Exploring the antimicrobial potency of gallium nanoparticles to combat *Acinetobacter baumannii*

By

Dimpy Sanjilkumar Sheth

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Principal Supervisor: Professor Melissa Brown

Co-Supervisor: Dr. Vi Khanh Truong

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ABSTRACT

Acinetobacter baumannii is becoming a significant global health concern due to its rise in hospital-acquired infections and its ability to resist multiple drugs. Infections caused by this bacterium can be deadly, with fatality rates ranging from 30% to 60%. *A. baumannii* can form biofilms, survive on medical surfaces, and develop resistance to antibiotics, making new treatment strategies essential. The bacterium has ability to form biofilms, acquire iron, and produce virulence factors contribute to its strength. Its protective capsule helps it evade antibiotics and the immune system, allowing it to thrive in various environments. The lack of new antimicrobial agents has led researchers to explore alternative approaches like using metals for antibacterial purposes. Gallium, a metal element from Group 13 of the periodic table, shows promise as an antimicrobial agent due to its unique properties such as low melting point and reactivity. Recent studies suggest that gallium-based technologies, including gallium liquid metal, can effectively combat drug-resistant bacteria like multidrug resistant bacteria. However, challenges remain in utilizing gallium safely and effectively in medical settings. Despite these challenges, gallium-based strategies offer a new avenue for combating antibiotic-resistant bacteria like MDR *A. baumannii.* This study aimed to develop and assess the efficacy of galliumbased liquid metal nanoparticles (GaLM NPs) against multidrug-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. The overarching hypothesis posits that GaLM NPs will demonstrate significant antibacterial activity against these resistant *A. baumannii* strains and will effectively reduce biofilm formation. The study is structured around several goals. Developing innovative agents such as GaLM NPs to counteract the ongoing threat posed by *A. baumannii,* which is resistant to several drugs, is the main objective. GaLM NPs are first synthesised and then thoroughly characterised to determine their physicochemical properties.

The second objective is to assess the antibacterial efficacy of GaLM NPs against strains of *A. baumannii,* with a particular emphasis on their capacity to impede bacterial growth and

destabilise formed biofilms. In-depth biofilm assays are used to evaluate this, offering information about the nanoparticles' mode of action and potential as a novel class of antibacterial medicines. This research aims to provide an essential understanding of GaLM NPs interactions with bacterial pathogens, with a focus on the developing problem of antibiotic resistance. A thorough assessment of the nanoparticle's antibacterial activity, capacity to destroy biofilms, and potential as a scalable remedy for getting cure from such infectious bacterial strains. The antimicrobial activity showed the inhibition to the agar by diffusing and creating the inhibition zones. Whereas the antibacterial activity was observed more in the *A. baumannii* ATCC 17978 which showed the higher resistance towards gallium liquid metal Nps. Moreover, the strains showed different OD in growth of biofilm as well due the presence of capsule in outer membrane of bacteria. This underscores the critical need for innovative treatment approaches in the face of increasing drug resistance.

DECLARATION

I certify that this thesis:

1. Does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university.

2. and the research within will not be submitted for any other future degree or diploma without the permission of Flinders University; and
3. to the best of my knowledge and belief, does not contain any material previously published or written by another person except where due reference is made in the text.

Signature of student

Dimpy Sanjilkumar Sheth

Date: 6TH May 2024

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LIST OF ABBREVIATIONS

Abbreviation	Full form

ATCC	American Type Culture Collection
CFU	Colony forming unit
ICU	Intensive care unit
LM	Liquid metal
LB	Luria broth
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
NPs	Nanoparticles
PBS	Phosphate buffered saline
Ps	Particles
ROS	Reactive oxygen species
SEM	Scanning electron microscope
TCP	Tissue culture plate

CHAPER 1: LITERATURE REVIEW

1.1 Classification of *Acinetobacter baumannii*

Acinetobacter baumannii is a major cause of hospital-borne illnesses and an opportunistic pathogen around the world. It is a Gram-negative, an aerobic, and non-fermentative coccobacilli

that is widely distributed in nature (Giamarellou, 2008). These bacteria freely grow to form smooth, creamy, white colonies that are 1.5–2 mm in diameter at 37 °C on commonly used media (Sudhir, 2009). *Acinetobacter* has more than 50 different species, the majority of which are isolates from non-pathogenic environments (Nemec *et al.,* 2011, Wong *et al.*, 2017). *A. baumannii* has been classified as an ESKAPE pathogen, which stands for *Enterobacter species, Pseudomonas aeruginosa, Acinetobacter baumannii, Staphylococcus aureus, Klebsiella pneumoniae, and Enterococcus faecium*. The very first pathogen is on the critical priority list of pathogens for novel antibiotics to be identified as a "red-alert" human pathogen is *A. baumannii* in particular (Gedefie *et al.,* 2021a). These bacteria are easily seen in the open environments such as soil, and water, and are often isolated from medical equipment (Bergogne-Bérézin *et al.*, 2008).

1.2 *A. baumannii:* A troublesome pathogen as an emerging threat to human health:

1.2.1 The significance of *Acinetobacter*

Understanding a particular bacterium's significance involves considering its unique traits, including morphology, growth requirements, frequency of clinical infections, pathogenicity, and antibiotic sensitivity or resistance (Martinez, 2009). The significance of *Acinetobacter's* is mostly associated with its notable characteristics, including its behavioural qualities, which include adaptability, diversity, ability for evolution, and changing virulence factors (Dijkshoorn *et al.,* 2007).

These bacteria were traditionally supposed to be parasitic organisms found in the environment. But due to several circumstances, *Acinetobacter* is now recognised as a significant nosocomial pathogen that causes serious infections and is primarily identified in intensive care units (ICUs) (Bergogne-Berezin and Towner, 1996). Furthermore, recent occurrences have demonstrated the

potential involvement of *Acinetobacter* as a community-acquired bacteria that can cause serious infections under a variety of conditions (Gedefie *et al.,* 2021a)

As per the recent reports from the WHO, it is noted that the mortality rate has increased and reached 30% - 60% due to infections caused by multi-drug resistant (MDR) *A. baumannii* and its resistance towards antibiotics (Runci *et al.,* 2017, Lăzureanu *et al.,* 2016). These traits, along with its ability to form capsules and biofilms, and its intrinsic and acquired resistance to antimicrobial agents, allow *A. baumannii* to persist in a variety of healthcare environments (Gallagher and Baker, 2020). Since antimicrobial treatments have begun to decrease in their effectiveness, it is critical to find novel approaches that will help in the creation of cutting-edge cures and tactics. Multidrug resistance is frequently linked to carbapenem-resistant *A. baumannii* (CRAB) it is an opportunistic pathogen that can cause a various kind of infections to humans in hospitals and it has colistin resistance that has been noted (Gedefie *et al.,* 2021b). There are several Gram-positive and Gram-negative bacteria which have resistance towards the antibiotics which are listed below in (Figure 1).

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Figure 1: List of Gram-negative and Gram-positive bacteria which have resistance towards the antibiotics (Ahmad *et al.,* 2023).

To combat *A. baumannii* infections, researchers have recently investigated several cuttingedge strategies, including nanomedicine, phage therapy, immunotherapy, CRISPR (Saeed *et al.,* 2023). Metal-based antibacterial approaches have drawn a lot of attention among these new tactics because of their distinct qualities and potential effectiveness (Cheeseman *et al.,* 2020). Gallium (Ga) is one such metal that has possible antibacterial characteristics. In the fight against drug-resistant bacteria, Ga-based technologies, especially Ga liquid metal, have demonstrated substantial potential. Gallium, a member of group 13 on the periodic table, possesses unique qualities that make it a desirable option for antibacterial applications (Alamri, 2020). Gallium offers a flexible platform for antimicrobial innovation due to its low melting point, nonvolatility, absence of reactivity with other compounds, and its amorphous solid state at room temperature (Truong *et al.,* 2023a, Nunes *et al.,* 2018).

1.2.2 *A. baumannii* importance

Acinetobacter's taxonomy history has been unorganised, and it is still challenging to identify individual species (Bergogne-Berezin and Towner, 1996). It was noted that only in 1968 a few previously identified bacteria including the Micrococcus calco-aceticus that was first discovered by the dutch microbiologist, Beijerinck (1911) that was grouped together and referred to as the *Acinetobacter* genus. Over the course of the next 30 years, a variety of distinct DNA hybridization groups, or genome species, have been described; however, not all of them have been given names (Bouvet and Grimont, 1986, Bouvet and Jeanjean, 1989). Although the genus *Acinetobacter* is now extensively established, it is still difficult to recognise and classify specific *Acinetobacter* species (Dijkshoorn and Nemec, 2008).

1.3 *A. baumannii* infections

Generally, the majority of *A. baumannii* infections used to occur in hospital settings around the world, specially to the patients who are treated in ICUs and has low immune system (Ansaldi *et al.,* 2011). Previously the use of antibiotics in major surgery, burns, immunosuppression, and the use of intrusive devices were at high risk factors for infections in humans. The majority of *A. baumannii* infections take place in ICUs, where it causes ventilator-associated pneumonia in 4% to 7% of cases. Meningitis and other diseases of the central nervous system can be brought on by this pathogen, especially in those who have had neurological surgery (Lăzureanu *et al.,* 2016) as shown in **FIGURE 1.** Additionally, *A. baumannii* has been identified as the cause agent in osteomyelitis, endocarditis, and soft tissue infections (McConnell *et al.,* 2013). Although healthcare settings account for the majority of *A. baumannii* infections, there is evidence that the organism also contributes significantly to community-acquired illnesses (Dexter *et al.*, 2015). Several risk variables such as male gender, old age, alcoholism, cancer, cerebrovascular illness, diabetes mellitus, renal disease, and liver cirrhosis, have been linked to fatal instances of community-acquired *A. baumannii* pneumonia (Chusri *et al.,* 2019), urinary tract infections (Eze *et al.,* 2018) also been caused by community-acquired infections of *A. baumannii*. According to a study by the National Naval Medical Centre (USA) on post-war wounds in US servicemen from Afghanistan, *A. baumannii* was the most prevalent organism isolated, accounting for 63% of all isolates. Furthermore, *A. baumannii* was the predominant source of infection for 65% of the Bali bombing victims who were treated at Perth Royal Hospital (Australia) (Heath *et al.,* 2003).

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Figure 2: Various types of infection caused by *A. baumannii* and *P. aeruginosa*. Taken from (Badescu *et al.*, 2022)

1.4 The adherence to abiotic surfaces and biofilm formation

1.4.1 The biofilm production

The biofilm formation occurs in a various steps, such as formation of layer, bacterial adhesion, bacterial growth and biofilm maturation (Kokare *et al.,* 2009). Different stages of microbial interactions with surfaces result in the production of extracellular microbial structures. These structures support the initial adhesion, biofilm structure maintenance, and biofilm communication (Zubair *et al.,* 2014).). *A. baumannii* can form biofilm on a range of different surfaces including glass, polypropylene and polycarbonate (Pour *et al.,* 2011), as well as at the air-liquid interface, where it is known as a pellicle (Pompilio *et al.,* 2021, Giles *et al.,* 2015). Even though the majority of *A. baumannii* isolates can generally produce very strong biofilms, various investigations reveal that strains differ greatly in their capacity to do so (Eijkelkamp *et*

al., 2013, Ghimire, 2020, McQueary and Actis, 2011). The location of *A. baumannii* infections may be associated with the ability to produce biofilm. For instance, *A. baumannii* strains isolated from infections associated with indwelling devices such as urinary catheters, venous catheters, and endotracheal tubes of ventilated patients, showed higher biofilm forming capabilities (Alamri *et al.,* 2020). Furthermore, increased biofilm forming capabilities may be linked with an increased severity of infections (McQueary and Actis, 2011).

1.4.2 Persistence of CRAB *A. baumannii*

The *A. baumannii* is one of the most severe bacteria in terms of antibiotic resistance among the "ESKAPE" pathogens (Vrancianu *et al.,* 2021, Navidinia, 2016). It is currently thought to be multidrug-resistant (MDR), extensively drug-resistant (XDR), and even because it exhibits resistance to nearly all dangerous first-line antibiotics used for healthcare-associated infections this risk was noted since 1970's (Agyeman *et al.,* 2022).

Moreover, the proteins, carbohydrates, nucleic acids, and other macromolecules make up the matrix of biofilms that grow on surfaces. Bacteria are protected by the biofilm from environmental harm caused by antibiotics, disinfectants, and host reactions. As a result, biofilms support bacterial effects that are more severe and persistent (Ibrahim *et al.,* 2021). *A. baumannii* strains that produce biofilms are more likely to survive than one that do not survive. Compared to other Gram-negative bacteria, these biofilm-producing strains in intensive care units demonstrated a 10-to-13-day survival rate on dry, undisturbed surfaces. Thus, this kind of bacteria can survive in the hospital even in humid environments.

According to the study by Yang and colleague's microscopic examination of biofilm indicated a relationship between the duration of the treatment and the total amount of antibiotic provided and the inhibition of biofilm formation. At a higher concentration and over a longer duration of treatment, the inhibition was considerably more noticeable (Yang *et al.,* 2019).

Accordingly, increase of biofilm formation as a result of exposure to sub-inhibitory concentrations of antimicrobial compounds has also been observed (Nucleo *et al.,* 2009).

1.4.3 *P. aeruginosa*: Adaptations for survival and the formation of biofilms

Similarly to *A. baumannii*, the ubiquitous Gram-negative bacterium *P. aeruginosa* can thrive on plant and animal tissues, in soil, marshes, and coastal marine habitats (Stover *et al.,* 2000). On moist surfaces like rocks and dirt, it grows biofilms (Costerton *et al.,* 1999). *Pseudomonas* is an opportunistic pathogen that is most linked to ventilator-related pneumonia and infections associated with healthcare settings as shown in Figure 1. Immunocompromised persons, cystic fibrosis sufferers, and, less frequently, healthy people are also affected (Pang *et al.,* 2019). Its genome is comparatively large in comparison to other MDR strains, which means that it can produce more regulatory enzymes involved in the metabolism, transport, and leakage of organic substances. As a result, this bacterium is highly versatile and adaptable to changes in its environment (Stover *et al.,* 2000). As a result, it has evolved the resistance to most existing antibiotics, just like *A. baumannii* (Thi *et al.,* 2020).

Persistent outbreaks of *P. aeruginosa* in clinical settings is thought to be caused by the bacterial synthesis of DNA, proteins, and exopolysaccharides, which leads to the formation of biofilms on the surfaces of lung epithelial cells (Pang *et al.,* 2019). These biofilms are distinguished by quorum sensing, an efficient cell-to-cell communication technique. It is known that the three primary quorum sensing systems LasR, RhlI-RhlR, and PQS-MvfR contribute to the development of mature and distinct *P. aeruginosa* biofilm types as described previously (Thi *et al.,* 2020).

1.5 Biofilm formation and its role in virulence

A biofilm is consider as complex community of microorganisms adhered to a surface and embedded in a self-produced matrix of extracellular polymeric substances (EPS) (Flemming and

Wingender, 2010). This matrix protects the bacteria from environmental stresses, including antimicrobial agents and the host immune response, thereby facilitating chronic infections, and increasing resistance to conventional treatments. Biofilm production in *A. baumannii* increases the pathogen's capacity to persist on abiotic surfaces, like hospital equipment and medical devices, increasing the risk of infection and transmission in clinical settings (Roy *et al.,* 2022). The biofilm growth mechanism of *A. baumannii* contributes to the organism's persistence in the host and environment by enabling the establishment of robust microbial communities that are resistant to immune cell phagocytosis and have an increased resistance to antibiotics (Ayoub Moubareck and Hammoudi Halat, 2020).

Because of the gradient of nutrients and oxygen provided by the structure of biofilms, bacteria can live in various metabolic states in these micro-environments. Given that cells in various physiological states may react differently to antimicrobial medication, it is thought that this heterogeneity contributes to the enhanced resistance of biofilm-associated bacteria to these agents (Mah and O'Toole, 2001). Furthermore, the EPS matrix may function as a physical barrier that prevents antibiotics from penetrating and reducing their effectiveness against bacteria within the biofilm (Fulaz *et al.*, 2019). In the nutshell, *A baumannii* has ability to form biofilms is a crucial component of its pathogenicity, aiding in the pathogen's perseverance, resistance, and ability to survive in medical settings. To effectively prevent and cure infections caused by this risky pathogen, it is imperative to comprehend the dynamics of biofilm development and its involvement in *A. baumannii* pathogenicity.

1.5.1 Virulence of *A. baumannii* in clinical environment

A. baumannii exhibits a range of virulence factors that facilitate its survival and pathogenicity under various growing conditions, their shape might range from coccoidal to rod (Peleg *et al.,* 2008). *A. baumannii* is on high risk for humans and is one of the leading causes of nosocomial infection worldwide (Gentile *et al.,* 2014). This bacterium is harmful in part because

it is multidrug-resistant (MDR) and can form biofilms on abiotic surfaces like medical equipment, which allows it to stay for a long time in hospital environments. (Pour *et al.,* 2011). The horizontal transfer of genes in between different species in the environment or in clinics, particularly in hospital settings, usually results in the acquisition of antibiotic resistance (Rumbo *et al.,* 2011). The emergence of MDR *A. baumannii* isolates has increased the frequency of untreatable infections which is resulting in a fatality rate since *A. baumannii* exhibits a variety of pathways for antibiotic resistance (McConnell *et al.,* 2012). According to (Chuang *et al.,* 2011) the mortality rate for bacteraemia by *A. baumannii* varies from 30% to 60%. *A. baumannii* infection also leads to patients who will need more medical treatments and longer hospital stays, which greatly raises the expense to the healthcare system. According to a study looking at CRAB. *A. baumannii* infections in intensive care units (ICU), these infections are linked to increased fatality rates as well as longer and more expensive stays in the hospital (Lemos *et al.,* 2014).

1.5.2 Pathogenicity of *A. baumannii* in clinical environments

There are numerous characteristics of *A. baumannii* strains contribute to their pathogenicity. Among these is its capacity to cling to both biotic and abiotic surfaces (Eijkelkamp *et al.,* 2013) *A. baumannii* can survive on the surface of all medical equipment and have resistance to antibiotics and several metallic compounds. The adherence and biofilm formation are due to host cells and devices are just two instances of the surfaces to which *A. baumannii* can cling (Babapour *et al.,* 2016). *A. baumannii* may become more immune- and antibiotic-resistant due to biofilms. Antibiotic resistance to the microorganism *A. baumannii* naturally resists a variety of antibiotics, and it also can quickly acquire resistance to new antibiotics. This makes treating *A. baumannii* infections challenging (Brossard and Campagnari, 2012).

A. baumannii is highly efficient at acquiring iron, a nutrient essential to the growth of bacteria. It allows *A. baumannii* to thrive in habitats insufficient in iron, such as the human body (McConnell *et al.,* 2013) adding to this. It also colonises biotic surfaces like human epithelial

cells as shown in Fig. 3a, which can be a target during respiratory infections, as shown in Fig.3 b; (Lee *et al.,* 2006). Virulence components of *A. baumannii* produces a variety of virulence components that support its capacity to spread disease rapidly. These consist of phospholipases, proteases, and outer membrane proteins (Thomas, 2020). The below Figure 3 represents the biofilm of *A. baumannii.*

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Figure 3: (a) Scanning Electron Microscopy Image of *A. baumannii* biofilm on Human Epithelial Cell Surface:

This image shows the biofilm formation of *A. baumannii,* shows its attachment to the surface of human epithelial cells. The intricate structure captured in this image provides visual insight into the adhesion and colonization of *A. baumannii* onto the host epithelial surfaces, highlights the pathogenicity. (b) Scanning Electron Microscopy of *A. baumannii* Cells on Candida albicans Filaments: This scanning electron microscopy image elaborates the *Acinetobacter baumannii* cells are observed attached to the surface of Candida albicans filaments. The detailed visualization offers a glimpse into the complex interactions between *A. baumannii* and Candida albicans within biofilm environments, light on potential synergistic or competitive relationships between these microbial species. Figure taken from: (McConnell *et al.,* 2013).

1.6 The capsule polysaccharide (CPS) of *A. baumannii:*

The production of capsule enhances antimicrobial resistance in many bacterial species.

The capsule of *A. baumannii* is a polysaccharide layer that surrounds the bacterial cell wall. It is made up of densely arranged, repeatedly occurring oligosaccharide units (K units), which typically contain 4-6 sugars (Allen, 2021). The capsule has the potential to increase the metallic compound resistance of *A. baumannii.* This is because metals cannot be able to break down the bacterial cell wall (Govindan *et al.*, 2022). Furthermore, the resistance against environmental stresses the capsule can also protect against environmental pressures like disinfectants for *A. baumannii.* This enables the bacterium to endure harsh conditions and remain active on surfaces for extended periods of time (McConnell *et al.,* 2013). For instance, an investigation conducted in 2019 discovered that the capsule can also increase *A. baumannii* resistance to antibiotics (Singh *et al.,* 2019), According to the research, a strain of *A. baumannii* that lacks capsules is less resistant to several compounds than a wild-type strain such as *A. baumannii* ATCC 17978.

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Figure 4: The construction and the export of capsule polysaccharides in *A. baumannii.*

The very first sugar of the repeating unit is transferred to a lipid carrier (Und-P; green circle) by the inner membrane (IM)-based initial transferase (Itr; maroon). Specific glycosyltransferases (Gtr; yellow) on the cytosolic side of the inner membrane after that they are add to the additional

sugars to the developing unit. The integral membrane protein Wzx (dark blue) then facilitates the transport of the capsule sub-unit (K unit) to the periplasm. The Wza/Wzb/Wzc complex (cyan, lime, red) coordinates high level polymerisation and export of the expanding chain, transporting the sugar subunits to the outer membrane (OM). The Wzy protein (purple) polymerizes the sugar subunits. PglL, which is orange, attaches the sugar subunit to certain outer membrane proteins (OMP; dark green).Taken from: (Singh *et al.,* 2019)

In total three primary cell envelope layers of Gram-negative bacteria into the inner membrane, the peptidoglycan layer, and the outer side membrane to which the capsule, (LPS/LOS) LPS is an integral component of the outer membrane of most Gram-negative bacteria

(Putker *et al.,* 2015). It is comprised with three distinct domains; a lipid A anchor, inner and outer core oligosaccharide, and O-antigen polysaccharide (Harding et al., 2018). Some bacteria, such as *A. baumannii*, have a surface glycoconjugate called LOS instead of the typical LPS (Kenyon and Hall, 2013). According to (Weber *et al.,* 2016) LOS is a crucial component of the cells outer membrane and is essential for the survival. Rather than having an O-antigen connected to a lengthy oligosaccharide, this molecule is like LPS in that it consists of lipid A, for inner and outer core saccharides, and a single repeat unit sugar or both are attached are covered by the capsule construction and export process (Singh *et al.,* 2019). Further they are comprised of the tightly organised repeating oligosaccharide subunits such as (K units), CPS then it creates the distinct cover which is coated on the upper surface of the bacteria which also helps it to escape the host immune response, to secure it gains the variety of environmental factors and become more resistance to several antimicrobial agents (Russo *et al.,* 2010). Moreover, *A. baumannii* has a capsule which assembly and exports through Wzy-dependent pathway (Woodward and Naismith, 2016). The initial sugar in the K unit is recruited by the inner membrane (IM) bound and then initial transferase (Itr), followed by the sequential addition of sugars to the growing K unit by specific glycosyl transferase (Gtr) enzymes Figure 4 (Woodward and Naismith, 2016).

Each K unit is then transferred to the other periplasmic side of the IM by the Wzx translocase and polymerized by Wzy, which transfers the growing polysaccharide chain from one (Und-P) carrier to the next for incoming subunit as shown in **Figure 4** (Collins *et al.,* 2007). After that the CPS which are the polymer is then synthesized, then they are further transported to the surface of the cell through a highly co-ordinated process involving the interaction of three proteins; Wza, Wzb, and Wzc, that comprise the export machinery as shown in **Figure 4**. CPS synthesis represents to the one arm of a pathway, as these K units are also used to decorate certain surface proteins via O-linked protein glycosylation (Lees-Miller *et al.,* 2013). In *A. baumannii*, the protein glycosylation contributes to biofilm formation by enhancing initial attachment and

maturation of biofilms, and pathogenicity as demonstrated in several animal infection models (Lees-Miller *et al.,* 2013, Scott *et al.,* 2014).

According to (Preston *et al.,* 1996) two more surface carbohydrates that are known to affect *A. baumannii's* pathogenicity are lip oligosaccharide (LOS) and the exopolysaccharide poly-β-(1-6)-N-acetylglucosamine (PNAG). After that, PNAG is a significant component of biofilms and serves as the cohesive "glue" between them and *A. baumannii* (Choi *et al.,* 2009). In contrast to most Gram-negative bacteria, produces a surface glyco-conjugate called LOS that is similar to lipopolysaccharide but consists of a lipid A core devoid of O-antigen (Kenyon *et al.,* 2015). At last when *A. baumannii* produces less LOS, the outer membrane becomes less stable, which reduces fitness (Moffatt *et al.,* 2010).

1.7 The need for development of novel antimicrobial therapies: Metals the next generation of antibiotics:

According to a study by (Murray and Vasilev, 2017) antimicrobial resistance (AMR) is expected to overtake infectious disease as the leading cause of mortality globally in the next decades (Doughty *et al.,* 2019). In 2019, 4.95 million fatalities were expected to be attributable to AMR, of which 1.3 million were due to resistant infections. By 2050, it is anticipated that there will be 10 million AMR-related deaths annually. In the ongoing battle against antibiotic resistance, metal-based nanoparticles (NPs) have shown immense potential as a viable substitute for conventional antibiotics (Mubeen *et al.,* 2021). The ability of metallic NPs like silver, gallium, gold, copper, and zinc oxide to fight different resistant bacterial strains has been demonstrated in a number of studies (Altun *et al.,* 2021). This highlights the need for novel methods to find the next generation of antibiotics. To assess the ability of metal compounds and wholly organic molecules to inhibit bacterial growth in 2020, Frei and colleagues made use of the enormous database of metal compounds screened as a component of the Community for Open

Antimicrobial Drug Discovery Initiative. The results of the study revealed that the hit rates of the complexes containing metals against important ESKAPE infections were nearly ten times greater than those of the other compounds in the database. This study talks about the myth that metal compounds are more hazardous than organic molecules by highlighting the potential of metal compounds as antibacterial agents (Frei *et al.,* 2023).

Due to the limitations of antibiotics, scientists are looking for inventive and efficient antimicrobial approaches, such as nanoparticles like gallium liquid metal, to increase the ability to kill the bacteria and reduce the possibility that resistance strains will emerge. In addition, reports of the antibacterial activity of metal compounds have been published more frequently, albeit unevenly and with a noticeable upsurge in the previous ten years. The study results on a few prospective antibacterial metals are shown in the figure below. Instead of conducting an indepth study into the mode of action of these bacteria or their potential as antibiotic medications, including toxicity and establishment investigations, most of these papers solely carried out in vitro action measurements against the bacterial strain (Terreni *et al.,* 2021).

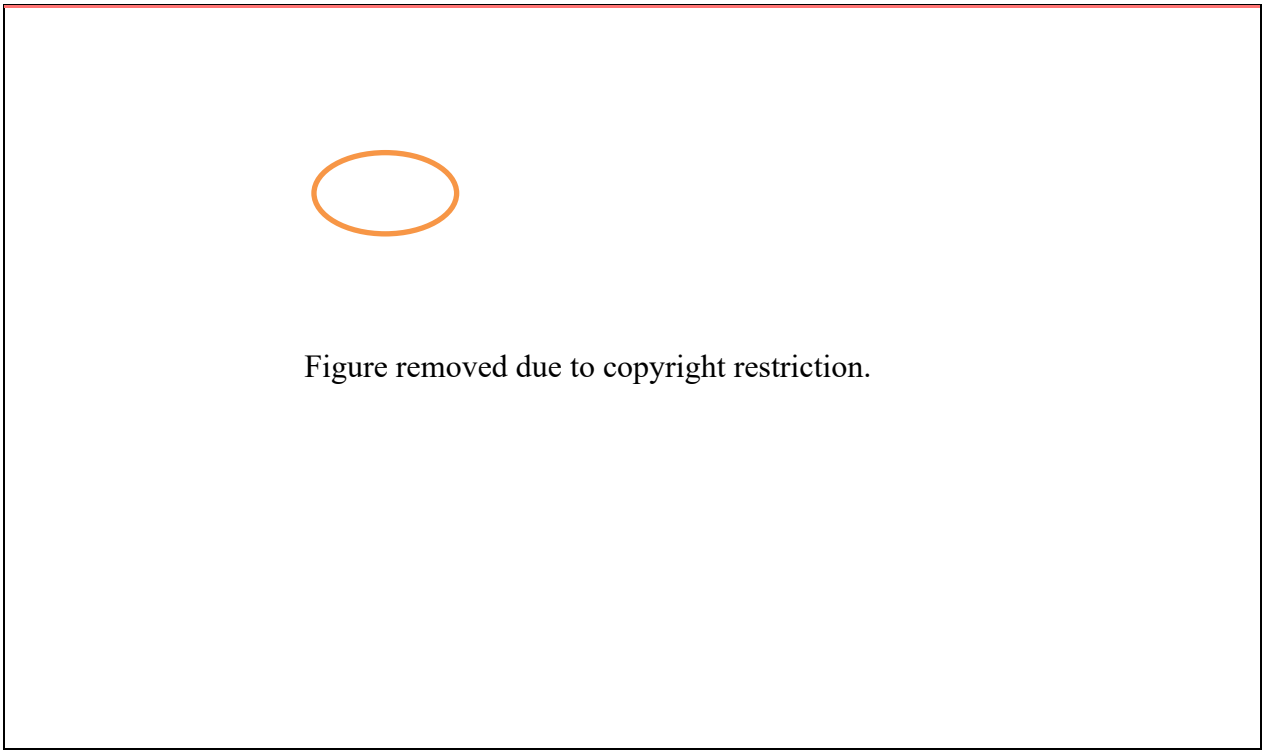

Figure 5: The periodic table which has several metals that are promising antimicrobials including gallium (Frei *et al.,* 2023).

1.8 Types of metals and their antibacterial properties:

1.8.1 Antibacterial activity of copper and copper ions:

According to (Butina, 2021), copper has a long history of use as an antibacterial agent, with tales of its promising properties going back hundreds of years. Despite this, it is unclear exactly how copper ions antibacterial properties work. The several distinct mechanisms, including damage to the cell wall, a shift in intracellular biochemical processes, and the induction of DNA destruction, ultimately result in non-viable bacterial cells, though several interconnected pathways have been proposed (Fang *et al.,* 2009). The are attachment of copper nanoparticles and ions to bacterial cell membranes, which compromises the integrity and function of the cell walls and associated proteins, is one of the main ways that these materials show antibacterial qualities (Godoy-Gallardo *et al.,* 2021). Cupper ions are also released from the matrix to cause this process, which alters the membrane's physicochemical properties and raises oxidative stress and membrane fluidity (Stanić and Tanasković, 2020). Moreover, Cu ions have a strong affinity for donor groups in proteins that are based on nitrogen, oxygen, and sulphur, which impairs protein function and causes structural damage (Dalecki *et al.*, 2017).

1.8.2 Antibacterial activity of silver and silver ion:

According to (Zivic *et al.,* 2018) silver, a well-known valuable metal used in jewellery, coins, and electronics, is also known to be toxic to a wide variety of microbes. This has led to a great deal of research into the development of new antibacterial systems to combat bacterial strains resistant to one or more antibiotics. Silver-based combinations have therefore been widely used for their antibacterial activities (Melaiye and Youngs, 2005). One of the most promising methods to carry out this function is to use systems containing silver, such as silver-nanoparticles

(Ag NPs) (Rai *et al.,* 2009). Despite the beneficial impacts of silver particles on bacteria are outstanding, the practical application of silver-based bactericidal agents is restricted to a small number of salt types and their subclasses. Previous research indicates that Ag+ ions firmly bind to the thiol (-SH) connections of essential components, making them inactive (Le Ouay and Stellacci, 2015). It has also been demonstrated that providing Ag-based particles to microbes decreases their capacity to reproduce DNA. Furthermore, a few analyses have revealed basic alterations in the cell layer as well as the development of tiny, electron-thick granules made of sulphur and silver (Ahmad *et al.,* 2020)

1.8.3 Nanoparticles based on gallium as a novel approach to antibacterial therapy.

Paul-Emile Lecoq de Boisbaudran made the first discovery of Ga in 1875 by using spectroscopy to identify the metal's appearance as two separate bands. Ga which is obtained as a byproduct during the processing of aluminium and zinc ores, is found in the crust of the Earth in amounts ranging from 5 to 15 mg/kg (Frenzel *et al.,* 2016). The novel antibacterial activity need to be discovered against metal resistant bacteria which will be the future challenge in treating and preventing infection (Turner *et al.,* 2020). Unlike metals which are currently used in various application, liquid metals have unique properties such as have metallic and fluidic properties simultaneously. Liquid metals are amorphous solids as of the low melting point (Yan *et al.,* 2018). Liquid metals and metal alloys are in the liquid form at room temperature.

Ga-based metal alloys are low viscosity, fluid nature at room temperature, low melting point, are non-volatile, don’t easily react with other substances and are non-toxic. Shape transformability, catalytic properties, magnetic properties, and self-healing properties are some other highlighted properties of gallium-based liquid metals (Yan *et al.,* 2018). A comprehensive yet brief overview of the current nanoscale antimicrobial uses of liquid metals based on gallium and the related antibacterial methods. Recently, metals like GaLM NPs and liquid metals have been allowed to form materials with broad-spectrum antibacterial properties. Moreover, due to

its molecular structure and iron-like ionic radius, gallium has the capacity to destroy the pathogens. Iron is an essential component of the metabolic and signalling processes because it plays a significant role in numerous bacterial activities, including cellular respiration, synthesis of DNA, oxygen transport, and defence against reactive oxygen species (ROS)as shown in (Figure 5) (Hijazi *et al.,* 2018).

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Figure 6: A schematic representation demonstrates that gallium (Ga) exhibits antibacterial properties. It has been demonstrated that Ga enters the cytoplasm of bacteria through transferrinmediated Fe absorption pathways such homophors and siderophore. Ga is resistant to reduction, which means that it interferes with the vital physiological processes like respiration, DNA synthesis, and oxidative stress that the bacteria are involved into the bacteria (Kurtuldu *et al.,* 2022).

Thus, iron metabolism is critical to bacterial existence and becomes more so during the infection phase because of enhanced high-affinity iron absorption mechanisms. However, because of very similar ionic radiuses, gallium and iron uptake are difficult for many microorganisms to distinguish between, and bacteria can absorb gallium through iron

metabolism. Unlike Fe3+, which inhibits certain iron-dependent chemical reactions, Ga3+ cannot be physiologically reduced (Kurtuldu *et al.,* 2022). As a result, insufficient iron for bacterial iron metabolism kills the bacteria. According to previous research, by interfering with iron-dependent metabolic pathways, Ga $(NO_3)_3$ and Ga (III)-maltolate suppressed the majority of multidrug-resistant ESKAPE infections (Hijazi *et al.,* 2018). So, for the new research into the field of metals alloys it will focus to medical applications. Thus, this says that gallium ions are safe for biomedical use. The results of the experiment revealed that *P. aeruginosa* activity was greatly inhibited by an extremely tiny concentration of gallium (4-6μM) (Goss *et al.,* 2018). GaLM may offer promise for use in biomedical applications, according to this proof. Ga is the first metal utilised in surgery for its antibacterial qualities. (Daeneke *et al.,* 2018). However, metals such as silver, gold, copper, and zinc were found to be able to kill *P. aeruginosa* and *A. baumannii* (Truong *et al.,* 2023b) (Rai *et al.,* 2009).

1.9 The side effect of metal in medical applications:

The use of metals encompassing silver, copper, and zinc in medical applications has become increasingly well-known in current years based on their antimicrobial characteristics (Truong *et al.,* 2023b). The following are a few possible adverse effects of using gallium metal in medicinal applications: Gallium can induce several symptoms, including skin rash, itching, and swelling, in those who are allergic to it. Gallium has the potential to be harmful to the kidneys, especially at high dosages. Failure and kidney damage may result from this. Gallium is also potentially harmful to the nervous system, especially in high dosages. Numerous neurological symptoms, including headache, vertigo, and seizures, may result from this. Other negative effects: Nausea, vomiting, diarrhoea, and hair loss are additional possible side effects of gallium metal used in medicinal applications. For example, once the body is exposed to silver nanoparticles through pathways such as skin contact, inhalation, ingestion, and injection. Various tissues and organs, including the cutaneous, respiratory, circulatory, neurological, hepatobiliary,

urinary, and reproductive systems, will receive GaLM NPs via these distribution pathways. (Liau *et al.,* 1997). Another main point is that the toxicity of metals could be influenced by their size, morphology, and chemical structure of the surface (Yan *et al.,* 2018).

1.10 Hypothesis and aims:

Hypotheses: GaLM NPs will show potent antibacterial activity against multi-drug resistant *A. baumannii*, also resulting in a reduction of biofilm.

General Aim: To test GaLM NPs as novel compounds to combat multi drug-resistant *Acinetobacter baumannii.*

Aim 1a: To synthesize GaLM NPs

Aim1b: To characterize the GaLM NPs.

Aim 2: To evaluate the antimicrobial effect of GaLM NPs against the *Acinetobacter baumannii.*

Aim2b: To evaluate static biofilm assay.

1.11 Scope of the thesis:

The scope of the thesis is to design the GaLM NPs by synthesizing, characterizing, and evaluating it as novel compound for targeting multi-drug-resistant *A. baumannii* strains and control group bacteria *pseudomonas aeruginosa*. The initial objective is to explore the effectiveness of GaLM NPs as a treatment against MDR *A. baumannii.* This involves assessing the mechanism of action of these nanoparticles and determining their practicality in combating these resistant strains.

This study investigates the synthesis of GaLM NPs using high-purity materials and efficient devices, particularly probe sonication procedures. The use of MIC and MBC and agar

well diffusion experiments to evaluate the antibacterial effectiveness of GaLM NPs against specific bacterial strains is utilized.In addition, the anti-biofilm capabilities of GaLM NPs are evaluated using a static biofilm assay. With a specific emphasis on the potential of GaLM NPs in tackling the global health challenge posed by metallic-compound resistance, the thesis hopes to provide important insights into the creation of novel techniques for combatting multi-drugresistant bacterial illnesses through these experiments.

Overall, the wide range of the experimental approaches and analyses utilised in this study generated a wealth of information on the *A. baumannii* persistence and resistance features. The results presented here have advanced the understandings about the factors that contribute to the success of *A. baumannii* as a human pathogen.

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials:

Various machines including sonics Vibra-Cell VCX750 Ultrasonic Liquid Processor, USA; Bio Tek Synergy HTX Multimode Reader; Eppendorf Centrifuge 5810/ 5810 R, Germany; Eppendorf Centrifuge 5425 R, Germany; FEI Inspect F50 Field Emission (SEM), USA. Gallium 99% obtain from Dongguan Wuchang company, China, Ga $(NO_3)_3$ (hydrate crystalline, 99% trace metals basis, Sigma-Aldrich). Ultrahigh purity water gained from a Milli Q system (Millipore Milli-Q Academic, USA), phosphate buffer saline (PBS) 10X, gallium nitrate, and common solvents were purchased from Sigma-Aldrich Australia and used without further purification**.**

2.2 Methodology:

2.2.1 Synthesis of GaLM NPs:

The production process of GaLM NPs and Ga $(NO_3)_3$ (hydrate crystalline, 99% trace metals basis, Sigma-Aldrich) involved the use of probe sonication through absolute ethanol. Liquid-metals (LMs) based on gallium can form an ultra-thin, self-passivating solid oxide layer under ambient conditions, resulting in an extraordinary liquid core and solid skin structure (Syed *et al.,* 2018). The production of LM droplets was achieved simply by ultrasonication of bulk gallium metal in a liquid environment including water or ethanol. Probe sonication was utilized as a well-known technique for generating gallium-particles (GaLM NPs) based on the property of gallium to undergo a solid to liquid transition above 29.76°C (Ramachandran *et al.*, 2021). The liquid gallium was broken down into smaller particles by ultrasound waves, followed by quenching in a water bath to transform into the solid state. This cycle was repeated, resulting in the formation of smaller GaLM NPs with extended probe sonication. However, gallium particles

in an aqueous environment exposed to temperatures above the sonication probe would 32 accelerate the hydroxylation process, causing the transformation of spherical particles into rod shaped Gao OH structures. To prevent this phenomenon, an ice bath was utilized as a cooling method to control the temperature during the probe sonication process.

The synthesis of GaLM NPs was performed as described previously (Nguyen et al., 2023b), using Milli-Q water as the solvent. First and foremost, 60 mg of Ga (99%, Dongguan Wuchang, China) was measured into a 15-milliliter tube, followed by the addition of 9 ml of Milli-Q water. Subsequently, an ice bath was prepared to maintain temperature control during the procedure. The solution was sonicated by using (Probe 1/8" (1-15 ml), sonicator, Q Sonica LLC, USA) with an amplitude of 30%, for 5 seconds, and a pulse time of 5 seconds, carried out over a duration of 35 minutes. Following synthesis, as required, 1 ml of F-127 (Sigma-Aldrich) at concentrations of 1% and 2%, or 1% and 2% polyethyleneimine (PEI) (Sigma-Aldrich), were separately added to the solution. The mixture was vortexed for two minutes to ensure thorough mixing of compounds. Finally, the solution was stored in a refrigerator for future use for up to one week.

2.2.2 Characterization of GaLM NPs:

The characterization was performed by (Nguyen *et al.,* 2023a) after the successful synthesis of solutions containing GaLM NPs, these solutions were diluted 10-fold with Milli-Q water. Then, drops of approximately 30 µl of each particle solution were added to clean silicon wafers, which were previously washed with acetone and ethanol, and then dried with nitrogen. The samples were left to dry totally in a fume hood. Once dry, the silicon wafer samples containing the particles were mounted onto a stage using conductive carbon adhesive tapes. The morphological features, size, and density of the particles were then analysed using scanning electron microscopy (FEI Inspect F50 Field Emission SEM, USA) at Flinders University.

2.2.3 Bacterial strains and growth conditions:

To evaluate the inhibitory effects of GaLM NPs on bacteria, several different bacterial species were sourced from the previous ongoing work on *A. baumannii*. The selected strains encompassed various capsule types, pathogenicity, and antibiotic susceptibility profiles as mentioned in (Table 2.1). Before each experiment, bacterial cultures were grown overnight at 37°C in medium Mueller-Hinton (MH) media were prepared according to the manufacturer's recommendations (BD, Bacto Laboratories Pty Ltd, Australia). A single bacterial colony was chosen from the culture using an inoculation loop and grown in MHB under static conditions overnight at 37°C. Cultures in tubes were incubated at a 45° angle with shaking at 200 rpm to ensure appropriate aeration. The density of the cultures was adjusted to an optical density at 600 nm (OD_{600}), and samples were collected during the logarithmic growth phase.

Table 1: Selected bacterial strains are stated below in table.

Bacterial strains	Description	Reference
***A. baumannii* ATCC 17978**	A lab-adapted avirulent reference strain originally isolated from cerebrospinal fluid over 50 years ago with a KL3type capsule locus type	(Adams and Brown, 2019)
***A. baumannii* HUMC1**	A hyper virulent clinical isolate from blood and lung with a KL22-type capsule locus type per the Kenyon classification	(Talyansky *et al.,* 2021)

***A. baumannii* ΔCPS ATCC 17978**	ΔCPS and gnaA-gtr9 inclusive deletion in ATCC 17978 background	(Jennifer, 2019)
***Pseudomonas aeruginosa* (PA01)**	Control out-group bacteria chosen to represent pathogenic Gram-negative bacteria.	(Nguyen *et al.,* 2023b)

2.2.4 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) assay

In this study, the antimicrobial activity of GaLM NPs was assessed using a microdilution assay to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC), respectively. The MIC represents the lowest concentration of an antibacterial agent required to completely inhibit the growth of the targeted microorganism and MBC represents the antibacterial agent which determines by subculturing the last clear MIC onto the growth medium and examining for bacterial growth (Wiegand *et al.,* 2008). For the MIC assays three different *A. baumannii* strains together with *P. aeruginosa* were used Bacterial strains Tabe 2.1 were routinely grown in Mueller Hinton (MH) broth at 37^{o}C with shaking (120 rpm) unless otherwise stated, Overnight cultures (ON) were initially adjusted to an optical density of OD_{600} = 0.5 and subsequently diluted 1:1000 in Mueller Hinton (MH) broth. Microtiter trays were prepared containing a 2-fold dilution series of the compound to be tested along with Ga-based liquid metal and compounds added to it such as F127 and PEI; 1% AND 2% respectively, and cultures were added at a 1:1 ratio, resulting in a final volume of 100 µl MH broth. Appropriate controls were included, such as medium alone and medium with the bacteria as negative and positive controls. The microtiter trays were then incubated overnight at 37°C

with shaking and were wrapped in foil to minimize evaporation. Bacterial growth was examined by measuring visually inspect each well for turbidity, indicating bacterial growth. Now, transfer the 10 µL from each well showing no visible growth to Mueller-Hinton agar plates using a multichannel pipette. Incubate the plates at 37°C for an additional 19-24 hours. Determine the MBC as the lowest concentration of the antimicrobial agent that results in no visible bacterial growth on the agar plates. The MIC and MBC assays were conducted on three separate occasions to ensure reproducibility This methodology ensures accurate evaluation of the inhibitory activity of GaLM NPs against the tested bacterial strains and provides valuable insights into their potential as antimicrobial agents.

2.2.5 Agar well diffusion assay:

In this study, the antimicrobial efficacy of GaLM NPs was performed as described previously (Nguyen et al., 2023b), the ON inoculations of *A. baumannii* and *P. aeruginosa* strains in MHB medium were prepared, and during the logarithmic phase of cell growth their densities were adjusted to OD_{600} = 0.5. Subsequently, 250 µl of the prepared bacterial strain solution was added into individual tube containing 25ml of MHB agar and then it was poured to petri plate. Wells with a diameter of 5 mm were created on the agar plates to accommodate solutions of GaLM NPs along with compounds which were prepared. Sterilized Milli-Q water served as the negative control, while Ga $(NO_3)_3$ (1 mg/ml) was used as the positive control, along with F-127 1% and 2% and PEI 1% and 2% as additional controls. Each well received 50 µl of the respective bacterial suspension on the MHB agar plates. The plates were then incubated for 24 hours at 37°C. Following incubation, the diameter of the inhibition zone produced by each solution was measured and recorded. Data analysis was performed using GraphPad Prism 9 software. This method provides a reliable means of assessing the antimicrobial activity of GaLM NPs against the tested bacterial strains, allowing for the comparison of inhibition zones and the determination of their effectiveness in inhibiting microbial growth.

2.2.6 Static biofilm formation assay:

A static biofilm formation assay was performed by following the protocol described previously (O'Toole *et al.,* 1999), with certain modifications implemented. A single bacterial colony was utilized to inoculate Luria Bertani (LB) medium, which was then incubated overnight at 37°C with shaking (120 rpm) unless otherwise stated, in a shaking incubator. Subsequently, 50 µl of the culture was diluted 1:100 into fresh LB broth. The cultures were then incubated statically for 72 hours at 37°C in the absence of light. Following incubation, the optical density at 600 nm (OD_{600}) of the culture containing planktonic growing bacteria was measured and compared to the wild-type (WT) *A. baumannii* ATCC 17978 to ascertain growth characteristics. The residual liquid culture was then discarded, and adherent cells were washed once with phosphate-buffered saline (PBS). Subsequently, the cells were stained by incubating in a solution containing 0.1% crystal violet for 30 minutes at 4°C, followed by three washes with PBS. To release the dye from the bound cells, 5 ml of an ethanol: acetone solution (4:1) was added, and the mixture was shaken at 200 rpm for 30 minutes at room temperature. The concentrations of compounds used to investigate the effect of stress on biofilm formation, such as bacterial conditions or supplementation with metallic compounds. The absorbance of the solution was measured at OD600 using a FLU Ostar Omega spectrometer (BMG Labtech, Germany). The biofilm data for each strain were calculated from the average of at least three independent experiments, each of which included two to three technical replicates. This methodology provides a robust approach for assessing biofilm formation and enables accurate comparisons between bacterial strains.

CHAPTER 3: RESULTS AND DISCUSSION

3.1 Preparation and characterization of GaLM NPs

The synthesis of GaLM NPs was facilitated by a high-frequency ultrasonic probe sonication method along with milli-Q water, based on the unique property of gallium which will undergo a solid to liquid transition above 29.7° C (Nguyen *et al.,* 2023a). Subsequently, the ultrasound waves broke down the solid gallium metal into smaller particles, which are then quenched in an ice bath to transform into the solid state to liquid state. This cycle was repeated, which resulted in the formation of smaller gallium particles in the form of gallium-based liquid metal as shown in APEENDIX 1. The surfactants were added to GaLM NPs for having resistance towards the bacteria and further the durability of the nanoparticles was significantly improved upon the addition of compounds, F-127 and polyethyleneimine (PEI) at different concentrations (1% and 2%) as shown in APPENDIX 1. The homogenization of the liquid through vortexing suggests that the surfactants were successfully incorporated into the suspension of nanoparticles. The final concentration of stock solution was 10:1 ratio resulting into 2.7mg/ml: 0.27mg/ml (GaLM NPS: compound (i.e. F127 and PEI). Moreover, the stability of GaLM NPs and compounds was checked by performing antimicrobial activity by agar well diffusion assay and antibacterial activity by performing MIC and MBC.

Followed by the synthesis of GaLM NPs, the characterization phase was started by determining their morphological features, size, and density. These silicon wafers were previously treated with acetone and ethanol to remove organic contaminants and dried using nitrogen to avoid any residues that might interfere with microscopic analysis. Each wafer was mounted on the sample stage using conductive carbon adhesive tapes to provide a stable and conductive base for scanning electron microscopy (SEM) analysis. The SEM image results of the samples illustrate that the Gallium-based metal ions have different shapes. The SEM images of Ga LM

NPs show uniformly rounded shape of the gallium particles Figure 7. ImageJ software was used to analyse the images and then measure the size of GaLM NPs. Moreover the gallium metal was converted from solid state to liquid state for performing the antimicrobial, and antibacterial activity against the bacterial strains to reduce the pathogenicity.

Figure(s) removed due to copyright restriction.

Figure 7: (A) The SEM images and nanoparticle size distributed by histogram of gallium-based liquid metal nanoparticle along with F127 compound deposited on the silicon wafer for 100s respectively. **(B)** This figure represents the SEM images and nanoparticle size distributed by histogram of gallium-based liquid metal nanoparticle. The SEM was performed by (Nguyen *et al.,* 2023a).

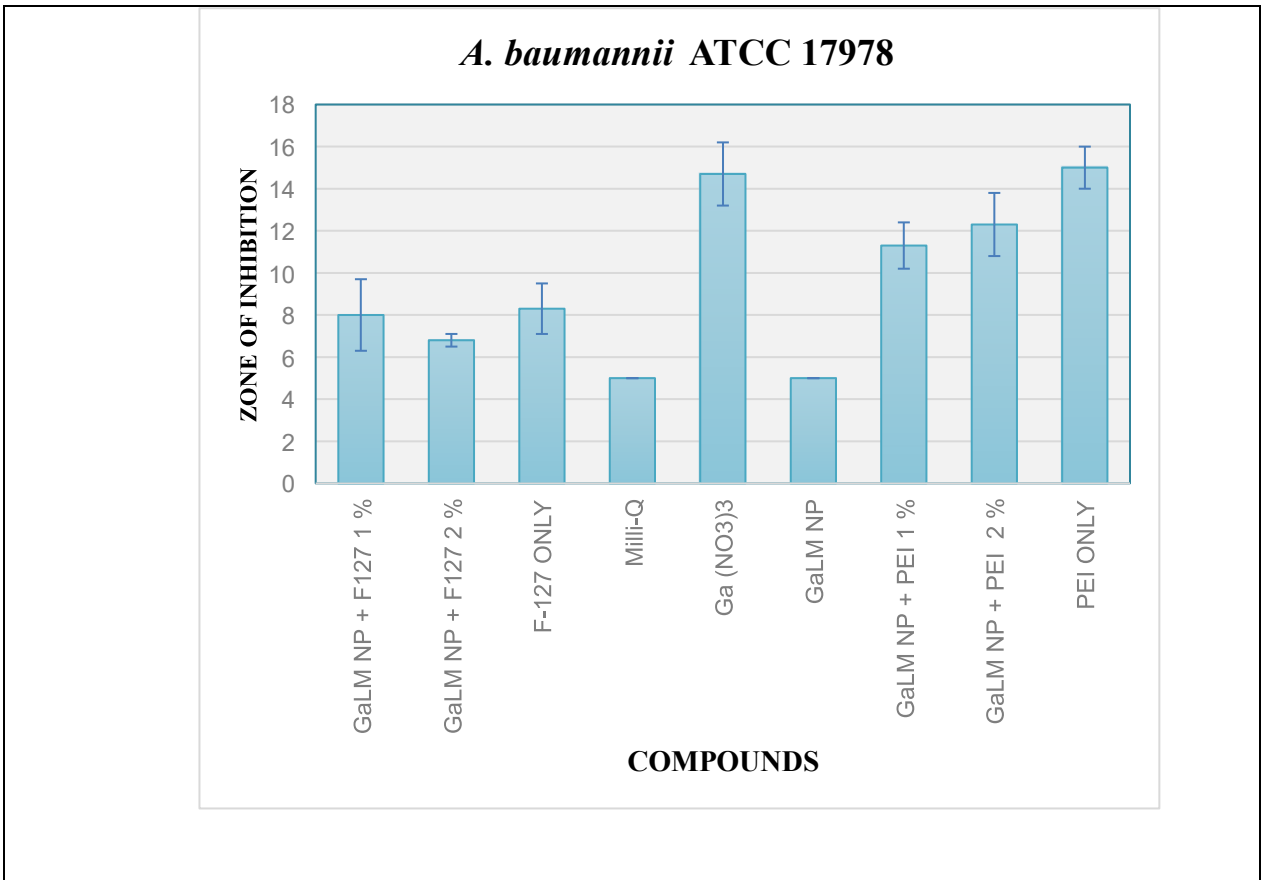
3.2 Preparation of selected bacterial strains

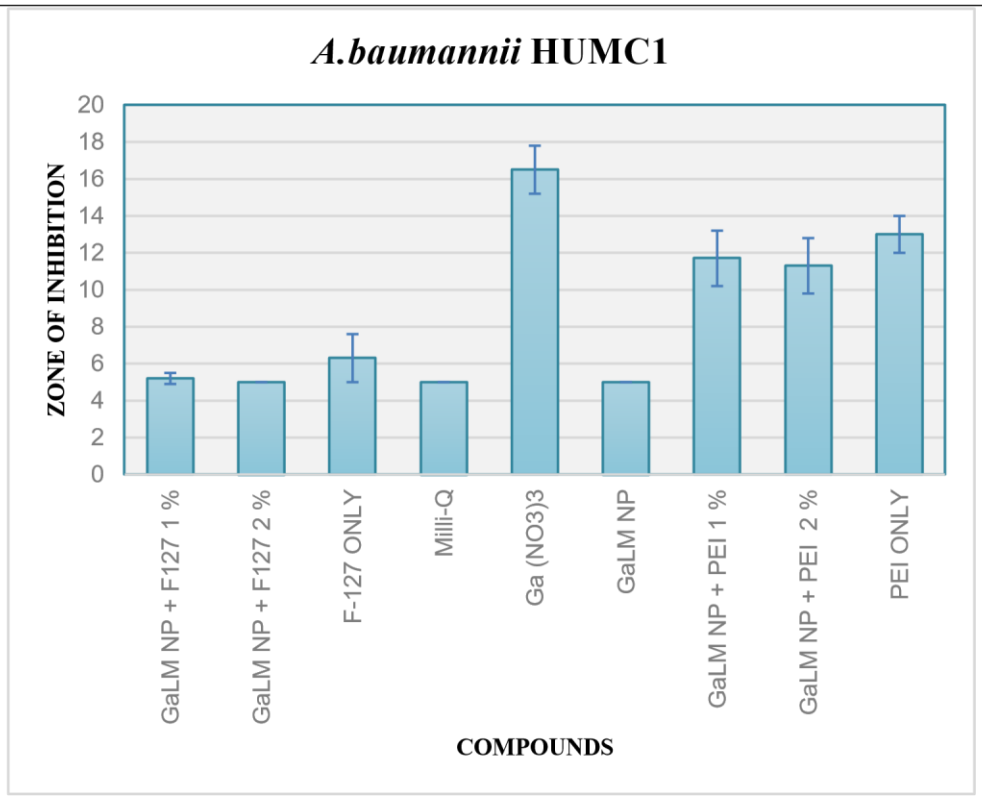
For the investigation of the study two different spp were selected namely *A. baumannii* and *P. aeruginosa*. The four different strains which were selected are *A. baumannii* ATCC 17978, *A. baumannii* Humc1, *A baumannii* Delta CPS ATCC17978, *P. aeruginosa* PA 01. They were streak on fresh MH agar plates and observed after 24hours. Single colony was used for inoculating the bacterial suspension over night for performing antimicrobial and antibacterial activity.

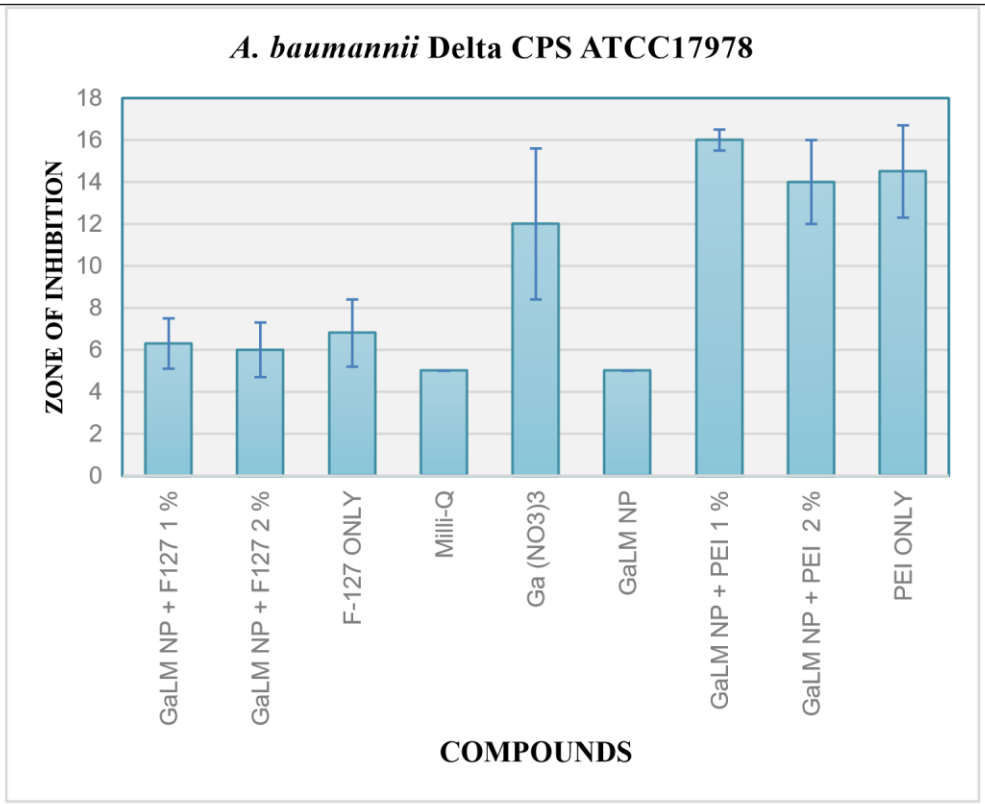
3.3 Antimicrobial properties of GaLM NPs

The current study investigated the antimicrobial activity of GaLM NPs against four bacterial strains was determined which includes *A. baumannii* ATCC 17978, *A. baumannii* HUMC1, *A. baumannii* Delta CPS, and *P. aeruginosa* PA01, by using the agar well diffusion method. In comparison to the negative control, which was sterilised Milli-Q water and showed no zone of inhibition (ZOI) to bacterial growth. The ZOI measured around the wells which contained GaLM NPs alone did not show any kind of inhibition whereas the GaLM NPs along with compounds such as F-127 and PEI showed considerable antimicrobial activity. The highest ZOI were seen in the positive control, Ga $(NO_3)_3$, at a concentration of 1 mg/ml, suggesting strong antibacterial activity with 14.6 ± 1.5 mm for *A. baumannii* ATCC 17978 whereas it was 16.5 ± 1.3 mm for *A. baumannii* HUMC1 as showed in APPENDIX 3. In this experiment wells were with GaLM NPs with compounds which had different inhibitions zone measurements, which suggest the different susceptibility between all different bacterial strains such as *A. baumannii* ATCC 17978, *A. baumannii* Delta CPS ATCC 17978, *A. baumannii* HUMC1 showed slightly larger ZOI compared *P. aeruginosa,* indicating a potentially higher sensitivity to the nanoparticles. Additionally, the inhibition zones for wells containing F-127 and PEI compounds by itself were observed larger than those with GaLM NPs, highlighting the enhanced

antimicrobial activity of the nanoparticle-based treatment as showed below in Figure 8. The ZOI resistant to *A. baumannii* and *P. aeruginosa* with F-127 and PEI concentration ratios, which were 2.7mg Ga + 0.27mg F127/ml, 2.7mgGa + 0.27mg PEI/ml respectively. The inhibition zone of GaLM NPs with two different concentrations of compounds, namely 2.7 mg/ml F-127 1% and 2%, 2.7 mg/ml PEI 1% and 2% against *A. baumannii* strains and *P. aeruginosa* were observed different and are mentioned in Figure 8, respectively. Whereas GaLM NP by alone did not show any inhibitory effect against bacteria. Furthermore, the ability to form ZOI of GaLM NPs increased in the presence of compound compared to a solution containing only GaLM NPs alone.




A.baumannii HUMC1
ZONE OF INHIBITION
20
18
16
14
12
10
8
6
4
2
0
GaLM NP + F127 1 %
GaLM NP + F127 2 %
F-127 ONLY
Milli-Q
Ga (NO3)3
GaLM NP
GaLM NP + PEI 1 %
GaLM NP + PEI 2 %
PEI ONLY
COMPOUNDS


A. baumannii Delta CPS ATCC17978
ZONE OF INHIBITION
18
16
14
12
10
8
6
4
2
0
GaLM NP + F127 1 %
GaLM NP + F127 2 %
F-127 ONLY
Milli-Q
Ga (NO3)3
GaLM NP
GaLM NP + PEI 1 %
GaLM NP + PEI 2 %
PEI ONLY
COMPOUNDS

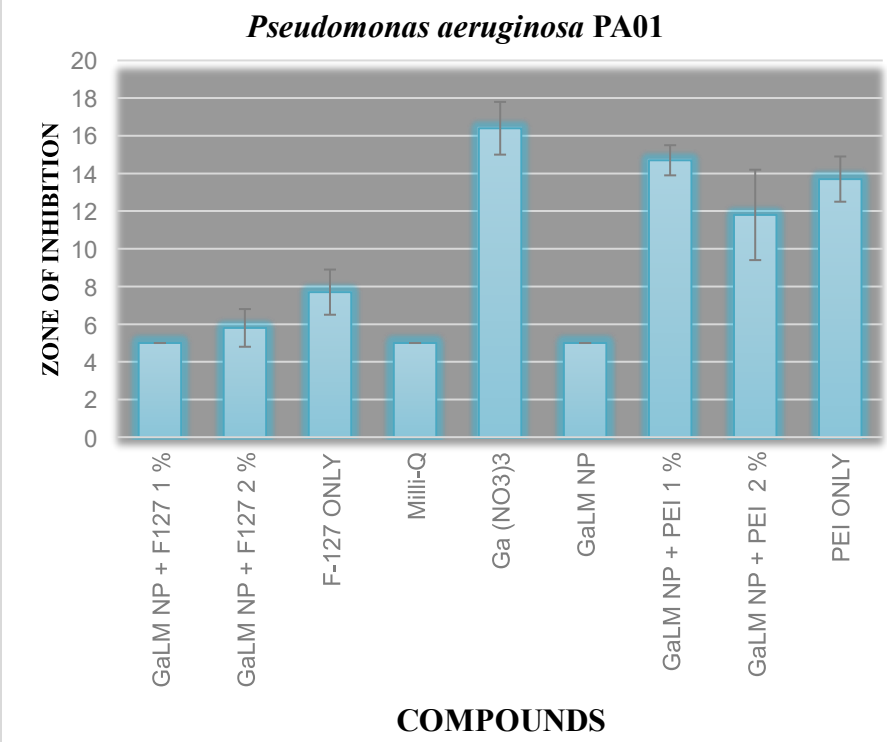


Figure 8: The antimicrobial activity against *A. baumannii* and *P. aeruginosa*. The error bars represent the standard error of the mean of triplicate ZOI data. The compounds were GaLM + F127 1% and 2%, GaLM + PEI 1% and 2%, Milli-Q water as negative control and Ga (NO3)3 as positive control against *A. baumannii* strains and *P. aeruginosa*. Data illustrates mean ± SD, error bars represent standard deviation.

The results of this investigation indicate that GaLM NPs and compounds provide the significant antimicrobial properties, which are particularly superior to standard controls and comparable to high-concentration gallium cures. The mechanism by which GaLM NPs inhibit bacterial growth is likely related to the sustained release of gallium metal ions, which disrupt bacterial iron metabolism with a critical pathway for bacterial survival and proliferation. The

differential susceptibility observed between *A. baumannii* and *P. aeruginosa* may be attributed to variations in cell wall composition and the inherent resistance mechanisms of these pathogens. These findings suggest that liquid metal enhances the delivery efficiency of gallium, thereby increasing its bioavailability and antimicrobial actions. Moreover, the antimicrobial activity of GaLM NPs showed greater inhibition in for compounds by itself which were F-127 and PEI compared to GaLM NPs + F-127/PEI.

3.4 Antibacterial properties of GaLM NPs

The current study investigated the inhibitory of GaLM NPs against four bacterial strains. As shown in Table 3.2, it is observed that the GaLM NPs were effective against capsulated strains of *A. baumannii* with MIC values of 0.084 and 0.337 mg/ml, respectively, and MBC values of 0.140 and 0.675 mg/ml, respectively. The MIC and MBC were measured in mg/ml as shown in **APPENDIX 4.** In addition, the concentration of GaLM NPs was about 2.7 mg/ml which were equal to the gallium-metal ion concentration which was utilised to synthesise GaLM NPs was used to test the inhibition and bactericidal ability. All MIC and MBC results were obtained was observed higher than 2.7 mg/ml and the growth of MBC in all the strains starts just before twofold dilution compared to MIC bacterial growth. Moreover, to sum up the results, the MIC of GaLM NP-F127 with 1%and 2% concentration is ranging from 0.084 mg/ml and for GaLM NPPEI 1%and 2% concentration it was inhibiting with similar values across various strains of *A. baumannii.* However, F127 1% and 2% alone and PEI 1% and 2% show no inhibitory effect on *A. baumannii* growth, even up to 2.7 mg/ml. Notably, the positive control, *P. aeruginosa* strain PA01, was inhibited by 0.337 mg/ml, indicating its higher sensitivity to gallium nanoparticles compared to *A. baumannii* ATCC 17978. Furthermore, when considering capsule presence, the MIC was >1.35 mg/ml in CPS and 0.337mg in capsulated strains. This suggests that the encapsulation decreases the *Acinetobacter's* resistance to gallium nanoparticles.

Table 2: The MIC and MCB concentration of GaLM NPs against *A. baumannii* [1]ATCC 17978, *A. baumannii* HUMC1, no capsule delta CPS ATCC 17978, *Pseudomonas aeruginosa* PA01. The data below represents the average of triplicate results.

Compounds	***A. baumannii* ATCC 17978**		***A. baumannii* HUMC1**	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Ga + F127 1%	0.084	0.140	0.337	0.675
Ga + F127 2%	0.084	0.140	0.337	0.675
F127 1%	>2.7	>2.7	>2.7	>2.7
F127 2%	>2.7	>2.7	>2.7	>2.7
Ga + PEI 1%	0.084	0.140	0.450	0.90
Ga + PEI 2%	0.084	0.140	0.450	0.90
PEI 1%	>2.7	>2.7	>2.7	>2.7
PEI 2%	>2.7	>2.7	>2.7	>2.7
Ga $(NO_3)_3$	0.337	0.675	0.337	0.675
Gallium	>1.35	>1.35	>1.35	>1.35

[1] The ratio of Ga:F-127/PEI is 10:1. The concentration of GaLM NPs is equivalent to the concentration of compounds used to synthesize GaLM NPs.

Compounds	*A. baumannii* Delta CPS		*P. aeruginosa*	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)
Ga + F127 1%	1.35	2.7	0.337	0.675
Ga + F127 2%	1.35	2.7	0.337	0.675
F127 1%	>2.7	>2.7	>2.7	>2.7
F127 2%	>2.7	>2.7	>2.7	>2.7
Ga + PEI 1%	1.35	2.7	0.337	0.675
Ga + PEI 2%	1.35	2.7	0.337	0.675
PEI 1%	>2.7	>2.7	>2.7	>2.7
PEI 2%	>2.7	>2.7	>2.7	>2.7
Ga $(NO_3)_3$	1.35	2.7	1.35	2.7
Gallium	>1.35	>1.35	>1.35	>1.35

The capsulated strains *A. baumannii* ATCC 17978 and HUMC1 strains showed lower MIC and MBC values than the non-capsulated strain for the GaLM NPs combined with F-127 and PEI, indicating a higher susceptibility to the gallium-liquid metal-based treatments to the *A. baumannii* Delta CPS and *P. aeruginosa* PA01 strains. This observation shows that the GaLM

NPs may be more effective against certain *A. baumannii* strains, due to differences in their cell structure, growth duration and the presences and absence of capsule in the bacteria. Interestingly, the F-127 and PEI compounds alone did not how any kind of inhibitory effects on the bacterial growth, even at the highest concentrations tested. This highlights the importance of the NPs to the antimicrobial activity of this compounds. The analysis of differences in MIC and MBC values between the *A. baumannii* and *P. aeruginosa* strains suggest that the GaLM NPs may have a different impact on the two bacterial strains. Overall, the results of this study provide valuable insights into the potential of GaLM NPs as a novel antimicrobial platform for the management of drug-resistant bacteria, particularly those caused by *Acinetobacter baumannii*. The enhanced antimicrobial activity observed when the nanoparticles are combined with the F-127 and PEI compounds warrants further investigation to optimize the formulation and explore its clinical applications.

3.5 Static biofilm production by capsular and acapsular strains

The static biofilm assay showed appropriate growth characteristics between various strains and the *A. baumannii* ATCC 17978. So before assessing the biofilm formation the growth character of bacterial strains was evaluated by performing same as MIC which was evaluated by doing serial dilution 1:1and then the growth was observed after 19-24 hour. Then the measurements were taken from optimal density OD_{600} showed a consistent growth pattern for all replicates which indicates the medium under experimental conditions. All the strains had difference in the growth of different concentration between wild-type and other strains that the modifications did not adversely affect the planktonic growth of the bacteria as shown in Figure 9. After the process of 72-hours the quantitative examination of biofilm formation was performed by measuring OD_{600} following crystal violet staining 0.1%. The investigated strains have different capacities for biofilm formation were shown by the results. Strains that were compared showed increased development of biofilm this could be related to growth-phase environmental

factors. According to the absorbance measurements performed by using spectrometer (OD), the growth media which had specific metal like GaLM NPs along with compounds such as F127/PEI had a major impact on the growth of the biofilm. As it was observed that the capsular polysaccharide mutant *A. baumannii* CPS had lower OD which highlights that no capsule strains cannot form biofilm compared to others. The GaLM NPs along with compound such as F-127 and PEI formed the higher biofilm and had higher OD compared to compounds alone, therefore it asserts that gallium-metal along with compound has a higher impact on bacterial strains.Moreover, there was significant increase in biofilm formation was seen in other strains cultured in the presence of these capsule which indicates that these conditions may cause stress responses or biofilm-promoting pathways in *A. baumannii.* Whereas the pathogenic bacteria *P. aeruginosa* showed equivalent growth to the biofilm formation to *A. baumannii* ATCC17978 as showed in Figure 10.

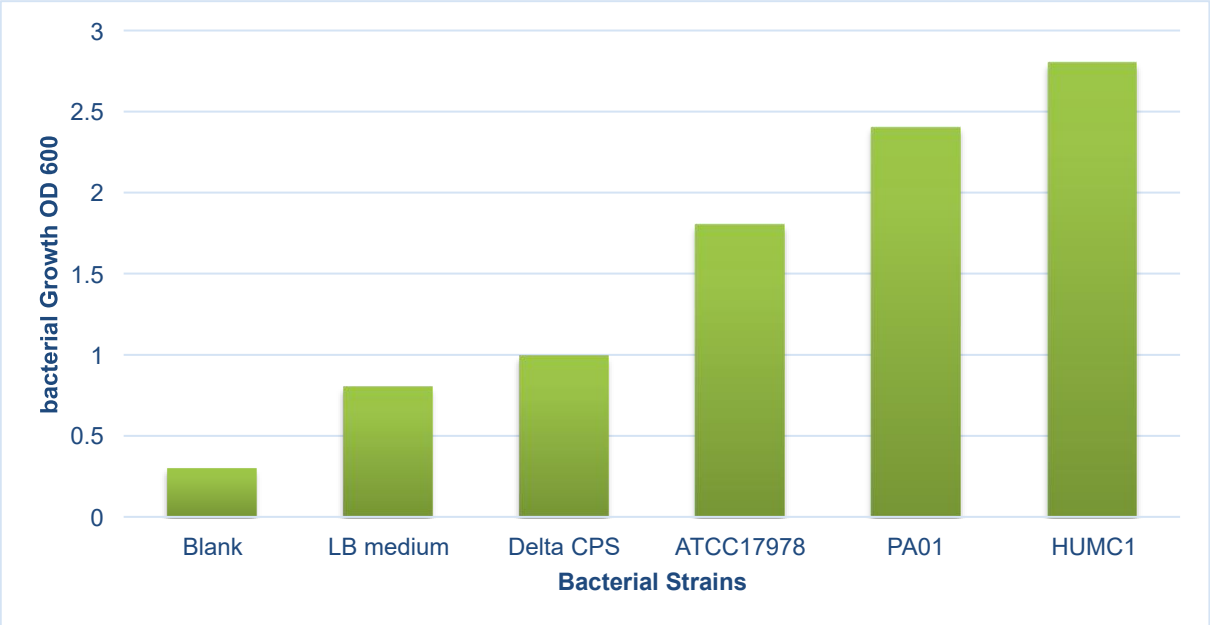
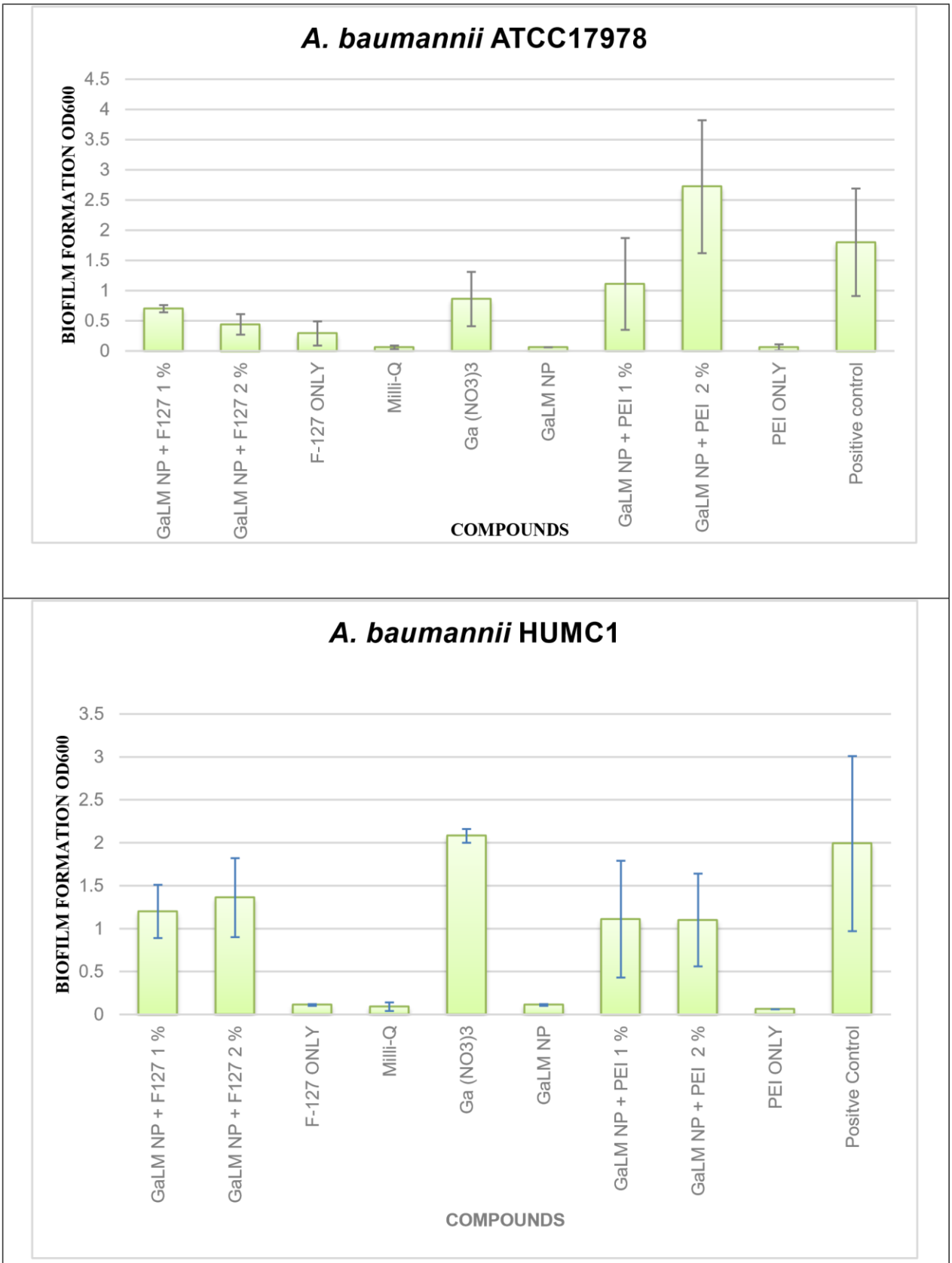
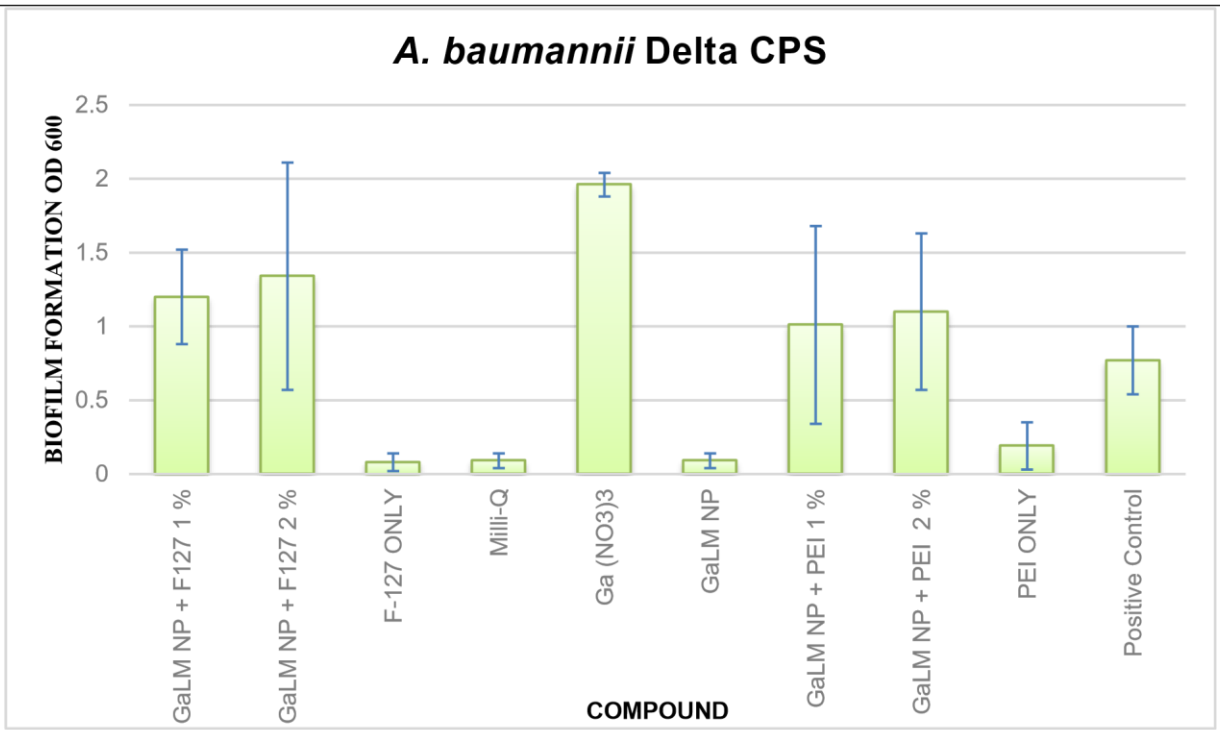


Figure 9: Comparison of biofilm formation among bacterial strains; Blank is Milli-Q water, LB medium, *A. baumannii* ATCC17978 CPS, *A. baumannii* ATCC17978, *A. baumannii* HUMC1, *P. aeruginosa* PA01.


A. baumannii ATCC17978
BIOFILM FORMATION OD600
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
GaLM NP + F127 1 %
GaLM NP + F127 2 %
F-127 ONLY
Milli-Q
Ga (NO3)3
GaLM NP
GaLM NP + PEI 1 %
GaLM NP + PEI 2 %
PEI ONLY
Positive control
COMPOUNDS
A. baumannii HUMC1
BIOFILM FORMATION OD600
3.5
3
2.5
2
1.5
1
0.5
0
GaLM NP + F127 1 %
GaLM NP + F127 2 %
F-127 ONLY
Milli-Q
Ga (NO3)3
GaLM NP
GaLM NP + PEI 1 %
GaLM NP + PEI 2 %
PEI ONLY
Positve Control
COMPOUNDS



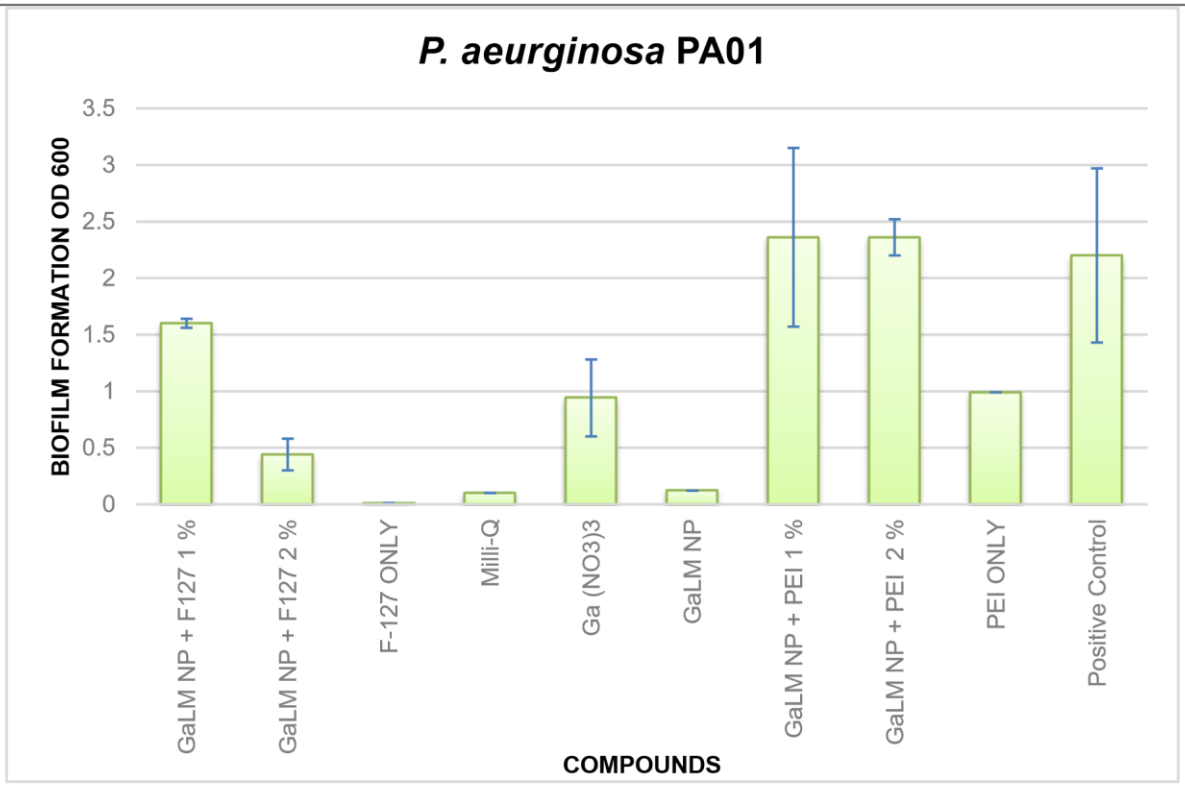


Figure 10: Static biofilm formation by GaLM NPs against four bacterial strains which includes *A. baumannii* ATCC 17978, *A. baumannii* ATCC CPS, *A. baumannii* HUMC1, *Pseudomonas aeruginosa* PA01.

The data shows different compounds such as F127, PEI alone, two different concentrations of F127 and PEI 1% and 2%, Ga $(NO_3)_3$, Ga alone and Milli-Q water which illustrates how differently the bacteria can grow biofilms. Data illustrates mean ± SD, error bars represent standard deviation.

CHAPTER 4: CONCLUSION AND FUTURE DIRECTIONS

4.1 Summary of the key findings of this study

This study investigated that Gallium was successfully prepared from a solid state to a liquid state above 29.7°C due to the high frequency ultrasonic probe sonication method used to create the GaLM NPs. The presence of the compounds such as F-127 and PEI at different concentrations (1% and 2%) greatly increased the GaLM NPs stability and durability. Further by using SEM the GaLM NPs were characterize by size and shape, it was discovered that the nanoparticles were uniformly rounded and had an average size of about 200 nm. For the antibacterial activity the ZOI measured around the wells showed that the GaLM NPs had significant antibacterial action against the strains of *A. baumannii* and *P. aeruginosa*, both when used alone and in combination with the F-127 and PEI compounds. At the concentration of 1 mg/ml, the positive control Ga $(NO_3)_3$ showed the greatest ZOI, indicating potent antibacterial action against *A. baumannii* ATCC 17978 and *A. baumannii* HUMC1. In contrast to the *P. aeruginosa* PA01 strain, the *A. baumannii* ATCC 17978, *A. baumannii* Delta CPS ATCC 17978, and *A. baumannii* HUMC1 strains displayed somewhat larger ZOI, suggesting that *Acinetobacter* may be more sensitive to the GaLM NPs.

It was observed that the inhibition zones for wells that only contained the F-127 and PEI compounds were larger than those with the GaLM NPs along with compounds, which highlights the enhanced antimicrobial activity of the GaLM Nps based treatment. For the MIC and MBC, the GaLM NPs showed inhibition against the capsulated strains of *A. baumannii*, showing MBC values of 0.140 and 0.675 mg/ml for the *A. baumannii* ATCC 17978 and HUMC1 strains, respectively, and MIC values of 0.084 and 0.337 mg/ml. In contrast to the capsulated strains, the non-capsulated *A. baumannii* Delta CPS strain displayed higher MIC and MBC values, indicating that encapsulation reduces *Acinetobacter's* resistance to the gallium nanoparticle. *P. aeruginosa* PA01, the positive control, was suppressed at a lower concentration than the *A.*

baumannii ATCC 17978 strain, suggesting that *P. aeruginosa* is more sensitive to the GaLM NPs. Even at the highest concentrations, neither the F-127 nor the PEI compounds by themselves showed any inhibitory effects on the bacterial growth tested (>2.7 mg/ml) because PEI was slightly turbid so could not conclude on that result, which highlights the importance of the nanoparticle formulation in the antimicrobial activity of these compounds.For the statistical analysis of biofilm growth by using serial dilution and optical density (OD600) measurements, the growth parameters of the several strains which includes the wild-type *A. baumannii* ATCC 1797 were tested . The findings demonstrated a regular growth pattern in each of the repetitions, suggesting that the planktonic growth of the bacteria was not negatively impacted by the experimental conditions. Following a 72-hour incubation period and crystal violet staining, a quantitative analysis of biofilm formation showed that the strains under investigation exhibited different capacity for biofilm production. Increased biofilm formation was observed in strains relative to the wild type, which may be shown to be significantly influenced by the metallic component content of the growth media, as indicated by the absorbance measurements (OD_{600}). Compared to the other strains, the capsular polysaccharide mutant *A. baumannii* CPS strain showed a decreasing optical density (OD), indicating that its incapacity to form biofilms was hampered. When the capsule was present during culture, the other *A. baumannii* strains demonstrated a substantial increase in the production of biofilms. This finding suggests that the capsule might cause *A. baumannii* to undergo stress reactions or activate pathways that promote the growth of biofilms.

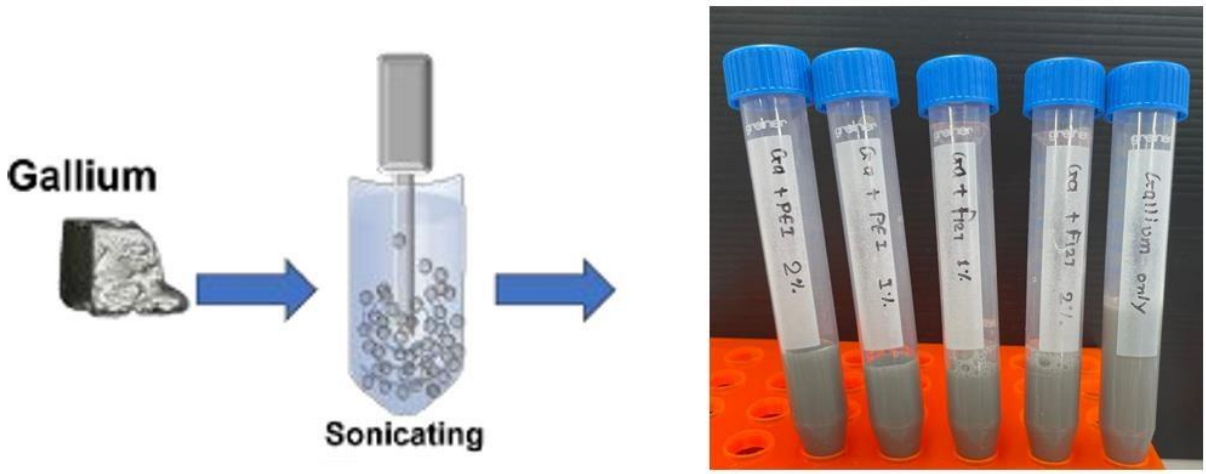
4.2 Future directions

The investigation needs to be examined the GaLM NP formulations long-term stability and storage circumstances to make sure they will remain live and effective over time. Need to learn more about the elements influencing the GaLM NPs increased stability and antimicrobial activity, carry out a more thorough characterisation of the particles, considering their surface

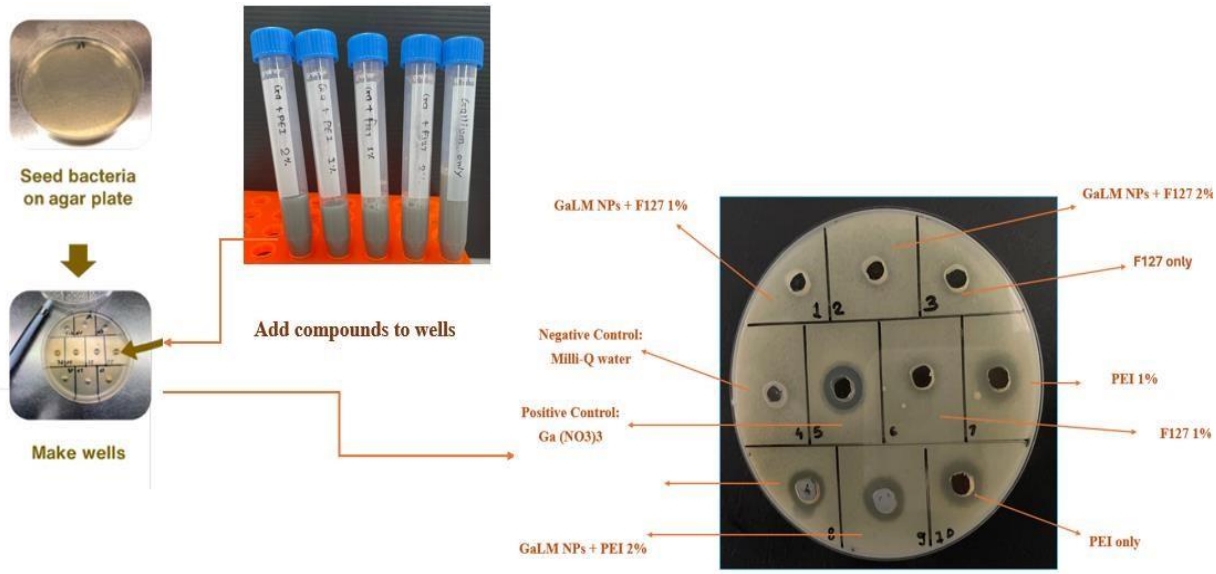
characteristics, chemical composition, and relation with interactive compounds such as with the F-127 and PEI compounds. The initial observations from the SEM images indicated that the GaLM NPs were not uniform in size with a relatively even distribution on the silicon substrates. This uniformity is indicative of successful synthesis and stabilization processes that prevented mixture. The size and density data collected from SEM analysis are expected to provide a quantitative measure of the nanoparticles dimensions, which are crucial for potential applications in targeted drug delivery and other biomedical fields. The future work should include further quantitative analysis of SEM images to observe the average size, size distribution, and density of the NPs across different samples. Additionally, it would be beneficial to correlate these physical characteristics with the nanoparticle's functional properties, such as their stability in biological environments and their efficacy in drug delivery applications. Such correlations are essential for optimizing the synthesis and modification processes to enhance the NPs suitability for specific clinical applications. Examine the specific mechanisms by which the GaLM NPs elicit their antimicrobial effects, paying attention to how they affect the of iron in bacteria and other important cellular functions. Then needs to create more potent combination therapies against drug-resistant *A. baumannii* and *P. aeruginosa*, assess the synergistic or additive effects of mixing the GaLM NPs with traditional antibiotics or other antimicrobial agents. Further it needs to be carried with the comprehensive research to ascertain how the bacterial capsule influences a subject's sensitivity to GaLM NPs. Investigate methods to disrupt and target the capsule to augment the antimicrobial efficacy. To guarantee the viability and efficacy of the optimised GaLM NPs preparation for prospective clinical applications, evaluate the formulations long-term usage and storage conditions. Further research is necessary to elucidate the specific molecular pathways involved in the enhanced biofilm formation observed under the influence of metallic compounds. Additionally, investigating the role of other environmental stressors could provide a more comprehensive understanding of the factors that promote biofilm development

in *A. baumannii*. This knowledge could be enough in designing targeted interventions to kill biofilm formation and reduce the persistence of infections caused by this pathogen.

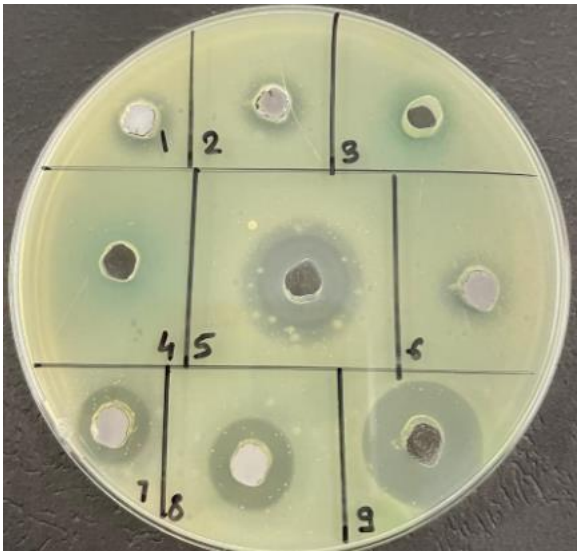
APPENDICES



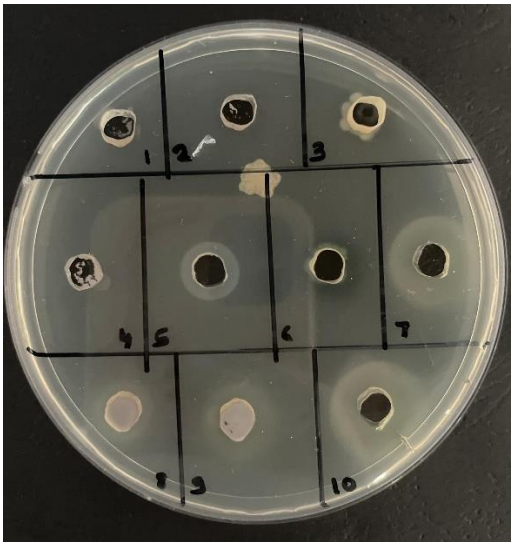
APPENDIX 1: The sonicated gallium-based metal from solid state to liquid state, which shows GaLM NP, GaLM NP + F127 1% and 2%, GaLM NPs + PEI 1% and 2%.



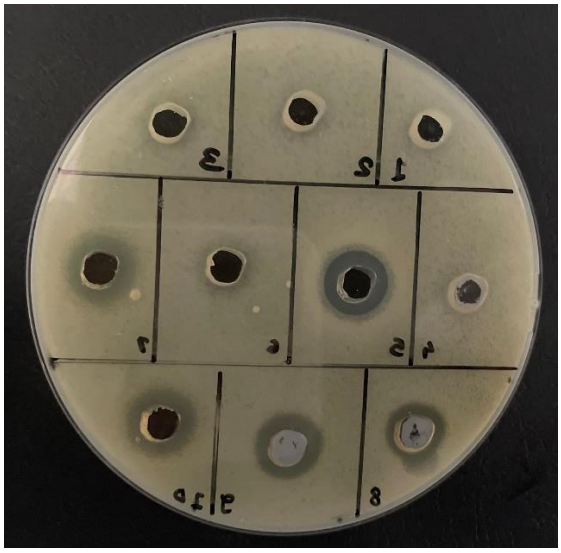
APPENDIX 2: The process of evaluating the antimicrobial activity of gallium metal and gallium metal along with compounds which shows the example of inhibition zones.

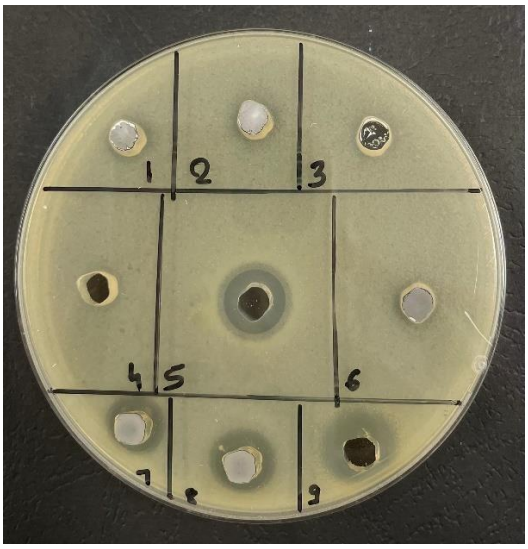


***P. aeruginosa* PA 01**



***A. baumannii* Delta CPS**





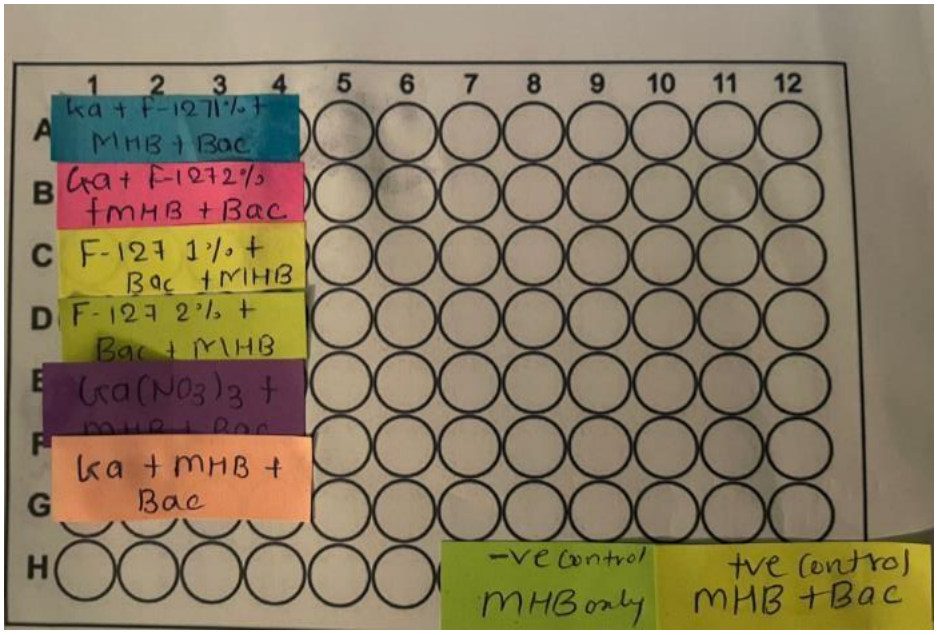
***A. baumannii* HUI** | ***A. baumannii* ATCC 17978**

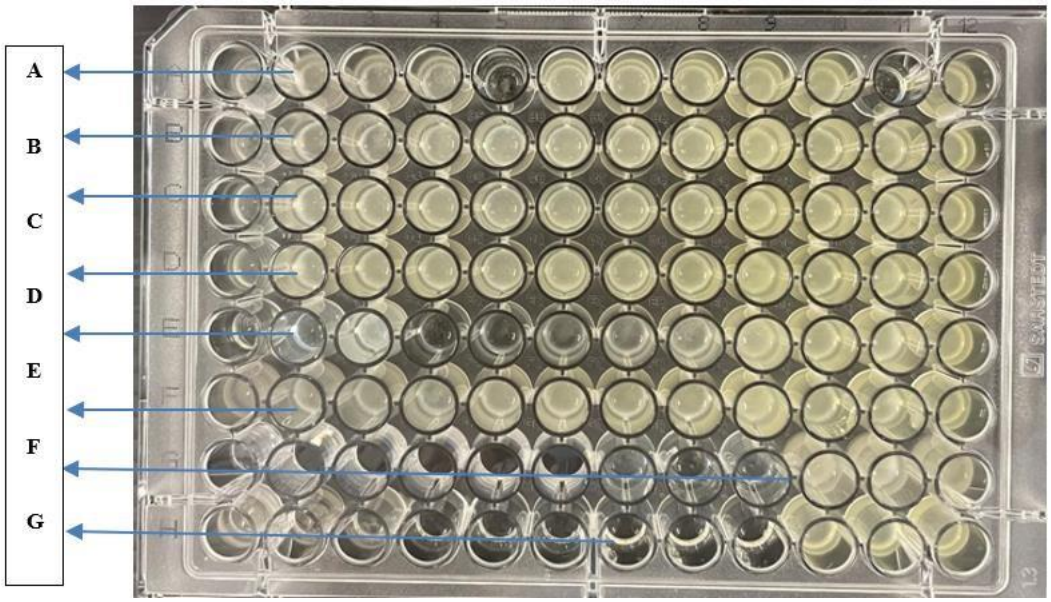
APPENDIX 3: The data shows the inhibition zone of all four strains tested in this study.

APPENDIX 4: The below table describes the average data and standard deviation of all compounds which were added to agar plates for antimicrobial activity to check the resistance against bacteria. The inhibition zone was measured in diameter (mm).

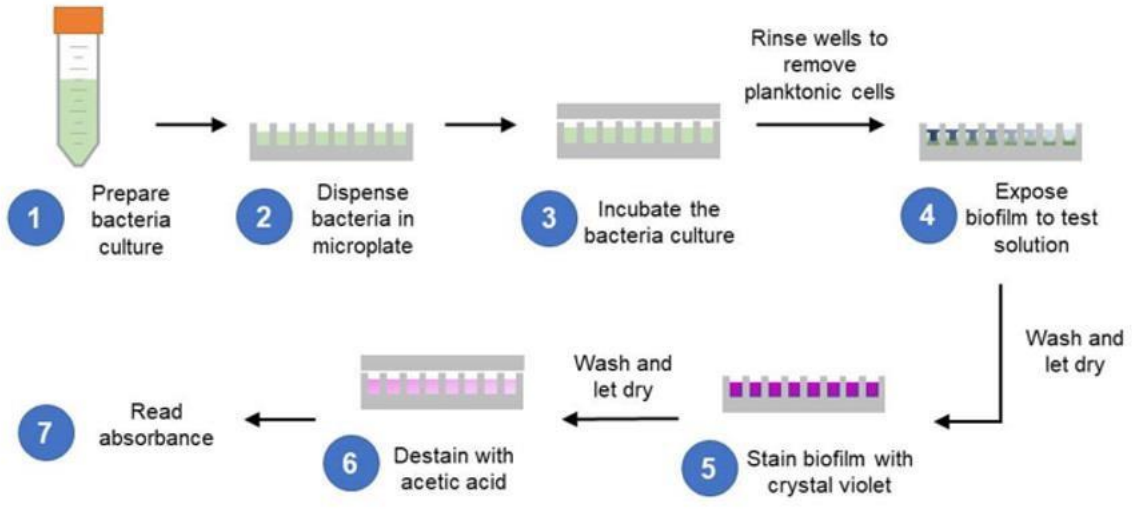
NO	COMPOUND	*A. baumannii* ATCC 17978	*A. baumannii* HUMC1	*A. baumannii* CPS	*P. aeruginosa* PA01
1	GaLM NP + F127 1 %	5 ± 4.3	5.1 ± 0.2	4 ± 3.6	3.3 ± 2.8
2	GaLM NP + F127 2 %	4.5 ± 3.9	5 ± 0	4.4 ± 3.8	4.1 ± 3.6
3	F-127 ONLY	6.3 ± 3.1	5.6 ± 2.3	6.1 ± 2.7	6.3 ± 3.1

4	Milli-Q (control -ve)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
5	$Ga(NO3)_3$ (con:+ve)	14.6 ± 1.5	16.5 ± 1.3	12 ± 3.6	16.4 ± 1.3
6	GaLM NP	0 ± 0	0 ± 0	0 ± 0	0 ± 0
7	GaLM NP + PEI 1 %	11.2 ± 1.1	11.6 ± 1.5	16 ± 0.5	14.7 ± 0.8
8	GaLM NP + PEI 2 %	12.2 ± 1.5	11.3 ± 1.5	14 ± 2.0	11.4 ± 2.4
9	PEI ONLY	15 ± 1.0	13 ± 1.0	14.5 ± 2.2	13.7 ± 1.1
The data is measured in diameter (mm)					



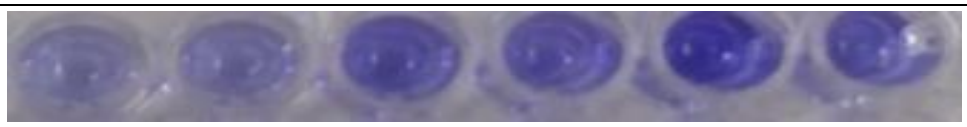


APPENDIX 5: The above template presents the 96-well plates from A to H, the compounds were F127/PEI into each bacterial plates with two plates per bacteria.

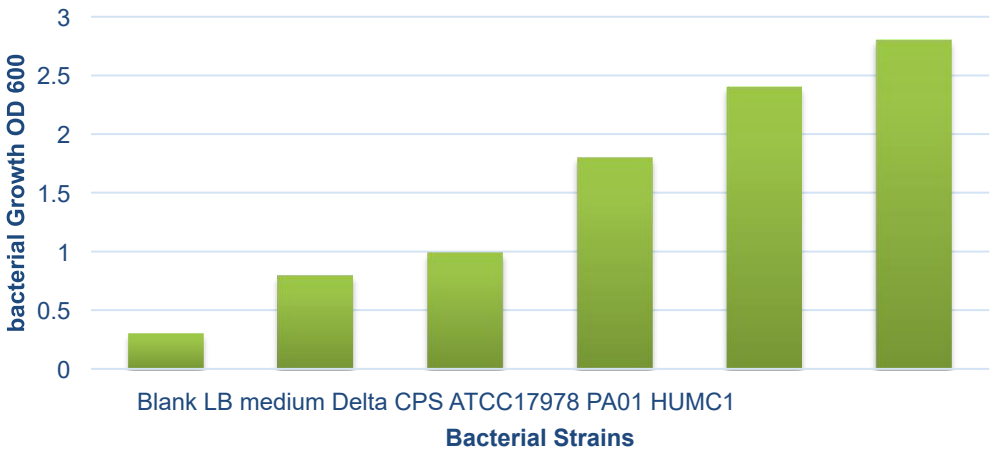


APPENDIX 6: The process of evaluating the antimicrobial activity of GaLM NPs which shows the example of static biofilm assay through crystal violet.

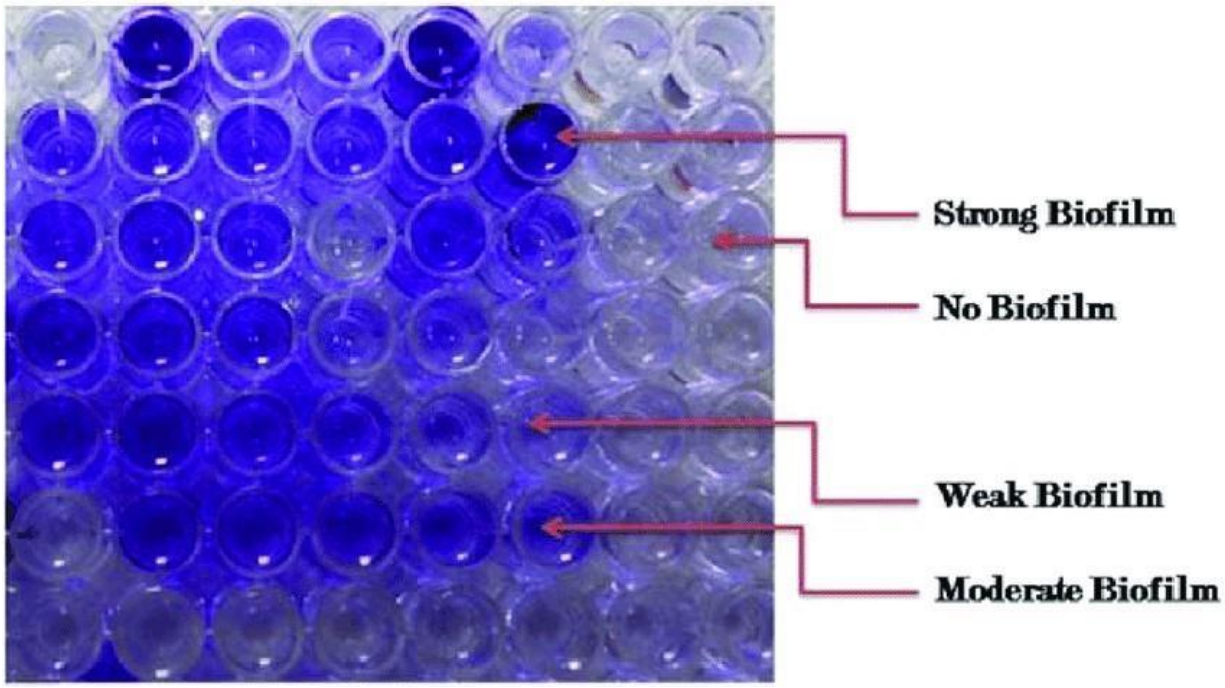
A



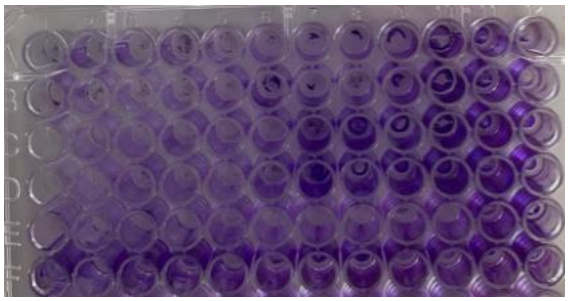
B



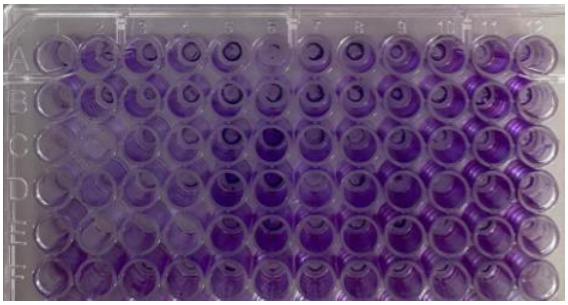
APPENDIX 7: The figure A represents the static biofilm growth of the blank and bacterial strains whereas the figure B represent the OD graph of strains.



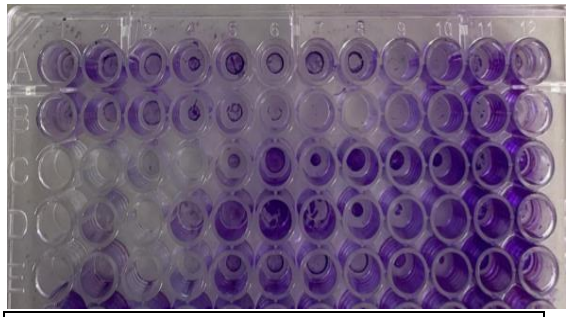
APPENDIX 8: The diagrams show the growth, no growth, strong, moderate, and weak biofilm formation.



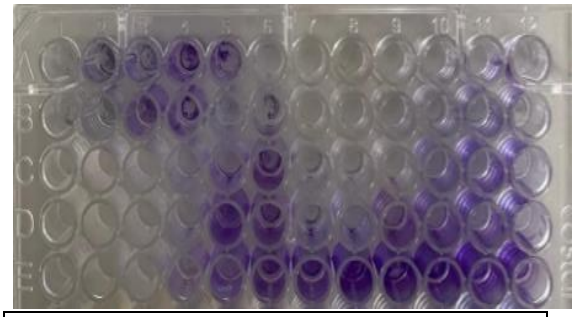
A. baumannii **ATCC 17978**



A. baumannii **HUMC1**



P. aeruginosa **PA 01**



A. baumannii **Delta CPS**

APPENDIX 9 The biofilm growth after crystal violet assay for all bacterial strains results.

A- GaLM NPs + F-127 2%

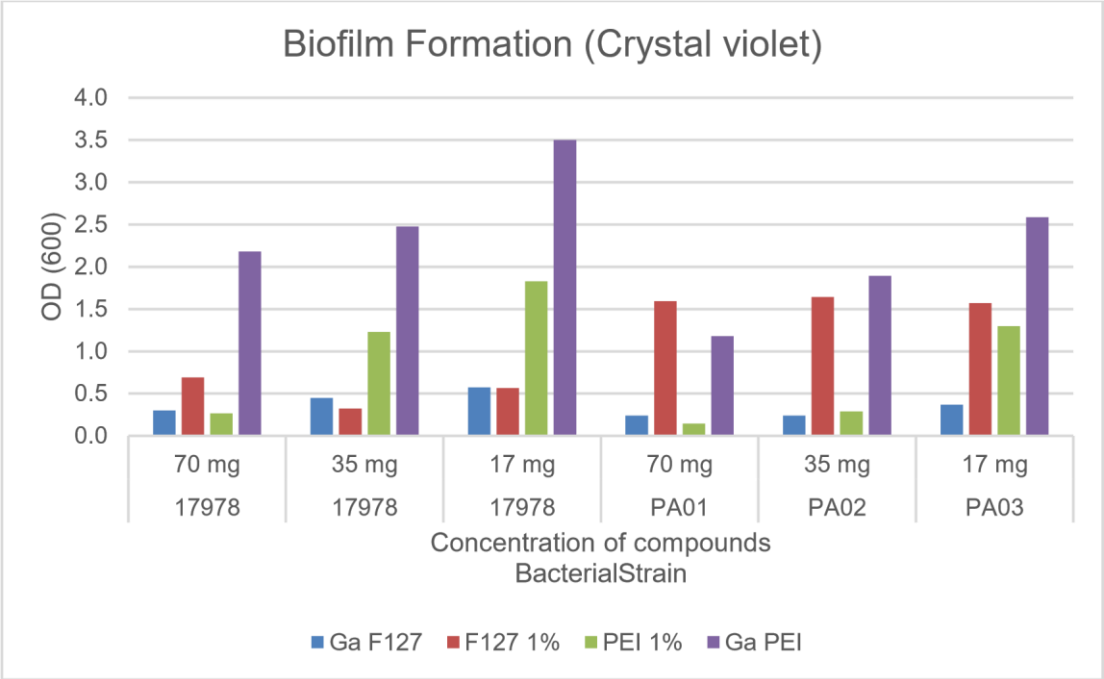
B- GaLM NPs + F-127 1%

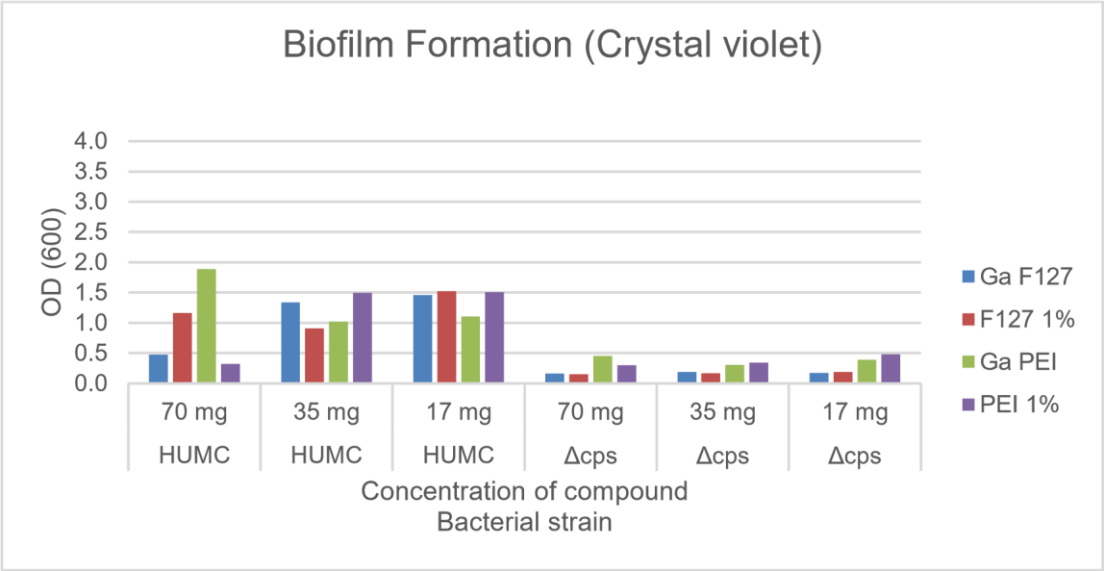
C- GaLM NPs + PEI 2 %

D- GaLM NPs + PEI 1%

E- Ga (NO3)3

F- (F1 TO F6 -GaLM NPs) (F7 TO F12 – Control +ve)





APPENDIX 10: Static biofilm formation of GaLM NPs against four bacterial strains.

It shows three different concentrations of planktonic growth which had grater OD compared to other wells.

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