12223-022-00979-0.

Mendame Ehya, Regis Ernest, Hao Zhang, Baiwen Qi, and Aixi Yu. 2021. "Application and Clinical Effectiveness of Antibiotic-Loaded Bone Cement to Promote Soft Tissue Granulation in the Treatment of Neuropathic Diabetic Foot Ulcers Complicated by Osteomyelitis: A Randomized Controlled Trial." *Journal of Diabetes Research* 2021: 1–12. https://doi.org/10.1155/2021/9911072.

Mensah, Lydia M., and Brian J. Love. 2021. “A Meta-Analysis of Bone Cement Mediated Antibiotic Release: Overkill, but a Viable Approach to Eradicate Osteomyelitis and Other Infections Tied to Open Procedures.” *Materials Science and Engineering: C* 123: 111999. https://doi.org/10.1016/j.msec.2021.111999.

Miragaia, M., J. C. Thomas, I. Couto, M. C. Enright, and H. de Lencastre. 2007. “Inferring a Population Structure for *Staphylococcus Epidermidis* from Multilocus Sequence Typing Data.” *Journal of Bacteriology* 189, no. 6: 2540–52. https://doi.org/10.1128/jb.01484-06.

Mishra, Meenu, Matthew S. Byrd, Susan Sergeant, Abul K. Azad, Matthew R. Parsek, Linda McPhail, Larry S. Schlesinger, and Daniel J. Wozniak. 2011. “*Pseudomonas* Aeruginosa PSL Polysaccharide Reduces Neutrophil Phagocytosis and the Oxidative Response by Limiting Complement-Mediated Opsonization.” *Cellular Microbiology* 14, no. 1: 95–106. https://doi.org/10.1111/j.1462-5822.2011.01704.x.

Moradali, M. Fata, and Bernd H. Rehm. 2020. “Bacterial Biopolymers: From Pathogenesis to Advanced Materials.” *Nature Reviews Microbiology* 18, no. 4: 195–210. https://doi.org/10.1038/s41579-019-0313-3.

Moradali, M. Fata, Shirin Ghods, and Bernd H. Rehm. 2017. “*Pseudomonas* Aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence.” *Frontiers in Cellular and Infection Microbiology* 7. https://doi.org/10.3389/fcimb.2017.00039.

Morita, Yuji, Lily Cao, Virginia C. Gould, Matthew B. Avison, and Keith Poole. 2006. “*Nald* Encodes a Second Repressor of the *Mexab-Oprm* Multidrug Efflux Operon of *Pseudomonas Aeruginosa*.” *Journal of Bacteriology* 188, no. 24: 8649–54. https://doi.org/10.1128/jb.01342-06.

Moynié, Lucile, Alexandre Luscher, Dora Rolo, Daniel Pletzer, Antoni Tortajada, Helge Weingart, Yvonne Braun, Malcolm G. Page, James H. Naismith, and Thilo Köhler. 2017. “Structure and Function of the Piua and PIRA Siderophore-Drug Receptors from *Pseudomonas* Aeruginosa and Acinetobacter Baumannii.” *Antimicrobial Agents and Chemotherapy* 61, no. 4. https://doi.org/10.1128/aac.02531-16.

Muhammad, M.H. *et al*. 2020 “Beyond risk: Bacterial biofilms and their regulating approaches,” *Frontiers in Microbiology*, 11. Available at: https://doi.org/10.3389/fmicb.2020.00928.

Murray, B E. 1990. “The Life and Times of the Enterococcus.” *Clinical Microbiology Reviews* 3, no. 1: 46–65. https://doi.org/10.1128/cmr.3.1.46.

Musser, J M, P M Schlievert, A W Chow, P Ewan, B N Kreiswirth, V T Rosdahl, A S Naidu, W Witte, and R K Selander. 1990. “A Single Clone of Staphylococcus Aureus Causes the Majority of Cases of Toxic Shock Syndrome.” *Proceedings of the National Academy of Sciences* 87, no. 1: 225–29. https://doi.org/10.1073/pnas.87.1.225.

Nuryastuti, Titik, Bastiaan P. Krom, Abu T. Aman, Henk J. Busscher, and Henny C. van der Mei. 2010. “ICA-Expression and Gentamicin Susceptibility of Staphylococcus Epidermidis Biofilm on Orthopedic Implant Biomaterials.” *Journal of Biomedical Materials Research Part A* 96A, no. 2: 365–71. https://doi.org/10.1002/jbm.a.32984.

O’Toole, George A. 2011. “Microtiter Dish Biofilm Formation Assay.” *Journal of Visualized Experiments*, no. 47. https://doi.org/10.3791/2437.

Oluyombo, Olubukola, Christopher N. Penfold, and Stephen P. Diggle. 2019. “Competition in Biofilms between Cystic Fibrosis Isolates of *Pseudomonas Aeruginosa* Is Shaped by R-Pyocins.” *mBio* 10, no. 1. https://doi.org/10.1128/mbio.01828-18.

O'Toole, George A., and Roberto Kolter. 1998. “Flagellar and Twitching Motility Are Necessary for*Pseudomonas Aeruginosa*Biofilm Development.” *Molecular Microbiology* 30, no. 2: 295–304. https://doi.org/10.1046/j.1365-2958.1998.01062.x.

Ottaviano, E. *et al.* 2021 “Candida albicans biofilm inhibition by two Vaccinium macrocarpon cranberry urinary metabolites: 5-3′,4′-dihydroxyphenyl-γ-

valerolactone and 4-hydroxybenzoic acid," *Microorganisms*, 97, p. 1492. Available at: https://doi.org/10.3390/microorganisms9071492.

Otto, Michael. 2009. "Staphylococcus Epidermidis — the 'Accidental' Pathogen." *Nature Reviews Microbiology* 7, no. 8: 555–67. https://doi.org/10.1038/nrmicro2182.

Ozdemir, Ozgun O., and Ferda Soyer. 2020. "*Pseudomonas Aeruginosa* Presents Multiple Vital Changes in Its Proteome in the Presence of 3-Hydroxyphenylacetic Acid, a Promising Antimicrobial Agent." *ACS Omega* 5, no. 32: 19938–51. https://doi.org/10.1021/acsomega.0c00703.

Parte, Aidan C. 2013. "LPSN—List of Prokaryotic Names with Standing in Nomenclature." *Nucleic Acids Research* 42, no. D1. https://doi.org/10.1093/nar/gkt1111.

Penner, Murray J., Bassam A. Masri, and Clive P. Duncan. 1996. "Elution Characteristics of Vancomycin and Tobramycin Combined in Acrylic Bone—Cement." *The Journal of Arthroplasty* 11, no. 8: 939–44. https://doi.org/10.1016/s0883-54039680135-5.

Pereira, David, Patrícia Valentão, José Pereira, and Paula Andrade. 2009. "Phenolics: From Chemistry to Biology." *Molecules* 14, no. 6: 2202–11. https://doi.org/10.3390/molecules14062202.

Pham, T. H. 2004. "The Role of Polyhydroxyalkanoate Biosynthesis by *Pseudomonas* Aeruginosa in Rhamnolipid and Alginate Production as Well as Stress Tolerance and Biofilm Formation." *Microbiology* 150, no. 10: 3405–13. https://doi.org/10.1099/mic.0.27357-0.

Piatek, Magdalena, Cillian O'Beirne, Zoe Beato, Matthias Tacke, and Kevin Kavanagh. 2023. "*Pseudomonas* Aeruginosa and Staphylococcus Aureus Display Differential Proteomic Responses to the Silveri Compound, SBC3." *Antibiotics* 12, no. 2: 348. https://doi.org/10.3390/antibiotics12020348.

Prestinaci, Francesca, Patrizio Pezzotti, and Annalisa Pantosti. 2015. “Antimicrobial Resistance: A Global Multifaceted Phenomenon.” *Pathogens and Global Health* 109, no. 7: 309–18. https://doi.org/10.1179/2047773215y.0000000030.

Prinz, Julia, Marianne Wink, Sonja Neuhaus, Markus C. Grob, Heinrich Walt, Philipp P. Bosshard, and Yvonne Achermann. 2023. “Effective Biofilm Eradication on Orthopedic Implants with Methylene Blue Based Antimicrobial Photodynamic Therapy in Vitro.” *Antibiotics* 12, no. 1: 118. https://doi.org/10.3390/antibiotics12010118.

Puhto, Ari-Pekka, Teija M. Puhto, Tuukka T. Niinimäki, Juhana I. Leppilahti, and Hannu P.T. Syrjälä. 2014. “Two-Stage Revision for Prosthetic Joint Infection: Outcome and Role of Reimplantation Microbiology in 107 Cases.” *The Journal of Arthroplasty* 29, no. 6: 1101–4. https://doi.org/10.1016/j.arth.2013.12.027.

Raney, Kevin D., Alicia K. Byrd, and Suja Aarattuthodiyil. 2012. “Structure and Mechanisms of SF1 DNA Helicases.” *Advances in Experimental Medicine and Biology*, 17–46. https://doi.org/10.1007/978-1-4614-5037-5_2.

Rasamiravaka, Tsiry, Quentin Labtani, Pierre Duez, and Mondher El Jaziri. 2015. “The Formation of Biofilms by*Pseudomonas Aeruginosa*: A Review of the Natural and Synthetic Compounds Interfering with Control Mechanisms.” *BioMed Research International* 2015: 1–17. https://doi.org/10.1155/2015/759348.

Rasigade, Jean-Philippe, and François Vandenesch. 2014. “Staphylococcus Aureus: A Pathogen with Still Unresolved Issues.” *Infection, Genetics and Evolution* 21: 510–14. https://doi.org/10.1016/j.meegid.2013.08.018.

Rehm, B. H., and S. Valla. 1997. “Bacterial Alginates: Biosynthesis and Applications.” *Applied Microbiology and Biotechnology* 48, no. 3: 281–88. https://doi.org/10.1007/s002530051051.

Rehm, Bernd H. 2010. “Bacterial Polymers: Biosynthesis, Modifications and Applications.” *Nature Reviews Microbiology* 8, no. 8: 578–92. https://doi.org/10.1038/nrmicro2354.

Rempe, Caroline S., Kellie P. Burris, Scott C. Lenaghan, and C. Neal Stewart. 2017. “The Potential of Systems Biology to Discover Antibacterial Mechanisms of Plant Phenolics.” *Frontiers in Microbiology* 8. https://doi.org/10.3389/fmicb.2017.00422.

Ren, Huan, Yiwei Liu, Jingyi Zhou, Yuqing Long, Chang Liu, Bin Xia, Jing Shi, et al. 2019. “Combination of Azithromycin and Gentamicin for Efficient Treatment of *Pseudomonas* Aeruginosa Infections.” *The Journal of Infectious Diseases* 220, no. 10: 1667–78. https://doi.org/10.1093/infdis/jiz341.

Rodríguez-Rojas, Alexandro, Ana Mena, Soledad Martín, Nuria Borrell, Antonio Oliver, and Jesús Blázquez. 2009. “Inactivation of the HMGA Gene of *Pseudomonas* Aeruginosa Leads to Pyomelanin Hyperproduction, Stress Resistance and Increased Persistence in Chronic Lung Infection.” *Microbiology* 155, no. 4: 1050–57. https://doi.org/10.1099/mic.0.024745-0.

Rogers, Kathie L., Paul D. Fey, and Mark E. Rupp. 2009. “Coagulase-Negative Staphylococcal Infections.” *Infectious Disease Clinics of North America* 23, no. 1: 73–98. https://doi.org/10.1016/j.idc.2008.10.001.

Rollet, Cécile, Laurent Gal, and Jean Guzzo. 2008. “Biofilm-Detached Cells, a Transition from a Sessile to a Planktonic Phenotype: A Comparative Study of Adhesion and Physiological Characteristics in *Pseudomonas* Aeruginosa.” *FEMS Microbiology Letters* 290, no. 2: 135–42. https://doi.org/10.1111/j.1574-6968.2008.01415.x.

Ryder, Cynthia, Matthew Byrd, and Daniel J Wozniak. 2007. “Role of Polysaccharides in *Pseudomonas* Aeruginosa Biofilm Development.” *Current Opinion in Microbiology* 10, no. 6: 644–48. https://doi.org/10.1016/j.mib.2007.09.010.

Sabater-Martos, Marta, Miguel A. Verdejo, Laura Morata, Ernesto Muñoz-Mahamud, Ernesto Guerra-Farfan, Juan C. Martinez-Pastor, and Alex Soriano. “Antimicrobials in Polymethylmethacrylate: From Prevention to Prosthetic Joint Infection Treatment: Basic Principles and Risk of Resistance.” *Arthroplasty* 5, no. 1 2023. https://doi.org/10.1186/s42836-023-00166-7.

Salgado-Pabón, Wilmara, Laura Breshears, Adam R. Spaulding, Joseph A. Merriman, Christopher S. Stach, Alexander R. Horswill, Marnie L. Peterson, and Patrick M. Schlievert. 2013. “Superantigens Are Critical for Staphylococcus Aureus Infective Endocarditis, Sepsis, and Acute Kidney Injury.” *mBio* 4, no. 4. https://doi.org/10.1128/mbio.00494-13.

Sánchez-Maldonado, A.F., A. Schieber, and M.G. Gänzle. 2011. “Structure-Function Relationships of the Antibacterial Activity of Phenolic Acids and Their Metabolism by Lactic Acid Bacteria.” *Journal of Applied Microbiology* 111, no. 5: 1176–84. https://doi.org/10.1111/j.1365-2672.2011.05141.x.

Schinner, Silvia, Florian Engelhardt, Matthias Preusse, Janne Gesine Thöming, Jürgen Tomasch, and Susanne Häussler. 2020. “Genetic Determinants of *Pseudomonas* Aeruginosa Fitness during Biofilm Growth.” *Biofilm* 2: 100023. https://doi.org/10.1016/j.bioflm.2020.100023.

Schleifer, K. H., and R. Kilpper-Balz. 1984. “Transfer of Streptococcus Faecalis and Streptococcus Faecium to the Genus Enterococcus Nom. Rev. as Enterococcus Faecalis Comb. Nov. and Enterococcus Faecium Comb. Nov..” *International Journal of Systematic Bacteriology* 34, no. 1: 31–34. https://doi.org/10.1099/00207713-34-1-31.

Schmidt, Axel, Cécile Batailler, Timothy Lording, Roger Badet, Elvire Servien, Sébastien Lustig, Michel P. Bonnin, et al. 2020. “Why Reintervention after Total Knee Arthroplasty Fails? A Consecutive Cohort of 1170 Surgeries.” *The Journal of Arthroplasty* 35, no. 9: 2550–60. https://doi.org/10.1016/j.arth.2020.04.043.

Schwarz, Edward M., Alex C. McLaren, Thomas P. Sculco, Barry Brause, Mathias Bostrom, Stephen L. Kates, Javad Parvizi, et al. 2020. “Adjuvant Antibiotic-loaded Bone Cement: Concerns with Current Use and Research to Make It Work.” *Journal of Orthopaedic Research* 39, no. 2: 227–39. https://doi.org/10.1002/jor.24616.

Scott, C.P. and Higham, P.A. 2003 “Antibiotic bone cement for the treatment of *pseudomonas* aeruginosa in joint arthroplasty: Comparison of tobramycin and

gentamicin-loaded cements," *Journal of Biomedical Materials Research*, 64B2, pp. 94–98. Available at: https://doi.org/10.1002/jbm.b.10515.

Sharma, D., Misba, L. and Khan, A.U. 2019 "Antibiotics versus biofilm: An emerging battleground in Microbial Communities," *Antimicrobial Resistance & Infection Control*, 81. Available at: https://doi.org/10.1186/s13756-019-0533-3.

Silo-Suh, Laura, Sang-Jin Suh, Paul V. Phibbs, and Dennis E. Ohman. 2005. "Adaptations of *Pseudomonas Aeruginosa* to the Cystic Fibrosis Lung Environment Can Include Deregulation of *Zwf*, Encoding Glucose-6-Phosphate Dehydrogenase." *Journal of Bacteriology* 187, no. 22: 7561–68. https://doi.org/10.1128/jb.187.22.7561-7568.2005.

Sloan, Matthew, Ajay Premkumar, and Neil P. Sheth. 2018. "Projected Volume of Primary Total Joint Arthroplasty in the U.S., 2014 to 2030." *Journal of Bone and Joint Surgery* 100, no. 17: 1455–60. https://doi.org/10.2106/jbjs.17.01617.

Sneath, Peter A., Nicholas S. Mair, M. Elisabeth Sharpe, John G. Holt, W. E. Kloos, and K H Schleifer. 1986. "Genus IV. Staphylococcus." Essay. In *Bergey's Manual of Systematic Bacteriology* 2, Vol. 2. London: Wiliams and Wilkins.

Sobel, Mara L., Didier Hocquet, Lily Cao, Patrick Plesiat, and Keith Poole. 2005. "Mutations in PA3574 *Nald* Lead to Increased Mexab-OPRM Expression and Multidrug Resistance in Laboratory and Clinical Isolates of *Pseudomonas Aeruginosa*." *Antimicrobial Agents and Chemotherapy* 49, no. 5: 1782–86. https://doi.org/10.1128/aac.49.5.1782-1786.2005.

Sønderholm, Majken, Kasper Nørskov Kragh, Klaus Koren, Tim Holm Jakobsen, Sophie E. Darch, Maria Alhede, Peter Østrup Jensen, Marvin Whiteley, Michael Kühl, and Thomas Bjarnsholt. 2017. "*Pseudomonas* Aeruginosa Aggregate Formation in an Alginate Bead Model System Exhibits *in Vivo* -like Characteristics." *Applied and Environmental Microbiology* 83, no. 9. https://doi.org/10.1128/aem.00113-17.

Staudinger, Benjamin J., Jocelyn Fraga Muller, Skarphéðinn Halldórsson, Blaise Boles, Angus Angermeyer, Dao Nguyen, Henry Rosen, et al. 2014. "Conditions

Associated with the Cystic Fibrosis Defect Promote Chronic *Pseudomonas Aeruginosa* Infection." *American Journal of Respiratory and Critical Care Medicine* 189, no. 7: 812–24. https://doi.org/10.1164/rccm.201312-2142oc.

Stepanek, Jennifer J., Tadeja Lukežič, Ines Teichert, Hrvoje Petković, and Julia E. Bandow. 2016. "Dual Mechanism of Action of the Atypical Tetracycline Chelocardin." *Biochimica et Biophysica Acta BBA - Proteins and Proteomics* 1864, no. 6: 645–54. https://doi.org/10.1016/j.bbapap.2016.03.004.

Strempel, Nikola, Anke Neidig, Michael Nusser, Robert Geffers, Julien Vieillard, Olivier Lesouhaitier, Gerald Brenner-Weiss, and Joerg Overhage. 2013. "Human Host Defense Peptide LL-37 Stimulates Virulence Factor Production and Adaptive Resistance in *Pseudomonas* Aeruginosa." *PLoS ONE* 8, no. 12. https://doi.org/10.1371/journal.pone.0082240.

Sukhodolets, Maxim V., and Ding Jun Jin. 1998. "Rapa, a Novel RNA Polymerase-Associated Protein, Is a Bacterial Homolog of swi2/SNF2." *Journal of Biological Chemistry* 273, no. 12: 7018–23. https://doi.org/10.1074/jbc.273.12.7018.

Sultan, Assem A., Linsen T. Samuel, Erica Umpierrez, Andrew Swiergosz, Jacob Rabin, Bilal Mahmood, and Michael A. Mont. 2019. "Routine Use of Commercial Antibiotic-Loaded Bone Cement in Primary Total Joint Arthroplasty: A Critical Analysis of the Current Evidence." *Annals of Translational Medicine* 7, no. 4: 73–73. https://doi.org/10.21037/atm.2018.11.50.

Sultana, Sujala T, Douglas R Call, and Haluk Beyenal. 2016. "Eradication of *Pseudomonas* Aeruginosa Biofilms and Persister Cells Using an Electrochemical Scaffold and Enhanced Antibiotic Susceptibility." *npj Biofilms and Microbiomes* 2, no. 1. https://doi.org/10.1038/s41522-016-0003-0.

Suvorova, Inna A., Yuri D. Korostelev, and Mikhail S. Gelfand. 2015. "GNTR Family of Bacterial Transcription Factors and Their DNA Binding Motifs: Structure, Positioning and Co-Evolution." *PLOS ONE* 10, no. 7. https://doi.org/10.1371/journal.pone.0132618.

Szymski, Dominik, Nike Walter, Paula Krull, Oliver Melsheimer, Alexander Grimberg, Volker Alt, Arnd Steinbrueck, and Markus Rupp. 2023. “Infection after Intracapsular Femoral Neck Fracture – Does Antibiotic-Loaded Bone Cement Reduce Infection Risk after Hemiarthroplasty and Total Hip Arthroplasty?” *Bone & Joint Research* 12, no. 5: 331–38. https://doi.org/10.1302/2046-3758.125.bjr-2022-0314.r1.

Tacconelli, Evelina, Elena Carrara, Alessia Savoldi, Stephan Harbarth, Marc Mendelson, Dominique L Monnet, Céline Pulcini, et al. 2018. “Discovery, Research, and Development of New Antibiotics: The Who Priority List of Antibiotic-Resistant Bacteria and Tuberculosis.” *The Lancet Infectious Diseases* 18, no. 3: 318–27. https://doi.org/10.1016/s1473-30991730753-3.

Tammam, Stephanie, Liliana M. Sampaleanu, Jason Koo, Kumararaaj Manoharan, Mark Daubaras, Lori L. Burrows, and P. Lynne Howell. 2013. “PILMNOPQ from the *Pseudomonas* Aeruginosa Type IV Pilus System Form a Transenvelope Protein Interaction Network That Interacts with Pila.” *Journal of Bacteriology* 195, no. 10: 2126–35. https://doi.org/10.1128/jb.00032-13.

Tang, Yi-Wei, Min Wu, and Xuefeng Li. 2015. “Klebsiella Pneumoniae and *Pseudomonas* Aeruginosa.” Essay. In *Molecular Medical Microbiology*, 2nd ed., 1547–64. London: Academic Press.

Tang, Yi-Wei, W Wu, Y Jin, F Bai, and S Jin. 2015. “*Pseudomonas* Aeruginosa.” Essay. In *Molecular Medical Microbiology,* 753–67. London: Academic Press.

Thi, Minh Tam, David Wibowo, and Bernd H.A. Rehm. 2020. “*Pseudomonas* Aeruginosa Biofilms.” *International Journal of Molecular Sciences* 21, no. 22: 8671. https://doi.org/10.3390/ijms21228671.

Tong, Steven Y., Joshua S. Davis, Emily Eichenberger, Thomas L. Holland, and Vance G. Fowler. 2015 “Staphylococcus Aureus Infections: Epidemiology, Pathophysiology, Clinical Manifestations, and Management.” *Clinical Microbiology Reviews* 28, no. 3: 603–61. https://doi.org/10.1128/cmr.00134-14.

Top, Janetta, Rob Willems, and Marc Bonten. 2008. “Emergence of CC17*Enterococcus Faecium*: From Commensal to Hospital-Adapted Pathogen.” *FEMS Immunology & Medical Microbiology* 52, no. 3: 297–308. https://doi.org/10.1111/j.1574-695x.2008.00383.x.

Tsakou, Foteini, Rosa Jersie-Christensen, Håvard Jenssen, and Biljana Mojsoska. 2020. “The Role of Proteomics in Bacterial Response to Antibiotics.” *Pharmaceuticals* 13, no. 9: 214. https://doi.org/10.3390/ph13090214.

Tsao, R., and Z Deng. 2004. “Separation Procedures for Naturally Occurring Antioxidant Phytochemicals.” *Journal of Chromatography B* 812, no. 1-2: 85–99. https://doi.org/10.1016/s1570-02320400764-0.

Tseng, Boo Shan, Wei Zhang, Joe J. Harrison, Tam P. Quach, Jisun Lee Song, Jon Penterman, Pradeep K. Singh, David L. Chopp, Aaron I. Packman, and Matthew R. Parsek. 2013. “The Extracellular Matrix Protects*Pseudomonas Aeruginosa*Biofilms by Limiting the Penetration of Tobramycin.” *Environmental Microbiology*. https://doi.org/10.1111/1462-2920.12155.

Tuon, F.F. *et al.* 2022 “Pathogenesis of the *pseudomonas* aeruginosa biofilm: A review,” *Pathogens*, 113, p. 300. Available at: https://doi.org/10.3390/pathogens11030300.

Turnbull, Lynne, Masanori Toyofuku, Amelia L. Hynen, Masaharu Kurosawa, Gabriella Pessi, Nicola K. Petty, Sarah R. Osvath, et al. 2016. “Explosive Cell Lysis as a Mechanism for the Biogenesis of Bacterial Membrane Vesicles and Biofilms.” *Nature Communications* 7, no. 1. https://doi.org/10.1038/ncomms11220.

Vaishya, Raju, Mayank Chauhan, and Abhishek Vaish. 2013. “Bone Cement.” *Journal of Clinical Orthopaedics and Trauma* 4, no. 4: 157–63. https://doi.org/10.1016/j.jcot.2013.11.005.

Van Staden, Anton Du Preez. 2011. "Developing bone cement implants impregnated with bacteriocins for prevention of infections Ph.D. diss., Stellenbosch: Stellenbosch University”.

Vrontou, Eleftheria, and Anastassios Economou. 2004. “Structure and Function of SECA, the Preprotein Translocase Nanomotor.” *Biochimica et Biophysica Acta BBA - Molecular Cell Research* 1694, no. 1-3: 67–80. https://doi.org/10.1016/j.bbamcr.2004.06.003.

Wang, Xu-Na, Kui-Yang Wang, Xue-Song Zhang, Chao Yang, and Xue-Ying Li. 2018. “4-Hydroxybenzoic Acid 4-HBA Enhances the Sensitivity of Human Breast Cancer Cells to Adriamycin as a Specific HDAC6 Inhibitor by Promoting HIPK2/P53 Pathway.” *Biochemical and Biophysical Research Communications* 504, no. 4: 812–19. https://doi.org/10.1016/j.bbrc.2018.08.043.

Webb, Jeremy S., Mathew Lau, and Staffan Kjelleberg. 2004. “Bacteriophage and Phenotypic Variation in *Pseudomonas Aeruginosa* Biofilm Development.” *Journal of Bacteriology* 186, no. 23: 8066–73. https://doi.org/10.1128/jb.186.23.8066-8073.2004.

Wei, Jian, Siqi Zhou, Hanwen Gu, and Kai Tong. 2022. “Meropenem-Loaded Cement Is Effective in Preventing Gram-Negative Osteomyelitis in an Animal Model.” *Clinical Orthopaedics & Related Research* 481, no. 1: 177–89. https://doi.org/10.1097/corr.0000000000002364.

Whiteley, Marvin, M. Gita Bangera, Roger E. Bumgarner, Matthew R. Parsek, Gail M. Teitzel, Stephen Lory, and E. P. Greenberg. 2001. “Gene Expression in *Pseudomonas* Aeruginosa Biofilms.” *Nature* 413, no. 6858: 860–64. https://doi.org/10.1038/35101627.

WHO. 2017. *Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics*.

Willyard, Cassandra. 2017. “The Drug-Resistant Bacteria That Pose the Greatest Health Threats.” *Nature* 543, no. 7643: 15–15. https://doi.org/10.1038/nature.2017.21550.

Wilton, Mike, Laetitia Charron-Mazenod, Richard Moore, and Shawn Lewenza. 2016 “Extracellular DNA Acidifies Biofilms and Induces Aminoglycoside Resistance in

Pseudomonas Aeruginosa." *Antimicrobial Agents and Chemotherapy* 60, no. 1: 544–53. https://doi.org/10.1128/aac.01650-15.

Wilton, Mike, Megan J. Wong, Le Tang, Xiaoye Liang, Richard Moore, Michael D. Parkins, Shawn Lewenza, and Tao G. Dong. 2016 "Chelation of Membrane-Bound Cations by Extracellular DNA Activates the Type VI Secretion System in *Pseudomonas* Aeruginosa." *Infection and Immunity* 84, no. 8: 2355–61. https://doi.org/10.1128/iai.00233-16.

Wong, Chi Chun, Ka Wing Cheng, Qing-Yu He, and Feng Chen. 2008. "Unraveling the Molecular Targets of Natural Products: Insights from Genomic and Proteomic Analyses." *PROTEOMICS - Clinical Applications* 2, no. 3: 338–54. https://doi.org/10.1002/prca.200880002.

Xia, Yushan, Yuding Weng, Congjuan Xu, Dan Wang, Xiaolei Pan, Zhenyang Tian, Bin Xia, et al. 2020. "Endoribonuclease Ybey Is Essential for RNA Processing and Virulence in *Pseudomonas* Aeruginosa." *mBio* 11, no. 3. https://doi.org/10.1128/mbio.00659-20.

Yang, Liang, Yifan Hu, Yang Liu, Jingdong Zhang, Jens Ulstrup, and Søren Molin. 2011. "Distinct Roles of Extracellular Polymeric Substances in *Pseudomonas* Aeruginosa Biofilm Development." *Environmental Microbiology* 13, no. 7: 1705–17. https://doi.org/10.1111/j.1462-2920.2011.02503.x.

Young, Crystal L., and Katrin Karbstein. 2011. "The Roles of S1 RNA-Binding Domains in RRP5's Interactions with Pre-Rrna." *RNA* 17, no. 3: 512–21. https://doi.org/10.1261/rna.2458811.

Zavascki, A. P., L. Z. Goldani, J. Li, and R. L. Nation. 2007. "Polymyxin B for the Treatment of Multidrug-Resistant Pathogens: A Critical Review." *Journal of Antimicrobial Chemotherapy* 60, no. 6: 1206–15. https://doi.org/10.1093/jac/dkm357.

Zeng, Bo, Chong Wang, Pansong Zhang, Zisheng Guo, Lin Chen, and Kangmin Duan. 2020. "Heat Shock Protein Dnaj in *Pseudomonas* Aeruginosa Affects Biofilm

Formation via Pyocyanin Production." *Microorganisms* 8, no. 3: 395. https://doi.org/10.3390/microorganisms8030395.

Zhu, Zikun, Shuai Wang, and Shu-ou Shan. 2022. "Ribosome Profiling Reveals Multiple Roles of Seca in Cotranslational Protein Export." *Nature Communications* 13, no. 1. https://doi.org/10.1038/s41467-022-31061-5.

Ziegler, Slava, Verena Pries, Christian Hedberg, and Herbert Waldmann. 2013. "Target Identification for Small Bioactive Molecules: Finding the Needle in the Haystack." *Angewandte Chemie International Edition* 52, no. 10: 2744–92. https://doi.org/10.1002/anie.201208749.

Žiemytė, Miglė, Miguel Carda-Diéguez, Juan C. Rodríguez-Díaz, Maria P. Ventero, Alex Mira, and María D. Ferrer. 2021. "Real-Time Monitoring of *Pseudomonas Aeruginosa* Biofilm Growth Dynamics and Persister Cells' Eradication." *Emerging Microbes & Infections* 10, no. 1: 2062–75. https://doi.org/10.1080/22221751.2021.1994355.

Zourob, Mohammed, Souna Elwary, Anthony Turner, Tracey Elizabeth Love, and Barbara Jones. 2008. "Introduction to Pathogenic Bacteria." Essay. In *Principles of Bacterial Detection: Biosensors, Recognition Receptors and Microsystems*, 3–13. New York, NY: Springer.

ZUMFT, Walter G., Andreas DREUSCH, Sabine LOCHELT, Heinrich CUYPERS, Barbel FRIEDRICH, and Beate SCHNEIDER. 1992. "Derived Amino Acid Sequences of the NOSZ Gene Respiratory N2O Reductase from *Alcaligenes eutrophus*, *Pseudomonas* Aeruginosa and *Pseudomonas* Stutzeri Reveal Potential Copper-Binding Residues. Implications for the CUA Site of N2O Reductase and Cytochrome-c Oxidase." *European Journal of Biochemistry* 208, no. 1: 31–40. https://doi.org/10.1111/j.1432-1033.1992.tb17156.x.

APPENDIX A

BOVINE SERUM ALBUMIN STANDARD CURVE

The protein quantification was performed using the Bradford assay. A standard curve was generated by measuring the absorbance of different concentrations of bovine serum albumin (BSA). BSA was prepared as a 1 mg/ml stock solution and then diluted to concentrations ranging from 0 µg/ml to 500 µg/ml for the assay. The dilutions were made by dissolving BSA in a resuspension buffer containing 7 M urea, 2 M thiourea, and 0.1 M Tris-HCl (pH 7.8).

In a 96-well plate, 180 µl of Bradford reagent (1X) was added to each well and mixed with 20 µl of each concentration of BSA. As a blank, 20 µl of phosphate-buffered saline (PBS) or resuspension buffer was mixed with the reagent. After a 10-minute incubation in the dark, the absorbance was measured at 595 nm. The absorbance values at 595 nm were plotted against the corresponding BSA concentrations (µg/ml) to create a standard curve for BSA. This standard curve, depicted in Figure A.1, was used to calculate the concentrations of isolated proteins during SDS-PAGE.

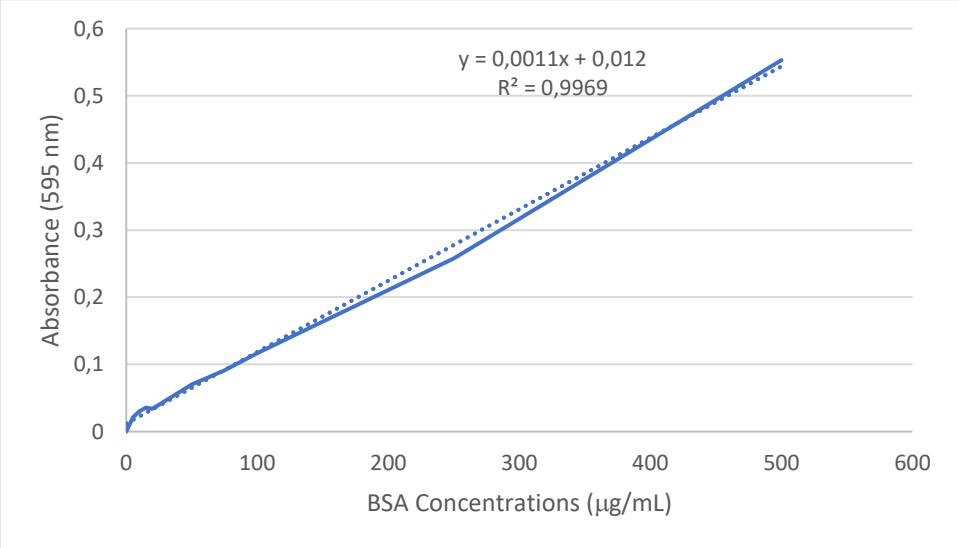

Figure A.1. Bovine Serum Albumin standard curve for proteomics.

APPENDIX B

SEM IMAGES OF *Pseudomonas aeruginosa* (10^1 CFU/mL) IN THE PRESENCE OF 4-HBA

To observe the morphological effects of 4-HBA treatment on *P. aeruginosa*, SEM images of overnight grown 10^1 CFU/mL of control and 1.6 mg/mL of 4-HBA treated *P. aeruginosa* cultures have been compared.

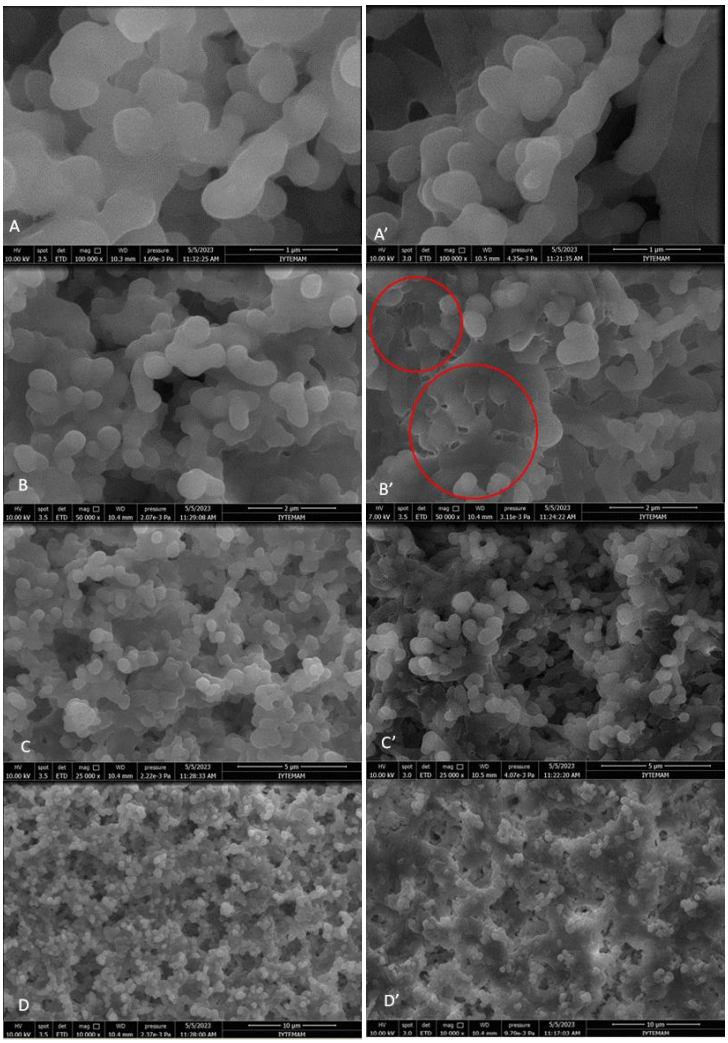

Figure B.1. SEM images of *Pseudomonas* aeruginosa (10^1 CFU/mL) in the presence of 4-HBA.

APPENDIX C

SEM IMAGES OF *Pseudomonas aeruginosa* (10^6 CFU/mL) IN THE PRESENCE OF 4-HBA

To observe the morphological effects of 4-HBA treatment on *P. aeruginosa*, SEM images of overnight grown 10^6 CFU/mL of control and 1.6 mg/mL of 4-HBA treated *P. aeruginosa* cultures have been compared.

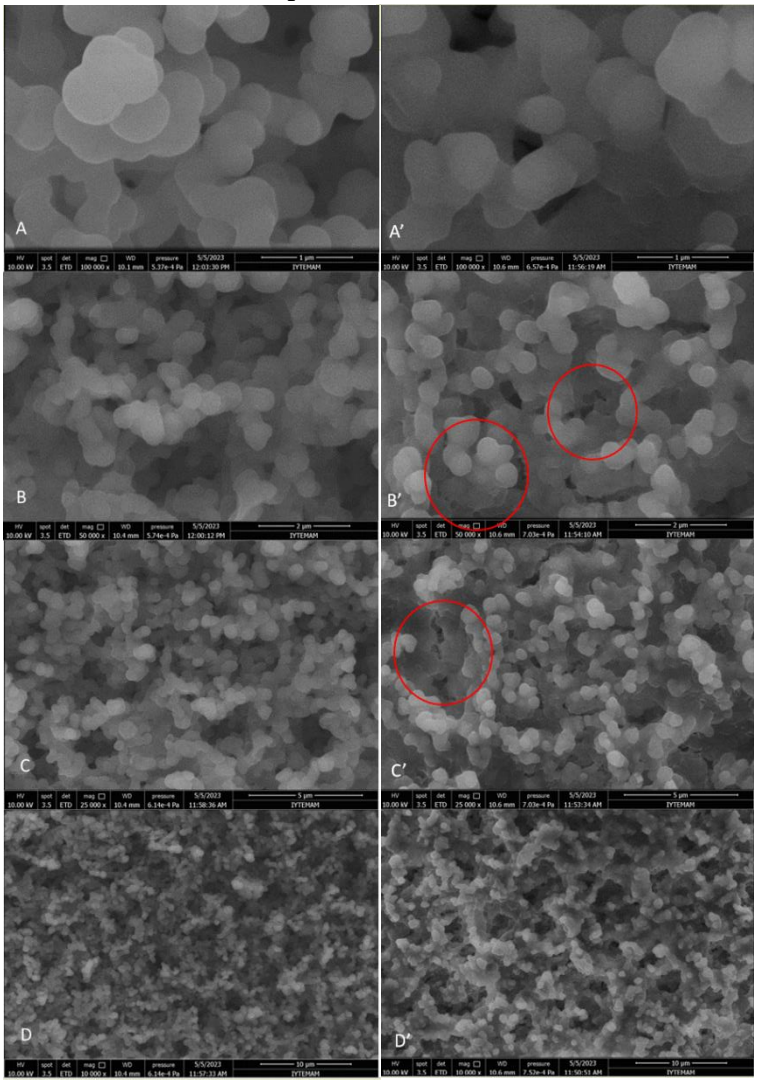

Figure C.1. SEM images of *Pseudomonas* aeruginosa (10^6 CFU/mL) in the presence of 4- HBA.