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Filipa Isabel Guimarães Macedo

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Dissertação de Mestrato
Mestrado em Biotecnologia

Trabalho efetuado sobre a orientação do
Professor Doutor Nuno Cerca
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Statement of Integrity

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism of any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code od Ethical Conduct of the University of Minho.

Resumo

Novas Estruturas Metal-Orgânicas (MOFs) Bioativas e Multifuncionais como Novos Agentes Antibiofilme

As infeções nosocomiais estão a tornar-se cada vez mais recorrentes devido ao aumento de estirpes bacterianas resistentes aos antibióticos. Muitas vezes estas infeções estão associadas a biofilmes microbianos, o que complica o tratamento destas infeções. Além disso, os biofilmes podem formar-se em muitos tipos de superfícies, especialmente nos dispositivos médicos invasivos. Há assim uma necessidade de desenvolver materiais bioativos funcionais novos e eficazes que possam prevenir a formação de biofilmes. Uma alternativa às estratégias antibacterianas convencionais é proporcionada por estruturas bioativas, sejam polímeros de coordenação (CPs) ou estruturas metal-orgânicas (MOFs). Assim, este projeto de dissertação visa estudar a atividade antibacteriana de novos CPs/MOFs e materiais biopoliméricos dopados com esses compostos a fim de descobrir novos materiais com potencial para técnicas de revestimento para o tratamento, prevenção e redução de infeções associadas a dispositivos médicos invasivos.

Os ensaios de triagem das estruturas bioativas em forma de pó revelaram ligandos orgânicos com potencial a serem estudados como elos de ligação na estrutura dos CPs e MOFs, uma vez que alguns deles apresentaram atividade antibacteriana contra *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* e *Staphylococcus epidermidis*, nomeadamente o ácido 2,5-furandicarboxílico (FDA), o ácido 5-sulfoisoftálico (5SIS) e o ácido dodecanodióico (DDDA). Testes mais detalhados demonstraram que os filmes de agarose e amido dopados com diferentes concentrações de $ZnCl_2$ apresentaram um maior efeito antibacteriano contra as bactérias Gram-positivas. Os filmes de agarose e amido dopados com diferentes concentrações de $[Ag_2(SDBA)]_n$ (TG1, MOF à base de prata) ação antibacteriana contra todas as espécies utilizadas, incluindo na prevenção da formação de biofilmes.

O presente trabalho demonstrou o potencial antibacteriano e antibiofilme dos CPs/MOFs, sugerindo o uso dos mesmos como alternativa aos antibióticos para o tratamento e prevenção de biofilmes.

Palavras-Chave: Biofilmes Microbianos; Estruturas Metal-Orgânicas; Infeções Nosocomiais; Inibição de Biofilme; Polímeros de Coordenação.

Abstract

Novel Multifunctional Bioactive Metal-Organic Frameworks (MOFs) as New Anti-Biofilm Agents

Nosocomial infections are becoming more recurrent due to the increase of antibiotic resistant strains. Often these infections are associated with microbial biofilms, which complicates the treatment of these infections. Furthermore, biofilms can form on many types of surfaces, especially on indwelling medical devices. Thus, there is a need to develop new and effective bioactive functional materials that can prevent biofilm formation. An alternative to conventional antibacterial strategies is provided by bioactive structures, either coordination polymers (CPs) or metal-organic frameworks (MOFs). Thus, this dissertation project aims to study antibacterial properties of novel CPs/MOFs and biopolymer materials doped with those compounds, in order to discover new potential materials for coating strategies for the treatment, prevention and reduction of infections associated with indwelling medical devices.

Starting from a large collection of CPs/MOFs, the screening assays performed revealed organic ligands with promising potential to be studied as linkers for the CPs and MOFs, since some of them had antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis*, namely 2,5-furandicarboxylic acid (FDA), 5-sulfoisophthalic acid (5SIS) and dodecanedioic acid (DDDA). Further testing showed that agarose and potato starch biopolymers films doped with different concentrations of $ZnCl_2$ demonstrated greater antibacterial action against Gram-positive bacteria. Agarose and potato starch films doped with different concentrations of $[Ag_2(SDBA)]_n$ (TG1, silver-based MOF) showed antibacterial activity against all tested bacteria, including in preventing biofilm formation.

The present work demonstrated the antibacterial and antibiofilm potential of CPs/MOFs, suggesting their used as an alternative to antibiotics for the treatment and prevention of biofilms.

Keywords: Biofilm Inhibition; Coordination Polymers; Metal-Organic Frameworks; Microbial Biofilms; Nosocomial Infections.

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List of Abbreviations

4PSB – 4-sulfobenzoic acid

5SIS – 5-sulfoisophthalic acid

ARGs - antibiotic resistance genes

CAUTI – catheter-associated urinary tract infections

CFU – colony-forming units

CLABSI – central line-associated bloodstream infections

CPs – coordination polymers

DL – malic acid

DMSO – dimethyl sulfoxide

ESOA – epoxidized soybean oil acrylate

EPS – extracellular polymeric substances

FDA - 2,5-furandicarboxylic acid

GL – gluten

GLHY – hydroxyapatite gluten

HGT – horizontal gene transfer

HY – hydroxyapatite

IMDs – indwelling medical devices

Lig. CH28 – dodecanedioic acid or DDDA

Lig. TG – 4,4′-sulfonyldibenzoic acid or SDBA

Lig. TG19 – 4,4'-oxydibenzoic acid or ODBA

MBC – minimum bactericidal concentration

MDR – multidrug-resistant

MHA – Mueller-Hinton II agar

MHB – Mueller-Hinton II broth

MIC – minimum inhibitory concentration

MOFs – metal-organic frameworks

NIs – nosocomial infections

RI – resin impression

SD – standard deviation

VAP – ventilator-associated pneumonia

WHO – World Health Organization

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Literature Review

This section is dedicated to the theoretical background related to this dissertation project. Antibiotic resistance and how it leads to the establishment of multidrug-resistant strains will be explored in this section, as will the importance of indwelling medical devices, their drawbacks, and what are nosocomial infections. It will be also addressed what are biofilms and how they are formed, what are bioactive coordination polymers, their applications, and their importance in the treatment of bacterial infections.

1. Antibiotic Resistance

Antibiotics are one of the most common forms of therapy in medicine, specifically in the treatment of bacterial infections (Davies & Davies, 2010). Antibiotics are small molecules that can kill bacteria or inhibit bacterial growth and, consequently, the increasing amounts of antibiotics for human applications and antibiotic abuse wield strong selective pressures on bacterial populations, leading to the appearance of antibiotic resistant strains (Ellermann & Sperandio, 2020). These strains result in infections difficult to treat with traditional antibiotics, which leads to increased morbidity[1] and mortality[2] in clinical settings (Akova, 2016). Thus, according to the World Health Organization (WHO), antibiotic resistance is the biggest threat and challenge to public health (WHO, 2020).

Bacterial resistance to antibiotics can be obtained by several strategies: prevention of antibiotic entry in the cell, modification of antibiotic targets, and modification or inactivation of antibiotic (Munita & Arias, 2016). These strategies can occur through mutations of chromosomal genes often associated with the mechanism of action of the antibiotic and by the acquisition of external DNA coding antibiotic resistance genes (ARGs) in a process called horizontal gene transfer (HGT). Mutations in the antibiotic target can result in decreased affinity for the pharmaceutical, decreased drug uptake, activation of effluxes that pump the antibiotic out of the cell, or overall changes in vital metabolic pathways. Moreover, it is important to mention that ARGs can be found not only in clinical isolates, but also in other pathogenic, commensal, and environmental bacteria. Additionally, bacteriophages and mobile genetic elements, such as plasmids, work as a ARGs storage from which bacteria can acquire resistance via HGT (Frieri *et al.*, 2017).

[1] Morbidity is any physical or psychological state of having a specific illness or condition. The term can refer to an acute or chronic condition.

[2] Mortality is often expressed in the form of mortality rate and refers to the number of deaths that have occurred due to a specific illness or condition.

Many antibiotic resistant bacteria have evolved into multidrug-resistant (MDR) strains. This phenotype is very common in clinical isolates, community acquired and nosocomial pathogens, and its prevalence has increased at an alarming rate over the past decades (Akova, 2016). As a result, MDR bacteria not only are difficult to treat, but can also be untreatable with traditional antibiotics, due to the current shortage effectiveness therapies, lack of successful prevention measures and lack of new antibiotics (Frieri *et al.*, 2017). Some examples of MDR bacteria include *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus.*

E. coli and *P. aeruginosa* are Gram-negative bacteria that cause a wide range of diseases, such urinary tract and bloodstream infections and chronic wounds. *P. aeruginosa* is also associated with respiratory tract infections, especially in patients with cystic fibrosis (Schulze *et al.*, 2021). These pathogens have high resistance to β-lactam, aminoglycoside, quinolone, carbapenem, among others, classes of antibiotics (Akova, 2016).

S. aureus is a Gram-positive bacterium associated with chronic wounds and infections related to indwelling medical devices. Furthermore, *S. aureus* is associated with bloodstream, respiratory tract, genitourinary tract infections and several diseases in the skin (Schulze *et al.*, 2021). Methicillin was introduced to treat penicillin-resistant *S. aureus*, however *S. aureus* also acquired resistance to methicillin, that have appeared not only in hospitals, but also in the community and among farmed animals, due to its capability to evolve and adapt to different environments (Frieri *et al.*, 2017). *S. aureus* strains that are sensitive to methicillin are usually susceptible to clindamycin, vancomycin and teicoplanin (Akova, 2016).

2. Bacterial Infections in Medical Devices

The most remarkable advance in modern medicine has been the development of medical devices, in particular indwelling medical devices (IMDs). A medical device is any instrument, apparatus, appliance, software, material, or other related objects, designed to be used alone or in combination for medical purposes (Ploeg, R. J., & Rakhorst, G., 2008).

The IMDs are essential in modern surgical practice that provides effective, cost-efficient, and simple solutions for the treatment of a variety of clinical settings, extending and/or enhancing many lives (Gilmore & Carson, 2015). Some examples of IMDs and their medical applications are presented in table 1.

Table 1. Examples of indwelling medical devices and their application in medical settings.

Indwelling Medical Devices	*Application*
Intravenous Catheter	Deliver fluids, blood products, nutritional solutions, or medications straight into the bloodstream, and for access in dialysis treatment (Cassoobhoy, 2020a).
Urinary Catheters	Measure urine output, collect urine during surgery, prevent urine retention or control urinary incontinence (Cassoobhoy, 2020a).
Prosthetic Heart Valves	Replace a heart valve that has become damaged due to heart valve disease (Vega, 2020).
Cardiac Pacemakers	Treat arrhythmias and help heart chambers beat in sync (Cassoobhoy, 2020b).
Endotracheal Tubes	Permit air to pass freely to and from the lungs to ventilate the lungs, especially when patients cannot maintain adequate respiratory function (Schiffman, 2021).
Joint Prostheses	Replace an arthritic or damaged joint (Fischer & Foran, 2021).
Contact Lenses	Vision correction, protect the cornea, relieve pain, and enhance healing of the corneal surface (Foulks, 2003).

Nosocomial infections (NIs) are infections that patients acquire while receiving health care, in other words, are infections for which there was no evidence that the infection was present or incubating at the time of hospital admission (Emori & Gaynes, 1993). It is important to mention that NIs include bacterial infections associated with IMD and represent a massive financial burden on healthcare services and lead to considerable morbidity and mortality (Percival *et al.*, 2016). Even though IMDs are provided as sterile, the instant their packages are opened, handled, and inserted into a patient, IMDs become exposed to microorganisms that colonise the human body and the healthcare environment itself. It is well-known that these environments are reservoirs of pathogenic microorganisms, therefore the employment of these devices must be driven by cautions healthcare policies (Percival *et al.*, 2016). Upon insertion of an IMD, it might be immediately coated with a layer of host-derived substances, which on one hand promotes the integration of the foreign material into the host body, but on the other hand enables microorganisms to attach to the device, and once attached, they can develop into a biofilm (Cooper, 2015). Thus, bacterial infections associated with IMD are often related to the formation of biofilms, which in turn can be

influenced by surface roughness, hydrophobicity, porosity and surface area of the devices (Bose & Ghosh, 2015).

Biofilms are communities of microorganisms that can attach to a biotic or abiotic surface and are integrated into a matrix of extracellular polymeric substances (EPS), which is produced by the communities themselves (Flemming, 2016). It is important to note that the community can be assembled by one or multiple species, thus the matrix composition may differ depending on the species (Faustino *et al.*, 2020). The matrix of EPS is the main characteristic of biofilms, which essentially consists of polysaccharides, proteins, and nucleic acids (extracellular DNA). The matrix allows the microbial attachment to surfaces, cell-to-cell adhesion, aggregation and communication, stabilizes the biofilm structure and functions as a barrier that help to protect the microorganisms from external threats, such as antibacterial treatments and immune system substances from the host (Percival *et al.*, 2016; Wi & Patel, 2019). Generally, bacterial biofilm development involves several stages, but can be summarize into three main stages: attachment, maturation, and dispersal (Figure 1). This process is mediated by auto-inducers that allow cell-to-cell communication, a process referred to as quorum sensing.

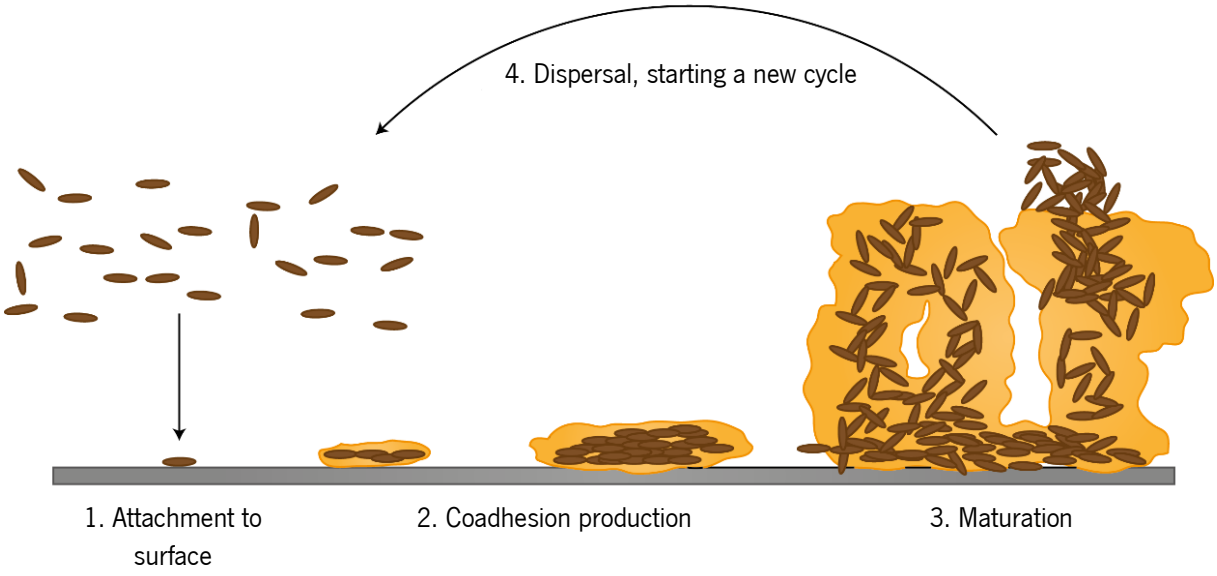

Figure 1. Representation of development cycle of biofilm formation, where surface is represented in grey, planktonic cells as brown ovals and extracellular matrix as yellow. Adapted from Hollmann *et al.* (n.d.).

In the first stage of biofilm formation, the attachment of planktonic bacteria to a surface involves several factors, such as bacterial mobility (pili, flagella and fimbriae), attractive physical forces and electrostatic interactions between bacteria and the surface (Faustino *et al.*, 2020). In addition, host-

derived proteins, released to help in the mending upon IMD insertion, enhance bacteria attachment, as mentioned above, through interactions between bacteria and these proteins (Wi & Patel, 2019).

Table 2. Overview of factors involved in the different stages of biofilm formation, in different bacterial pathogens (*E. coli* (Jackson *et al.*, 2002), *P. aeruginosa*, *S. aureus* and *S. epidermidis* (adapted from Schulze *et al.* (2021)).

Stage of biofilm formation	***Bacterial pathogen***		
	E. coli	*P. aeruginosa*	*S. aureus* and *S. epidermidis*
Attachment	Flagella, type I pili, curli fimbriae	Flagella, twitching motility mediated by type IV pili, WspA protein, CdrA adhesin and cup fiambrial adhesins	Teichoic acids, autolysin Atl, protein Bap, MSCRAMMs, SERAMs
Maturation	DNABII binding proteins, exopolysaccharides (colanic acid), OmpR, Rpos	Exopolysaccharides (alginate, Psl and Pel), eDNA (EndA), proteins (Psl binding protein CdrA, LecA and LecB), rhamnolipids	Exopolysaccharide (PIA), eDNA (Nuc1 and Nuc2), proteins (SasG and other adhesins [see above]), teichoic acids
Detachment	N-acetyl-heparosan lyase, CsrA	Rhamnolipids, cell lysis, alginate lyase	Exoproteases (SspA, SplA-F, SspB, ScpA, Aur), PSMs

Once the bacteria have irreversibly attached to the surface, the stage of maturation takes place, where bacteria begin to produce a matrix of EPS. At this stage, bacteria start a process of multiplication and division, which leads to the formation of microcolonies (Faustino *et al.*, 2020). As the biofilms matures, it acquires a three-dimensional structure that provides protection from external threats, as previously mentioned (Wi & Patel, 2019).

Finally, the dispersal stage starts, during which portions of the biofilms detach and release planktonic bacteria from the surface. The cyclic di-GMP secondary messenger is one of the intracellular

mechanisms responsible for the detachment of bacteria within the biofilm. However, there are other factors that contribute for this stage, such as changes in nutrients, temperature, pH, oxygen levels and other stressors, or through chemical signals between microorganisms. Dispersed bacteria can then attach to new surfaces and start a new biofilm formation cycle (Percival *et al.*, 2016). Besides this general process of biofilm formation, there may be some factors that are different according to the bacterial pathogen. These differences are illustrated in table 2.

In clinical settings, biofilms are very difficult to treat due to their high tolerance to antibiotics, causing constant reinfections and chronic inflammations, leading to a general resistance to therapies, which poses a challenge in the treatment of NIs (Schulze *et al.*, 2021). Antibiotic tolerance in biofilm is associated with the presence of EPS matrix, which protects the bacteria from external factors, decreases penetration of antibiotics into the biofilms and inactivates the antibiotic through substances released by the matrix of EPS (Faustino *et al.*, 2020). It is also associated with metabolic alterations in bacteria and exchange of bacterial resistance mechanisms between bacteria (Schulze *et al.*, 2021).

The most common NIs associated with IMD are central line-associated bloodstream infections (CLABSI), catheter-associated urinary tract infections (CAUTI) and ventilator-associated pneumonia (VAP), which are presented in table 3, along with their most common bacterial agents (Faustino *et al.*, 2020).

Table 3. Examples of indwelling medical devices and their nosocomial infections and most common bacterial agents associated (Faustino *et al.*, 2020).

Indwelling Medical Devices	*Nosocomial Infection*	*Common Bacterial Agents*
Intravenous Catheter	CLABSI	*S. epidermidis*, *S. aureus*, *P. aeruginosa*
Urinary Catheters	CAUTI	*E. coli*, *Proteus mirabilis*, *Klebsiella pneumoniae*, *P. aeruginosa*, *Enterococcus facecalis*, *S. aureus*, *S. epidermidis*
Endotracheal Tubes	VAP	*Pseudomonas* spp., *Klebsiella* spp., *S. aureus*

As a result, there is a constant demand not just for new and more effective antibiotics, but also for bioactive functional materials that can inhibit bacteria accumulation and biofilm growth on their surface. Nowadays, there are several approaches to prevent infections associated with IMD, the most fundamental being hand cleansing with alcohol-based hand rub, since it was established to control the propagation of

infections (Sartelli *et al.*, 2018). Other approaches are based on decreasing and preventing bacterial adhesion to IMDs and to eliminate adhering microorganisms (Zander & Becker, 2018).

3. Bioactive Coordination Compounds

Bioactive coordination compounds, including coordination polymers (CPs) and metal-organic frameworks (MOFs) provide a potentially effective alternative to common antibacterial strategies, as mentioned in literature (Giliopoulos *et al.*, 2020; Golmohamadpour *et al.*, 2018; Wyszogrodzka *et al.*, 2016; Zhao *et al.*, 2020). These compounds can be made by combining a metal center with organic linkers, resulting in metal-organic structures that can also incorporate antibacterial guest molecules (metal nodes or bioactive organic molecules) (Figure 2).

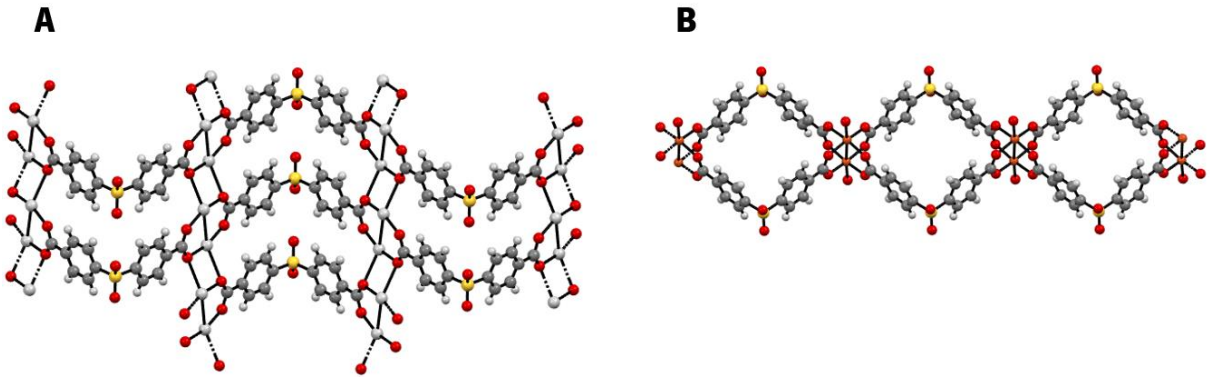

Figure 2. 2D layer-pillared (A) metal-organic network of $[Ag_2(sdba)]_n$ and (B) coordination polymer structure of $\{[Cu(sdba)\cdot H_2O]\cdot 1.5H_2O\}_n$.

MOFs, in particular, are crystalline materials with high porosity, large internal surface areas, typically low density and moderate thermal and mechanical stability (Keskin & Kizilel, 2011; Shen *et al.*, 2019). The active center of CPs or MOFs are stabilized by the establishment of strong chemical bonds which make the material robust, but fragile enough not to block their functionality. Such bonds include metal coordination, hydrogen bonding, electrostatic interactions and π-π stacking (Wyszogrodzka *et al.*, 2016). Therefore, one of the advantages of these structures is the ample choice of the components, structural features and physical properties, such as pore sizes and shapes. The different building-blocks interaction and their spacial geometries provide a theoretically limitless number of materials with a wide range of unique properties, enabling the production of different and suitable CPs or MOFs for specific applications (Shen *et al.*, 2019). MOFs can be categorized as rigid or flexible: rigid MOFs have a defined porosity and robust porous; on the other hand, flexible MOFs change according to external factors, such

as guest molecules, temperature and pressure, which allows the MOF to reversibly modulate their pores (Keskin & Kizilel, 2011).

Other advantage of coordination compounds, CPs and MOFs, is the capability to readjust the structure and functionality during their synthesis (Keskin & Kizilel, 2011). These synthetic methods essentially include hydro/solvothermal, ionothermal, microwave-assisted, sonochemical, electrochemical, mechanochemical and microfluid synthesis (Yang & Yang, 2020). As may be expected, each of these methods has advantages and disadvantages in terms of efficiency, scale-up production, physiochemical properties, amongst others (Yang & Yang, 2020).

After the synthesis, the following characterization techniques are frequently employed to characterize MOFs: powder and single crystal X-ray diffraction (because they are highly crystalline and porous), infrared and/or Raman spectroscopy (to characterize functional groups and incorporated guest molecules in the pores), elemental analysis, thermogravimetric analysis and scanning electron microscopy. The surface areas of MOFs are evaluated using nitrogen absorption assays (Keskin & Kizilel, 2011).

As previously mentioned, AR is rapidly increasing due to the appearance of resistant bacteria, therefore the development of new antibacterial agents is required to address this global issue. The application of MOFs and CPs in biomedicine is becoming a topic of high interest due to their potential antibacterial activity (Shen *et al.*, 2019). The antibacterial activity of CPs and MOFs is caused by several features, such as (1) encapsulation of antibacterial molecules, in the case of MOFs, due to their high surface areas and pores; (2) properties of the metal ion in the structure, since some metal ions reveal antibacterial activity (such silver (Ag), copper (Cu), zinc (Zn), etc.) and their maintained released throughout the degradation of the CP/MOF structure provides a highly effective and durable antibacterial effect; (3) the organic ligands in their structure can also have antibacterial properties. Consequently, the metal ion and the organic ligand can form a synergistic effect (Shen *et al.*, 2019; Yang & Yang, 2020). Moreover, the antibacterial mechanisms of action of CPs or MOFs are very distinctive to those of antibiotics, considering that their mechanisms are associated with the physical damage of bacteria, instead of metabolic processes. As a result, CPs or MOFs provide a possible alternative to common antibacterial strategies (Wyszogrodzka *et al.*, 2016).

The cytotoxicity, particle size and porosity of MOFs are properties that must be taken into consideration in their application in biomedicine. Firstly, MOFs incorporate metal ions which can exhibit some cytotoxicity; however, the gradual degradation of the MOF and the release of their components will

have less toxicity than the toxicity caused from the administration of the pure component. Additionally, choosing components with low cytotoxicity is most likely to form a less toxic MOF. On the other hand, the control of MOF particle size in the nanometric range allows their incorporation into the bacteria cells and their structure preservation. Also, the reduction of MOF size improves their surface affinity, allowing greater interaction with the surroundings, and prevents their aggregation; therefore, the antibacterial activity of many metal ions can be enhanced by the reduction of their size to the nanoscale (Wyszogrodzka *et al.*, 2016).

4. Applications of Bioactive Coordination Compounds in Biofilm Formation

In this section, it will be discussed some examples of MOFs applicated to biofilm formation. It is important to mention that there are two types of bacteria, Gram-negative (such as *E. coli* and *P. aeruginosa*) and Gram-positive (such as *S. aureus* and *S. epidermidis*), which often results in different tolerance to CPs or MOFs. The distinction between both types is their cell wall composition, where the peptidoglycan layer of the Gram-negative bacteria is thinner than the Gram-positive, and the presence or absence of an outer membrane, which is characteristic of Gram-negative bacteria (Shen *et al.*, 2019).

In 2017, Neufeld and colleagues reported the water-stable MOF Cu-BTTri in a chitosan matrix (chitosan/Cu-BTTri material film) for the evaluation of bacteria attachment in solution. The results exhibited a considerable reduction, approximately 85%, in bacterial attachment within 24 hours, where the threshold for biofilm inhibition began at 5% w/w incorporation of the material film. However, increasing the chitosan/Cu-BTTri material film from 5% to 10 and 20% w/w did not increase the antibacterial activity, suggesting a saturation point of this activity. Furthermore, this functionality was retained after a second round of bacterial attachment studies, indicating reusability of these material as antibacterial surfaces. This report presents a novel biomaterial as a biofilm inhibitor to be used as a passive antibacterial surface in settings with regular bacterial infections.

In 2019, Arenas-Vivo and co-workers reported the synthesis of a multi-active silver-containing nanoscaled MOF composite – (AgNP@nanoMOF) – as a potential surface coating against *S. aureus* biofilm. The AgNPs were successfully loaded into the nanoMOF and the biocidal activity of AgNP@nanoMOF was significantly higher than the nanoMOF alone and the nanoMOF impregnated with already pre-synthesized AgNPs (AgNPs+nanoMOF), exhibiting 99,98% growth inhibition. Surprisingly, the biocidal activity of the AgNP@nanoMOF was promoted after 2 hours of ultraviolet-a irradiation, which

prevented more than 99% of the viable bacterial growth. Furthermore, the AgNP@nanoMOF film favoured 80% of bacterial detachment from the biofilm after UV-a irradiation, while in the irradiated nanoMOF and AgNPs+nanoMOFs it was only 40% and 15%, respectively. This anti-adherent property might be associated with the generation of reactive oxygen species, which leads to bacterial death and their detachment from the biofilm. Therefore, these results demonstrated the potential use of AgNP@nanoMOF composite-coated surfaces for biofilm treatment on nosocomial infections. In addition to the application of MOFs as an antibacterial material, they can be used as well as delivery systems for cancer therapy, bioimaging nanoplatforms, biosensors and biocatalyst (Yang & Yang, 2020). MOFs can also be utilized for wastewater treatment (Abdelhameed *et al.*, 2020), gas storage, adsorption and separation (Long *et al.*, 2009; Morris & Wheatley, 2008).

Materials and Methods

1. Bacterial Cultures and Growth Conditions

Bacteria used in these experiments were two Gram-positive bacteria (*S. aureus* ATCC 25923 and *S. epidermidis* RP62A) and two Gram-negative bacteria (*E. coli* ATCC 25922 and *P. aeruginosa* PA14). The growth mediums used were Mueller-Hinton II agar (MHA) and Mueller-Hinton II broth (MHB) for both antibacterial and antibiofilm assays, minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays and were from Liofilchem. In addition, a solution of sodium chloride (NaCl) at 0.9% was used in antibiofilm assays for the washing and dilution steps. The bacteria grew in aerobic conditions, with agitation at 120 rpm and at 37 °C.

2. Powdered Compounds

These compounds were synthesized in the Department of Chemical Engineering of Instituto Superior Técnico de Lisboa. Table 4 lists the film matrices used herein. The powdered compounds tested are listed in table 5. Each row of the table consists in the given reference name of the compound, the respective metal source and organic ligand, and each colour represents compounds that are relatable with each other (structural or building-block similarities). The table 6 lists the compounds that were tested for MIC and MBC assays.

Table 4. List of powdered compounds for film matrices tested.

Film Matrices		
GL	HY	GLHY
Casein	Cellulose Acetate	Sodium Alginate
Potato Starch	Agarose	

GL – gluten; HY - hydroxyapatite; GLHY – hydroxyapatite gluten;

Table 5. List of powdered compounds tested.

Complexes	*Metal Source*	*Organic Ligand*
TI108, $[Cu_2(FDA)\cdot 4H_2O]_n$	$Cu(CH_3CO_2)_2$	FDA
TI104, $[Ag(FDA)]_n$	$AgNO_3$	FDA
CH28, $\{[Cu(DDDA)(NH_3)_2]\cdot H_2O\}_n$	$Cu(NO_3)_2$	Lig. CH28
TG19, $[Cu(ODBA)(NH_3)_2]_n$	$Cu(NO_3)_2$	Lig. TG19
TG1, $[Ag_2(SDBA)]_n$	$AgNO_3$	Lig. TG
TG2, $\{[Cu(SDBA)\cdot H_2O]\cdot 1.5H_2O\}_n$	$Cu(CH_3CO_2)_2$	Lig. TG
CHTF06	$AgNO_3$	Lig. CHTF
CHTF08	$Cu(NO_3)_2$	Lig. CHTF
Nd5SIS	$Nd(CH_3CO_2)_3$	5SIS
TG58	Zinc	Lig. TG19
TG59	Zinc	Lig. TG
-	-	$C_9H_{16}O_4$
-	-	$C_7H_{12}O_4$
-	-	4PSB
-	-	DL

FDA – 2,5-furandicarboxylic acid; Lig. CH28 – dodecanedioic acid; Lig. TG19 – 4,4'-oxydibenzoic acid;
Lig.TG – 4,4'sulfonyldibenzoic acid; 5SIS – 5-sulfoisophthalic acid; 4PSB – 4-sulfobenzoic acid; DL – malic acid

Table 6. List of powdered compounds for MIC and MBC assays.

Powdered Compounds			
C7	C8	C9	$ZnCl_2$

3. Biopolymer Films Tested

These biopolymeric films were synthesized in the Department of Chemical Engineering of Instituto Superior Técnico de Lisboa. Tables 7 and 8 list the biopolymeric films that were completely characterized and demonstrated potential to be used as an antibacterial material, accordingly to the results obtained

from screening assays. Tables 9 and 10 list biopolymeric films doped with metal sources that demonstrated antibacterial activity in their powder form.

Table 7. List of biopolymeric films of TG1 and TG2 tested.

Biopolymeric Films of Agarose		*Biopolymeric Films of Potato Starch*	
TG1 1%	$AgNO_3$ 1%	TG1 1%	$AgNO_3$ 1%
TG1 2.5%	$AgNO_3$ 2.5%	TG1 2.5%	$AgNO_3$ 2.5%
TG1 5%	$AgNO_3$ 5%	TG1 5%	$AgNO_3$ 5%
TG2 1%	$Cu(CH_3CO_2)_2$ 1%	TG2 1%	$Cu(CH_3CO_2)_2$ 1%
TG2 2.5%	$Cu(CH_3CO_2)_2$ 2.5%	TG2 2.5%	$Cu(CH_3CO_2)_2$ 2.5%
TG2 5%	$Cu(CH_3CO_2)_2$ 5%	TG2 5%	$Cu(CH_3CO_2)_2$ 5%
Lig. TG 1%	Agarose (blank)	Lig. TG 1%	Potato Starch (blank)
Lig. TG 2.5%		Lig. TG 2.5%	
Lig. TG 5%		Lig. TG 5%	

Table 8. List of biopolymeric films of Impression Resin (RI) and Eposidized Soybean Oil Acrylate (ESOA) tested.

Biopolymeric Films of RI		*Biopolymeric Films of ESOA + 0.5% Benzophenone*	
TG19 0.5%	Lig. TG19 0.5%	TG19 0.5%	Lig. TG19 0.5%
TG19 1%	Lig. TG19 1%	TG19 1%	Lig. TG19 1%
CH28 0.5%	Lig. CH28 0.5%	CH28 0.5%	Lig. CH28 0.5%
CH28 1%	Lig. CH28 1%	CH28 1%	Lig. CH28 1%
$Cu(NO_3)_2$ 0.5%	$Cu(NO_3)_2$ 5%	$Cu(NO_3)_2$ 0,5%	$Cu(NO_3)_2$ 5%
$Cu(NO_3)_2$ 1%	$Cu(NO_3)_2$ 10%	$Cu(NO_3)_2$ 1%	$Cu(NO_3)_2$ 10%
$AgNO_3$ 0.5%	RI (blank)	ESOA (blank)	
$AgNO_3$ 1%			

Table 9. List of doped biopolymeric films with $ZnCl_2$.

Biopolymeric Films of Agarose		*Biopolymeric Films of Potato Starch*	
$ZnCl_2$ 1%	Agarose (blank)	$ZnCl_2$ 1%	Potato Starch (blank)
$ZnCl_2$ 2.5%		$ZnCl_2$ 2.5%	
$ZnCl_2$ 5%		$ZnCl_2$ 5%	

Table 10. List of doped biopolymeric films with $Cu(NO_3)_2$.

Biopolymeric Films of Agarose	*Biopolymeric Films of Potato Starch*
$Cu(NO_3)_2$ 1%	$Cu(NO_3)_2$ 1%
$Cu(NO_3)_2$ 2.5%	$Cu(NO_3)_2$ 2.5%
$Cu(NO_3)_2$ 5%	$Cu(NO_3)_2$ 5%

4. MIC and MBC Assays

These experiments were performed for the compounds mentioned in the table 6 and were carried out accordingly to the Clinical and Laboratory Standards Institute by the method involving microdilution. The required solutions of each compound were prepared with distilled water on unsterile conditions, in addition the reagent dimethyl sulfoxide (DMSO) was added at a concentration of around 7%. The maximum and minimum concentration of each compound tested was 1.024 and 0.002 mg/mL, respectively. In the MIC experiments, it was used microtiter plates with 96 wells with flat bottom. The wells were filled with a total volume of 200 µL to a final concentration of approximately 5×10^5 CFU/mL, which was made up of 100 µL of the compound solution at a range of concentrations and 100 µL of the bacterial solution (figure 3). Furthermore, it was used two different controls, one with 200 µL of MHB and another with 100 µL of MHB, 100 µL of bacterial solution and DMSO at 7%. After 20 hours of incubation with agitation at 37 °C, bacterial growth was determined by observing turbidity in the wells, since the MIC value is defined as the minimum concentration of the antibacterial substance necessary to inhibit bacterial growth. Afterwards, to determined MBC values, which is defined as the lowest concentration of the antibacterial substance that inhibit the formation of bacterial colonies, 10 µL was removed, in triplicate, from the wells where no growth was observed and subsequently inoculated onto MHA plates, which were incubated at 37 °C for around 20 hours. These assays were performed independently three times per bacteria.

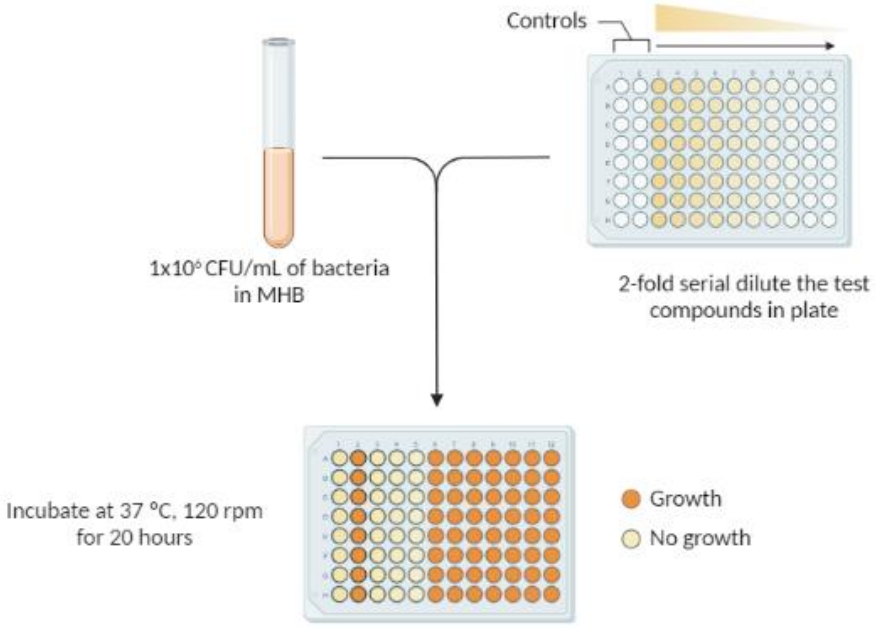

Figure 3. Schematization of the MIC assays procedure. Created in BioRender.

5. Soft Agar Overlay Assays

The antibacterial properties of powdered compounds and doped biopolymer films, mentioned above, were assessed in a soft agar overlay assay for *E. coli*, *P. aeruginosa*, *S. aureus* and *S. epidermidis,* separately. Similar to experiments previously realized by Fernandes and co-workers (2021), in this assay (illustrated in figure 4), the bacteria that grew overnight (for about 18 hours) in MHB were transferred to soft MHA (0.5%, m/V agar), at a final concentration of 1×10^6 colony-forming units per millilitre (CFU/mL). The final concentration was obtained by measuring the absorbance of the bacterial suspension at 620 nm in a microplate reader after diluting the inoculum with a dilution factor of 1:4, this dilution was performed to ensure that the absorbance values were within the detection limit and corresponded to CFU/mL values of around 5 to 10×10^8. The dilution was performed taking into consideration previously established correlation between absorbance and bacterial concentration for each species, which were also used to further dilute the inoculum to the final concentration. Afterwards, a volume of 3 mL of soft MHA II with bacterial suspension was placed on top of 10 mL solidified MHA (1.7%, m/V agar) on a 9 cm diameter Petri dish. Then, 2.0 mg of the powdered compounds or biopolymer films of 1 cm diameter were placed on top of the soft MHA II and incubated at 37 °C for about 20 hours. The antibacterial properties were analysed by photographing the plates in a Bio-Red ChemicDoc Imager and measuring the minimum inhibition radius with the support of the image editing software GIMP (The GIMP Development Team, 2021). The results were analysed using Excel software. In these assays were performed at least three independent experiments per bacteria, apart from some of the powdered compounds, where only two independent assays were carried out.

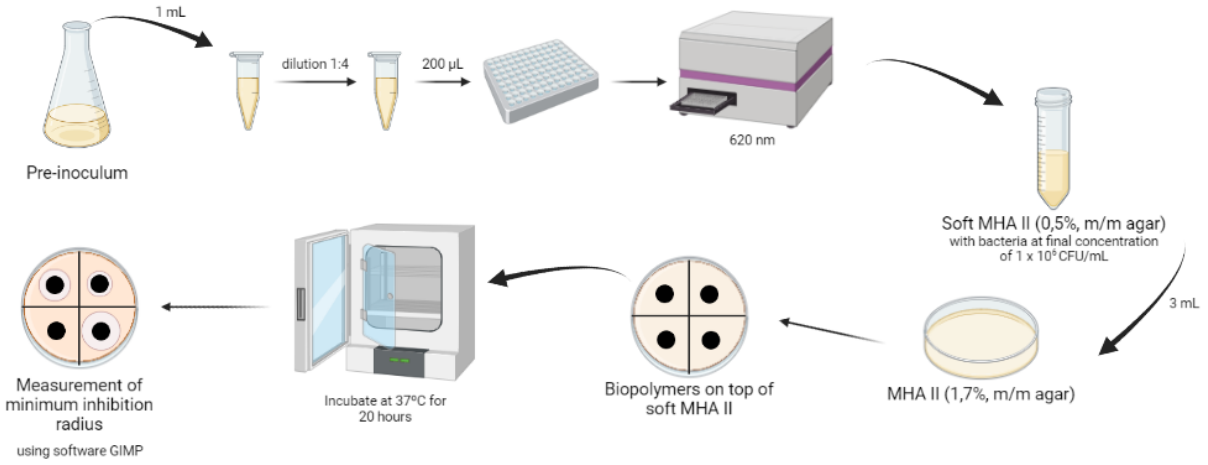

Figure 4. Schematization of the soft agar overlay assays procedure. Created in BioRender.

6. Biofilm Inhibition Properties of Biopolymer Films

The selected biopolymer films were used in the biofilm inhibition against the previously mentioned bacteria. Biofilms were assembled in the presence of the biopolymer films to test for biofilm formation on their surface and for cell adhesion. This assay (illustrated in figure 5) was similar to experiments carried out before by Fernandes and colleagues (2021), where the biomass was transferred to fresh media at a final concentration of 1×10^6 CFU/mL after growing overnight in MHB II. The biopolymer films and 1 mL of the bacterial suspension were placed into a 24 well plate and incubated for 24 hours with agitation at 120 rpm. The bacterial suspension that has developed was discarded and the biopolymer films were transferred into clean wells and cleaned twice with NaCl (0.9%) solution, to remove non-adhered cells. The bacteria attached were released in 1 mL of NaCl solution in an ultrasonic bath (220 V, 50/60 Hz) for 15 minutes, followed by vortex for 30 seconds in eppendorfs. Afterwards, serial dilutions in NaCl solution were performed for each sample with 10 µL of each dilution being plated in MHA II, in order to quantify the detached cells by CFU counting. Finally, after the 20 hours of incubation, at 37 °C, of the MHA II plates, the colonies formed were counted. The results were analysed using Excel software and are presented as the logarithm of the amount of detached cells per cm^2 of biopolymer film. These experiments were performed at least three times per bacteria with one replicate (due to the limited amount of samples, it was preferable to perform a larger number of assays than more technical replicates).

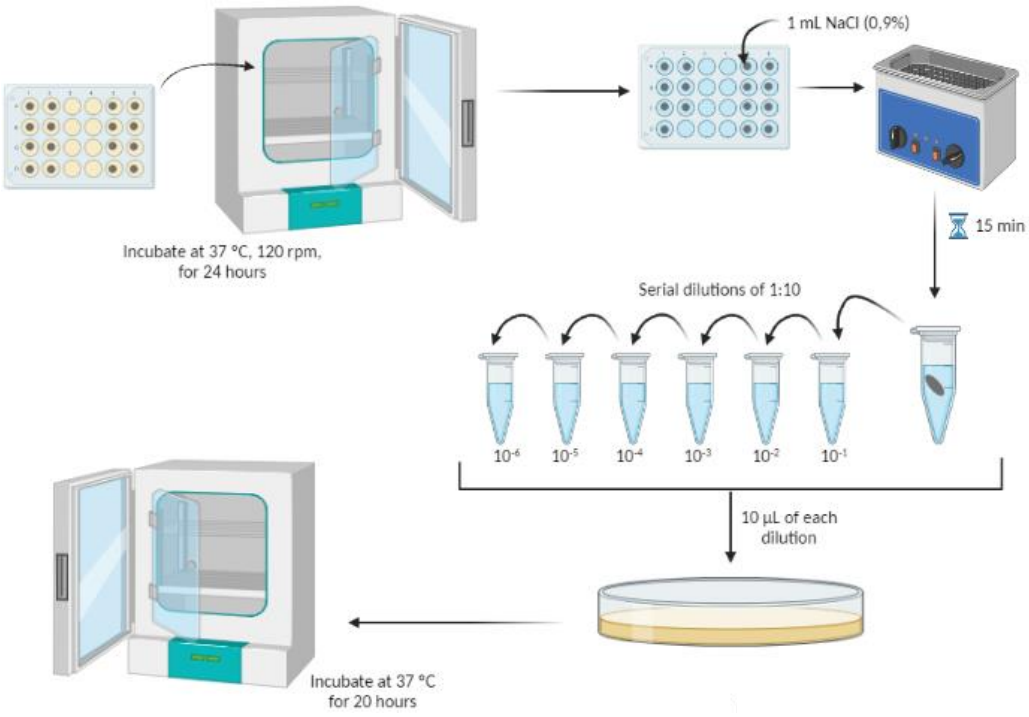

Figure 5. Schematization of the biofilm inhibition activity assays procedure. Created in BioRender.

7. Statistical Analysis

Data were analyzed by t-test using Excel software, to compare the mean of the sample with the mean of the respective control in order to evaluate if there is a statistical difference between them. Results are presented as the mean ± standard deviation (SD), being $p \leq 0.05$ considered statistically significant.

Results and Discussion

1. Powdered Compound Screening

The screening of powdered compounds offers an overview of the synthesized compounds' antibacterial activity, allowing to choose the compounds with the highest activity for further biopolymer film synthesis. The screening tests also gave insight on the bacterial activity that results from the metal source, the organic ligand or from the interaction between the components. Figures 6 and 7 show the screening outcomes from the compounds listed in table 5. The screening results of the matrices are present in appendix 1.

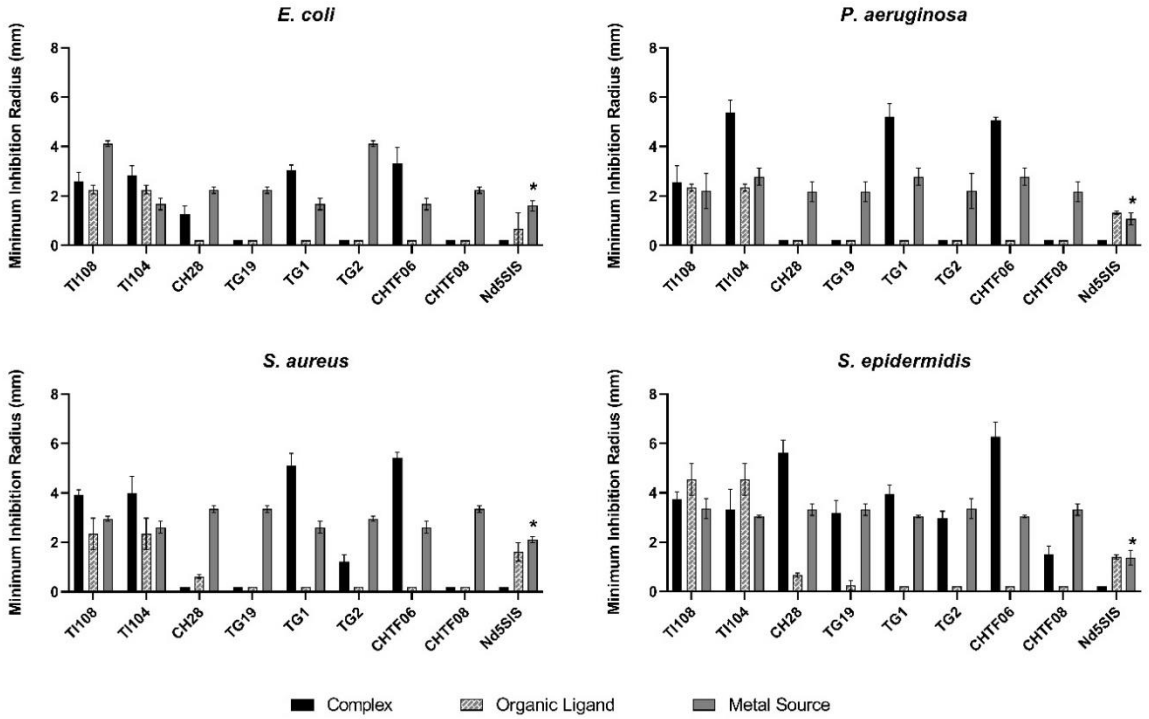

Figure 6. Qualitative assessment of antibacterial activity of powdered compounds against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD). The results that are not normalized for the molar content of the metal centre are represented with *.

This pilot experiment revealed that some of the tested organic ligands, including FDA (from the TI104 and TI108-CPs), 5SIS (from Nd5SIS-MOF) and DDDA (from CH28-CP), had antibacterial activity against the bacterial species tested. However, DDDA only exhibited activity towards Gram-positive bacteria (*S. aureus* and *S. epidermidis*).

The Nd5SIS-MOF, a 3D niobium-based MOF with formula $\{[Nd(5SIS)]\cdot 4H_2O\}_n$, did not demonstrate antibacterial activity for none of the tested bacteria, even though its metal source (neodymium acetate) and organic ligand (5SIS) exhibited some activity (Figure 6). It may be interesting to apply the ligand with different metal sources as well as to apply the metal source with different organic ligands to determine if the new CP will have antibacterial activity.

The TI104 (Ag-based CP), TI108 (Cu-based CP), TG1 (Ag-based MOF) and CHTF06 (Cu-based) exhibited antibacterial properties against all the bacteria tested. However, the CH28, TG19 and CHTF08 (all Cu-based) only exhibited against *S. epidermidis* bacteria, and TG2 (Cu-based CP) against Gram-positive bacteria (*S. aureus* and *S. epidermidis*) (Figure 6).

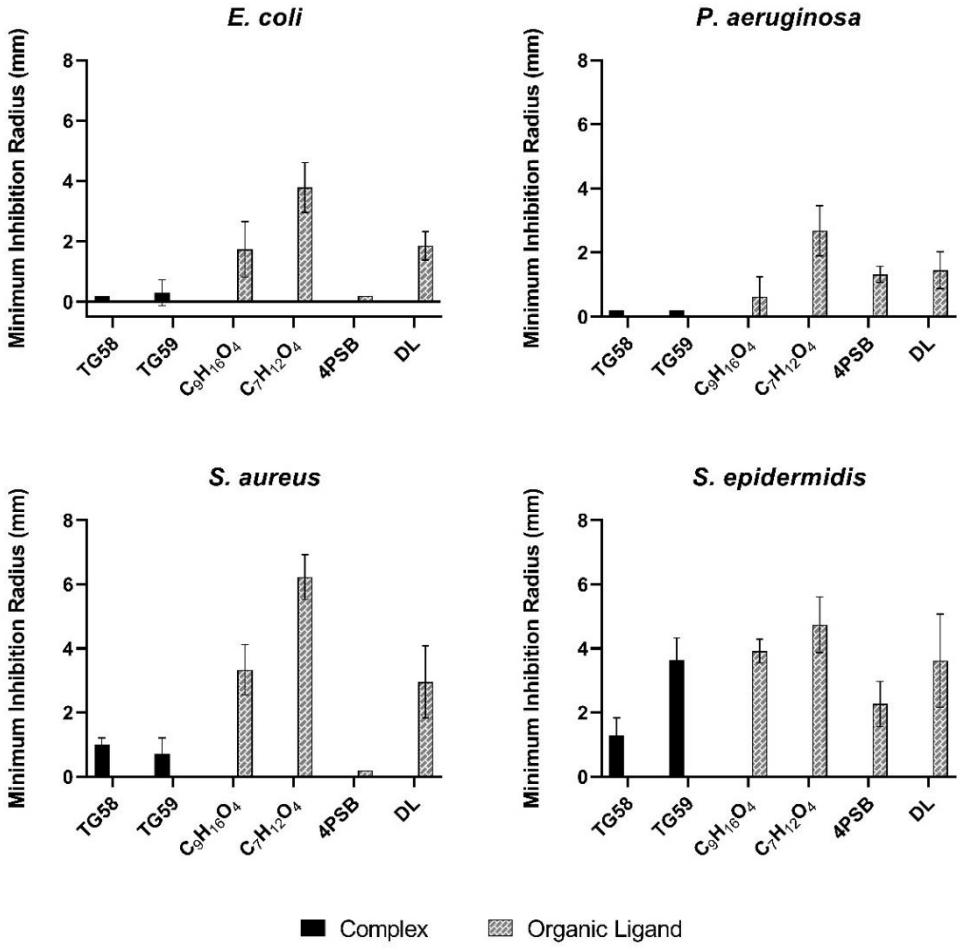

Figure 7. Qualitative antibacterial activity of other powdered compounds against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD).

As expected, all the metal sources analyzed (listed in table 5) demonstrated antibacterial properties (Rai *et al.*, 2012; Subha *et al.*, 2022). For this reason, it would be interesting to combine these metals with different ligands to determine if the resulting MOFs demonstrate improved antibacterial properties.

According to the figure 7, the CPs TG58 and TG59 (Zn-based) only exhibited antibacterial activity against Gram-positive bacteria (*S. aureus* and *S. epidermidis*), however it would be better to apply MIC and MBC assays in these compounds. Importantly, these powders were difficult to manipulate, they spread out when placing it on the Petri dish and most dissolved in the medium (Figure 8(B)), and often the accuracy of the screening was not very high, with a lot of variability between assays. Additionally, in general, the organic ligands (Figure 6) demonstrated greater antibacterial properties against Gram-positive bacteria.

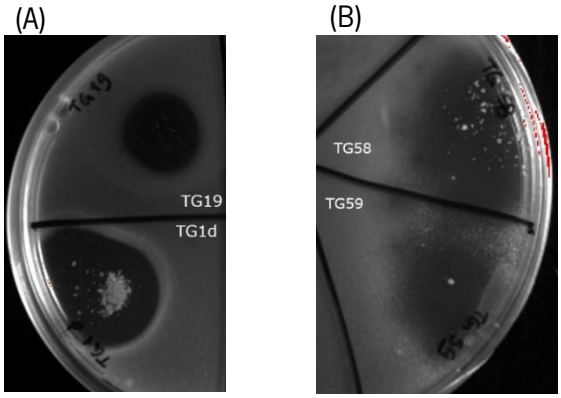

Figure 8. Examples of inhibition halos obtained from powdered compounds, demonstrating (A) good inhibition halos with a clean edge, and (B) inhibition halos diffused, without a clean edge.

None of the chemicals used in the production of film matrices had antibacterial properties against tested bacteria (Appendix 1), indicating that the substrate used in the biopolymer synthesis will not have affect the antibacterial activity of metal complexes used, either CPs or MOFs.

2. Soft Agar Overlay Assays

The technique of soft agar overlay was used to assess the antibacterial activity of biopolymer films doped with CPs/MOFs by observing bacterial growth inhibition, similarly to the Kirby-Bauer disk diffusion assay. This is a semi-quantitative method, showing only the presence or absence of antibacterial activity. Halo measurement is not reliable, since it is not possible to guarantee spatial homogeneity or full contact of the sample with the bacteria. It is important to note that only the CPs/MOFs that are fully characterized can be doped in the biopolymer films, therefore not all the bioactive structures tested in the screening assays were used for this experiment.

a. Biopolymer Films of TG1 and TG2 Compounds

Biopolymer films of agarose and potato starch were doped with TG1 (Ag-based MOF), TG2 (Cu-based CP) and their respective metal sources and organic ligands in different concentrations (1, 2.5 and 5%) were tested against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria. These biopolymers have the same organic ligand (4,4'-sulfonyldibenzoic acid - SDBA), their distinction is in the metal source. It is important to note that the results are normalized for the molar content of the metal centre.

All tested agarose biopolymer films doped with TG1 presented antibacterial activity against all bacteria of interest (Figure 9), contrasting with the agarose biopolymer films doped with TG2, which only exhibited activity against *E. coli* at 2.5%. The absence of antibacterial activity from the metal source of TG2-CP doped in this type of matrix is a plausible explanation to justify this observation.

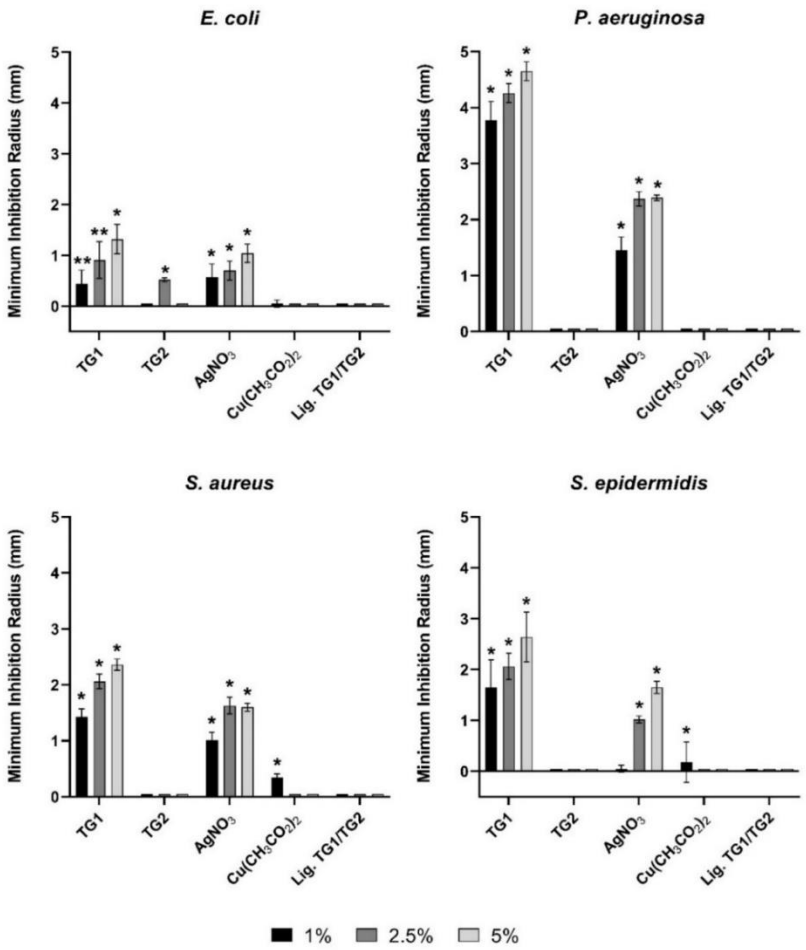

Figure 9. Normalized antibacterial activity of agarose biopolymer films doped with $AgNO_3$-based MOF (TG1) and $Cu(CH_3CO_2)_2$-based CP (TG2) at different percentages (1, 2.5 and 5%) against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD). Statistical significance $p \leq 0.05$ is represented by *.

Accordingly to the literature, silver ions exhibit stronger antibacterial action than copper ions (Ruparelia *et al.*, 2008). Additionally, the agarose biopolymer films doped with the organic ligand did not demonstrate any antibacterial activity, which was unsurprising given that they exhibited the same results in the screening tests (Figure 6). Overall, antibacterial activity was concentration dependent with all biopolymer films showing the highest antibacterial properties at 5% concentration of the compound (Figure 9).

Curiously, *S. epidermidis* was more susceptible to biopolymer film of agarose (without any added compounds), even though agarose in powder form did not showed any activity, as mentioned in the previous section (Appendix 1). This result may be due to the formation or degradation of some chemicals or polymers after thermal treatment of agarose film, leading to the formation of inhibition halos. Further experiments should be done to analyse and understand this outcome. Regardless, figure 9 was established taking that into account, therefore the results with *S. epidermidis* were normalized to the baseline activity of the agarose biopolymer films.

In accordance with the previous results, the potato starch biopolymer film doped with TG1-MOF showed greater antibacterial activity when compared to the biopolymer film doped with TG2-CP. However, with this matrix, the TG2-CP demonstrated antibacterial activity against Gram-positive bacteria (*S. aureus* and *S. epidermidis*), with greater action against *S. epidermidis* (Figure 10).

In addition, the biopolymer films doped with the copper metal source [$Cu(CH_3CO_2)_2$] and the organic ligand did not exhibit antibacterial properties, similar to the results with agarose biopolymer films (Figure 9). Furthermore, with potato starch biopolymer films, antimicrobial activity was only found at concentrations above 2.5% of the silver metal source ($AgNO_3$) (Figure 10). It is possible that this occurs because the potato starch matrix can be restricting the release of the silver ions to the medium, and only the matrices doped with higher percentages can do so. All potato starch biopolymers films demonstrated higher antibacterial activity at 5% of the compound (Figure 10), demonstrating that the antibacterial action is also concentration dependent.

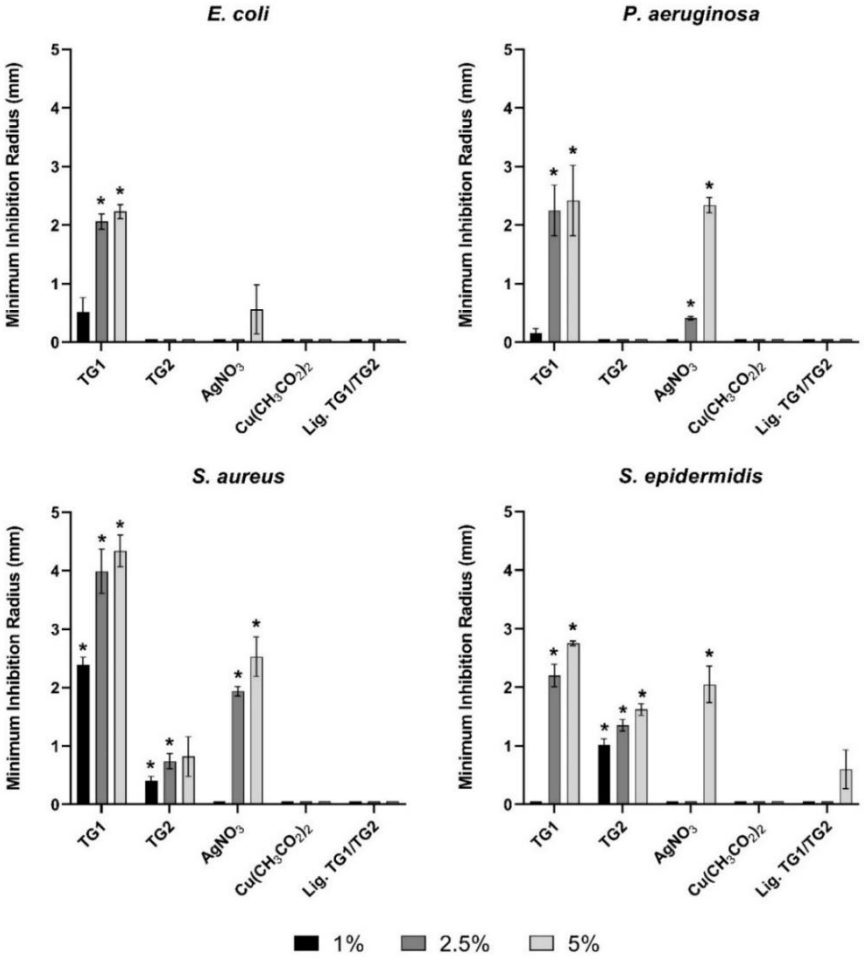

Figure 10. Normalized antibacterial activity of potato starch biopolymer films doped with $AgNO_3$-based MOF (TG1) and $Cu(CH_3CO_2)_2$-based CP (TG2) at different percentages (1, 2.5 and 5%) against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD). Statistical significance $p \leq 0.05$ is represented by *.

b. Biopolymer Films of RI and ESOA

These biopolymer films (Table 8) are doped with TG19 and CH28 compounds in different concentrations (0.5 and 1%). The results obtained with these films of RI and ESOA are presented in figures 12 and 13, respectively. Both of these new CPs are copper based, being $Cu(NO_3)_2$ their metal source. According to the literature, copper has been utilized as an inexpensive and efficient substance to sterilize textiles, liquids and even human tissue, since it has shown antibacterial properties (Borkow & Gabbay, 2009). For instance, copper raises the quantity of cellular reactive oxygen species in *E. coli*, which causes DNA deterioration, lipid peroxidation and protein oxidation, all of which result in cell death (Chatterjee *et al.*, 2014).

The RI and ESOA matrices (without active compounds) are not shown in the figures, however they did not yield any antibacterial activity, which is an essential aspect to note. Given that these CPs are copper-based and when compared to other reported compounds that have similar properties (Jadhav *et al.*, 2011), it was expected that they would exhibit some antibacterial activity, which was not observed under our tested conditions, as shown in figures 11 and 12.

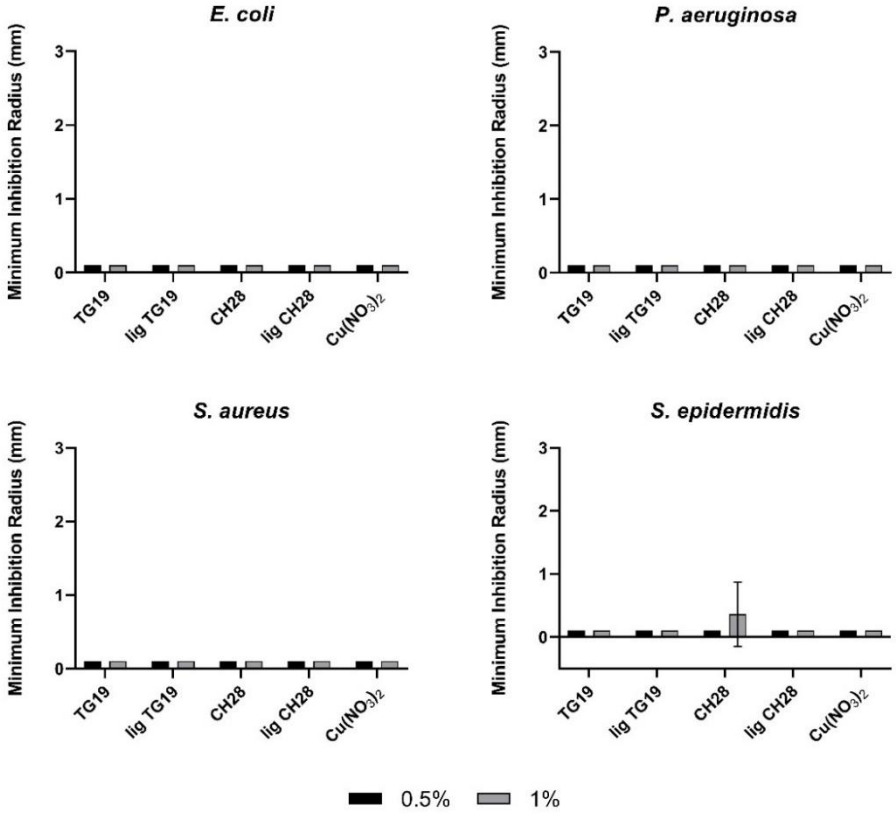

Figure 11. Antibacterial activity of RI biopolymer films doped with different concentrations (0.5 and 1%) of TG19 and CH28 compounds against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD).

In order to ascertain whether the matrices themselves are preventing the release of the compounds into the medium, it was decided to test the metal source at higher concentrations (5 and 10%) for both matrices (Figure 13), and even a different compound ($AgNO_3$) at the same concentrations (0.5 and 1%) for RI matrices (Figure 14).

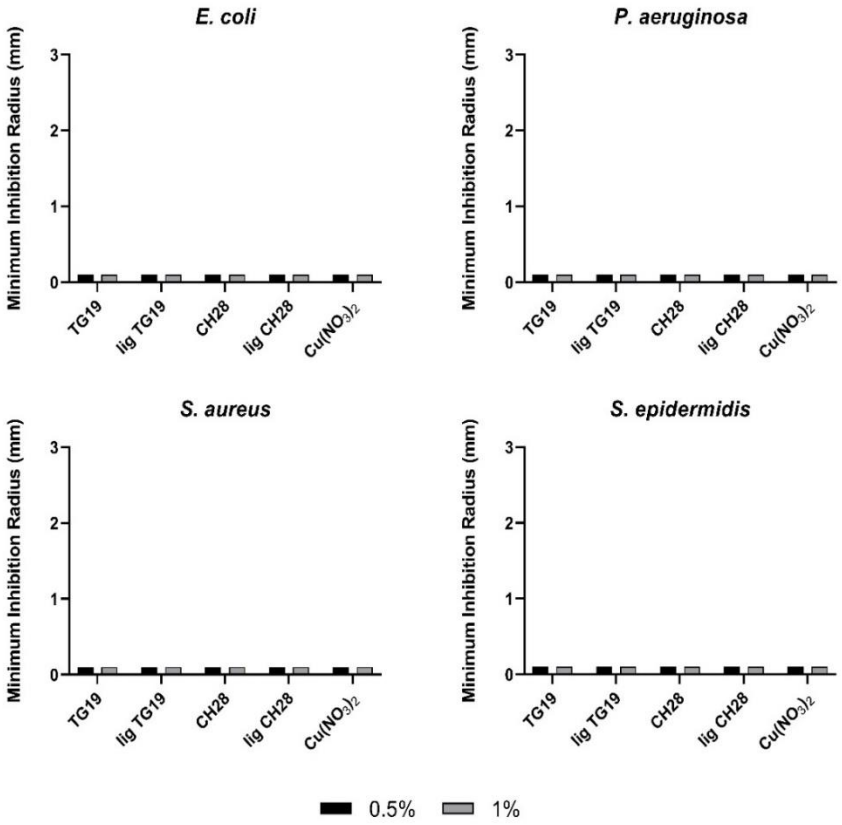

Figure 12. Antibacterial activity of ESOA biopolymer films doped with different concentrations (0.5 and 1%) of TG19 and CH28 compounds against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD).

The idea that these matrices may be limiting the release of the compounds is supported by the fact that only the biopolymers at higher concentrations demonstrated antibacterial activity, as can be seen in the figure 13. Additionally, it is noted that whereas ESOA biopolymers dopped with 5 and 10% of $Cu(NO_3)_2$ only display activity against the Gram-positive bacteria, the biopolymer of RI dopped with 10% of the compound displays antibacterial activity against all four species.

However, as shown in figure 14, there is no problem in the releasing of the compounds to the medium, which disproves the idea stated previously for the other compounds. This can happen because the coordination of Ag metal centres is considerably different from that of Cu, so the interaction with the matrices will also be different and will depend on the functional groups that will be present in the matrices. Additionally, it can also be observed that there is a correlation between the concentration of the compound in the biopolymer film and its antibacterial activity, given that in higher concentrations greater activity is observed.

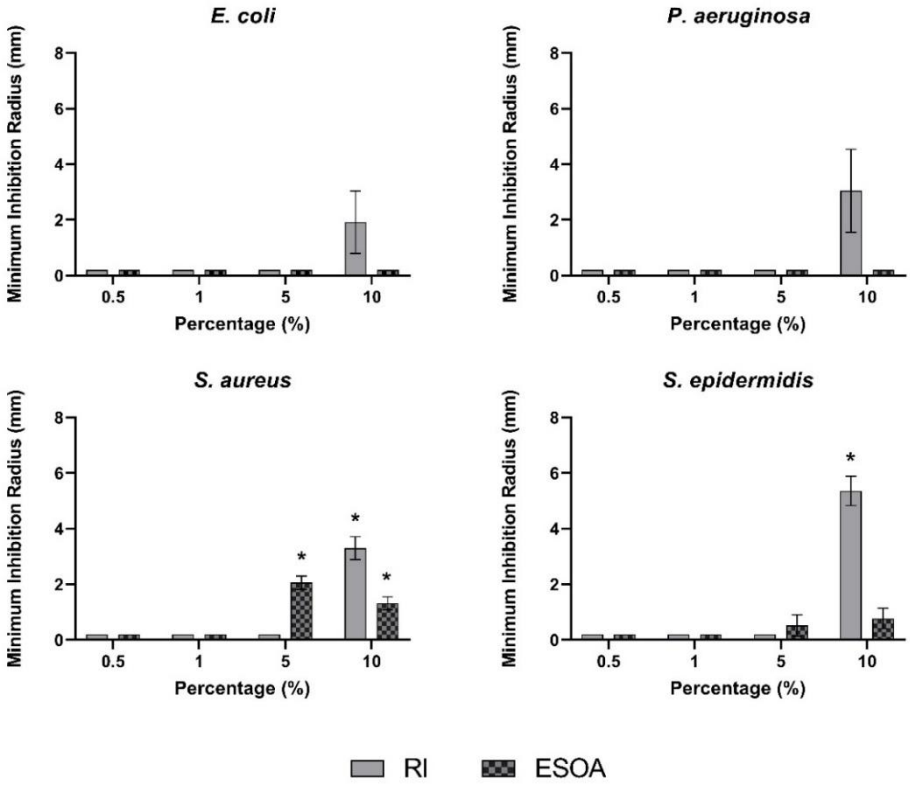

Figure 14. Antibacterial activity of RI and ESOA biopolymer films dopped with different concentrations (0.5, 1, 5 and 10%) of $Cu(NO_3)_2$ compound against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD). Statistical significance $p \leq 0.05$ is represented by *.

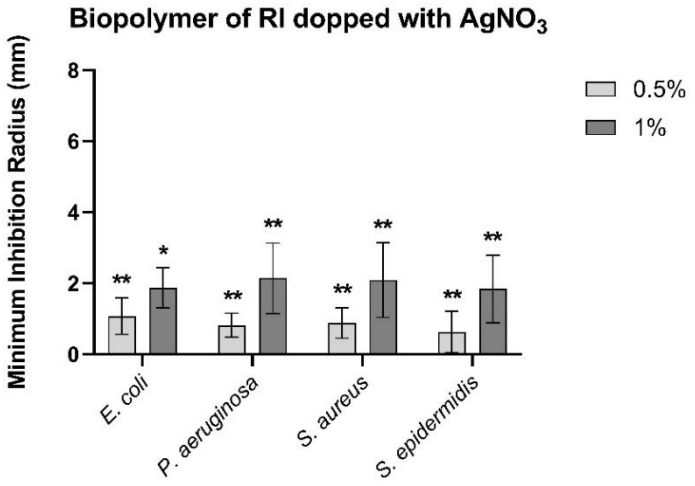

Figure 13. Antibacterial activity of RI biopolymer films dopped with different concentrations (0.5 and 1%) of $AgNO_3$ against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD). Statistical significance $p \leq 0,05$ is represented by * and ** represents biological differences, although there are no statistically significant differences.

c. Biopolymer Films of $ZnCl_2$

Accordingly to the literature, zinc is used as an antibacterial agent, and it has been incorporated to mouthwashes and toothpastes to control dental plaque and suppress the growth of calculus (Lynch, 2011). In fact, there are several studies that demonstrate great antibacterial activity of zinc against *Streptococcus* sp. (Almoudi *et al.*, 2018; Barma *et al.*, 2021). In this sense, it was decided to test a variable of this element doped in agarose and potato starch matrices, and in different concentrations (1, 2.5 and 5%), against species different from those that reside in the mouth environment.

Analysing these results (Figure 15), it is possible to infer that the zinc compound is not being released from the agarose biopolymers or not being released in enough quantities to inhibit bacterial growth, because none of them exhibited antibacterial properties.

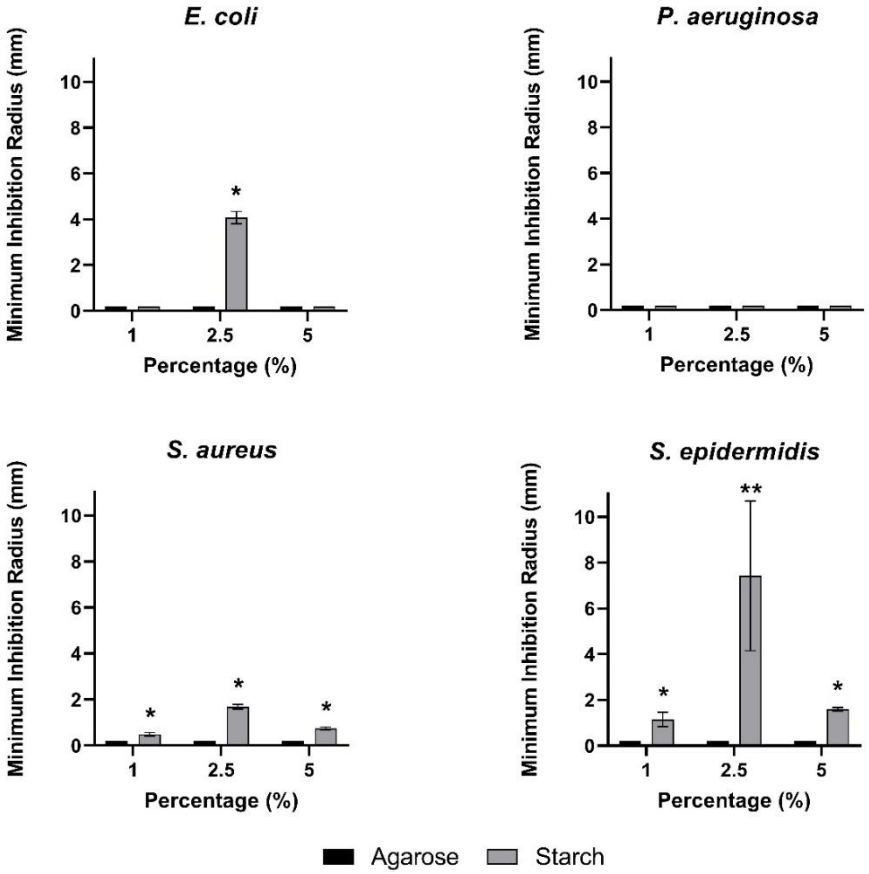

Figure 15. Antibacterial activity of agarose and starch biopolymer films doped with different concentrations (1, 2.5 and 5%) of $ZnCl_2$ against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD). Statistical significance $p \leq 0.05$ is represented by * and ** represents no statistically significance with biological differences.

Regarding the starch biopolymers, superior outcomes were obtained against Gram-positive bacteria, most notably in *S. epidermidis*, with the exception of the starch biopolymer containing 2.5% of $ZnCl_2$ against *E. coli*. Additionally, inductively coupled plasma mass spectrometry should be done in order to quantify the amount of Zn that is released from the biopolymer films to a buffer solution, since this will ascertain the relation between the biopolymer's action and the concentration.

d. Biopolymer Films of $Cu(NO_3)_2$

Agarose and potato starch biopolymer films doped with $Cu(NO_3)_2$ (Table 10) at different concentrations (1, 2.5 and 5%) were tested against the bacteria of interest (Figure 16). It is important to note that $Cu(NO_3)_2$ compound was also tested doped in RI and ESOA matrices, previously mentioned (Figure 13).

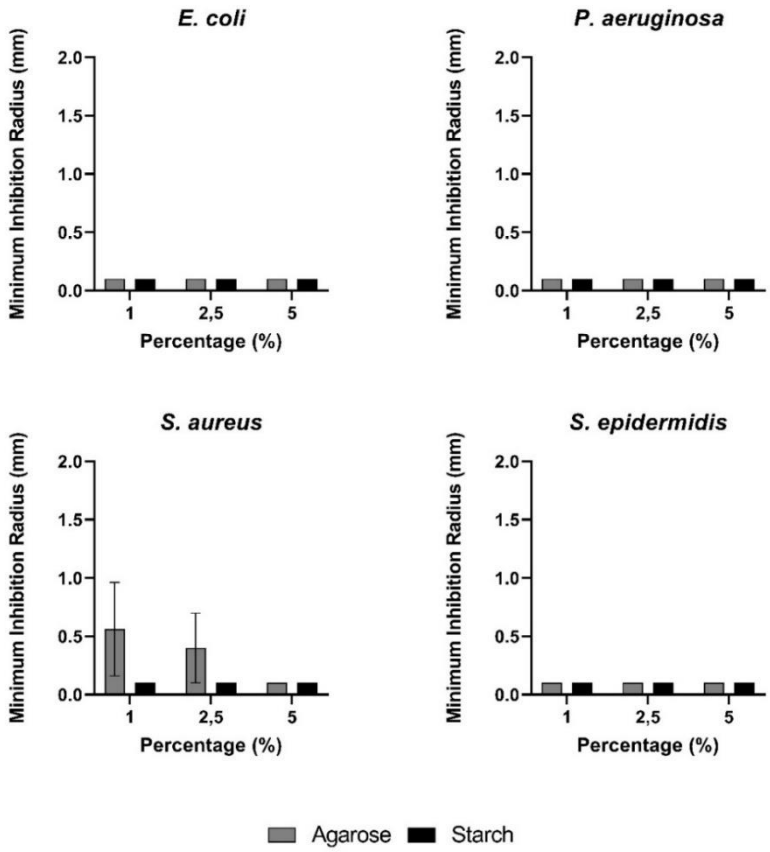

Figure 16. Antibacterial activity of agarose and potato starch biopolymer films doped with different concentrations (1, 2.5 and 5%) of $Cu(NO_3)_2$ against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean±SD).

Figure 16 depicts the results obtained, which are comparable to the results found with the RI and ESOA matrices. Apart from the experiments against *S. aureus* (with no statistically significance),

none of the biopolymer films exhibited antibacterial activity, which means that the compound does not have sufficient activity to inhibit bacterial growth. Therefore, this assay also confirms that there is not complication in the release of the compounds from the RI and ESOA matrices.

Moreover, it was also obtained some results against *S. epidermidis*, but since the biopolymer film of agarose exhibit antibacterial properties against this bacterium, the results presented in figure 16 were obtained by subtracting the value from agarose biopolymer film (without any bioactive compound).

3. Biofilm Inhibition Properties of Biopolymer Films of TG1

Based on the outcomes from the aforementioned soft agar overlay assays of the biopolymer films with TG1 and TG2 (Figures 9 and 10), it was decided to proceed with biofilm inhibition assays utilizing agarose and potato starch biopolymer films of TG1 (Ag-based MOF) at 5% of concentration (Figure 17).

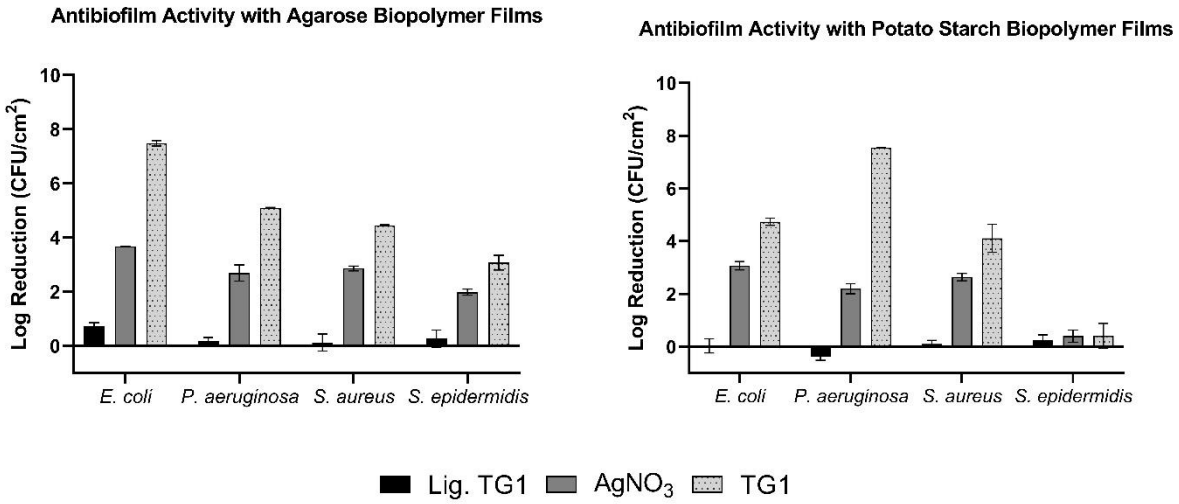

Figure 17. Normalized antibiofilm activity of agarose and potato starch biopolymer films doped with 5% of TG1-MOF, and its respective organic ligand and metal source, against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria. Graphs show log reduction when compared with the respective controls.

To ascertain whether any of the tested biopolymer films exhibited properties that inhibited the formation of biofilms, the amount of cells adhering to the different biopolymer films was assessed. Both biopolymer matrices doped with 5% of TG1 were able to prevent the growth of biofilms on their surface in different extents, depending on the species and the matrix type (Figure 17). Compared to both controls (films doped with only organic ligand or metal ion), this antibiofilm activity was substantially higher, except

for potato starch biopolymer film doped with 5% of TG1 against *S. epidermidis*. In general, Gram-negative species showed greater susceptibility than Gram-positive ones, with *E. coli* and *P. aeruginosa* being the most susceptible to agarose biopolymer film doped with TG1 and potato starch biopolymer film doped with TG1, respectively. It is worth noting that some results confirm almost total biofilm inhibition, namely agarose film with TG1 against *E. coli* and potato starch film with TG1 against *P. aeruginosa*.

Additionally, agarose biopolymer film doped with 5% of TG1 revealed great potential as a candidate for coating techniques to prevent infections (especially infections related with surface contamination), because it was highly effective and showed a broad spectrum of activity among species.

4. MIC and MBC Assays

The tested concentrations of the compounds ranged from 0.002 to 1.024 mg/mL since they are new compounds and there is no information relative to their MIC and MBC values. The reagent DMSO was added at a concentration of around 7% to facilitate de dilution of the carbon compounds (C7, C8 and C9). The results from these assays are presented in table 11.

Table 11. Antibacterial activity of C7, C8, C9 and $ZnCl_2$ compounds against the bacteria of interest.

Bacterial Specie		Compounds			
		C7	C8	C9	$ZnCl_2$
E. coli	MIC	≥ 1.024	≥ 1.024	> 1.024	0.512 – 1.024
	MBC	> 1.024	> 1.024	> 1.024	1.024
P. aeruginosa	MIC	> 1.024	> 1.024	> 1.024	≥ 1.024
	MBC	> 1.024	> 1.024	> 1.024	> 1.024
S. aureus	MIC	1.024	1.024	1.024	0.256 – 0.512
	MBC	> 1.024	≥ 1.024	> 1.024	1.024
S. epidermidis	MIC	≥ 1.024	1.024	≥ 1.024	0.128
	MBC	> 1.024	≥ 1.024	> 1.024	0.512 – 1.024

Both MIC and MBC are expressed in mg/mL.

The control with the bacterial suspension and DMSO alone allowed to confirm that the eluent had no impact on the results. In a brief assessment, it was observed that, for most of the bacteria species, MIC and MBC values for the compounds C7, C8 and C9, were higher than 1.024 mg/mL (Table 11), suggesting a low or non-existent antimicrobial activity, under our tested conditions. The values obtained

with $ZnCl_2$ compound are in agreement with the values obtained with the corresponding biopolymer films, since lower MIC and MBC values were obtained in Gram-positive bacteria, and better results were also obtained with Gram-positive bacteria in the soft agar overlay assays (Figure 14). A new range of concentrations must be done in order to assess the exact antibacterial activity of these compounds.

Conclusions and Future Studies

The development of antibiotic-resistant bacteria has increased the incidence of nosocomial infections, which are typically accompanied by bacterial biofilms, making their treatment more challenging. In addition, biofilms are able to grow on a wide variety of surfaces, particularly those found on indwelling medical devices. Thus, the development of novel and effective bioactive functional materials that can inhibit the biofilm formation is essential. CPs or MOFs are examples of bioactive structures that offer an alternative to traditional antibacterial approaches. Additionally, the polymeric matrix has a fundamental role in the application of bioactive structures, since it allows slow and controlled release of the compounds.

The screening assays revealed organic ligands with promising potential to be studied as linkers for the CPs and MOFs, since some of them displayed antibacterial activity against the bacteria tested, namely FDA, 5SIS and DDDA. Variable degrees of antibacterial activity were observed for the CPs/MOFs assessed in their powder form, depending on the species. None of the chemicals used in the production of film matrices exhibited antibacterial action.

Regarding the soft agar overlay assays, RI and ESOA biopolymer films doped with TG19 and CH28 (both Cu-based CPs) did not showed antibacterial activity under our tested conditions. Agarose and potato starch biopolymer films doped with $ZnCl_2$ demonstrated overall greater action against Gram-positive bacteria. The antibacterial activity of the biopolymer films of agarose and potato starch doped with TG1 (Ag-based MOF) was superior in comparison to similar samples where their correspondent metal source was applied as dopants. In addition, agarose and potato starch biopolymer films doped with TG1 showed antibacterial activity against all the species tested and were more effective than those that had been doped with TG2. Thus, of the three different concentrations tested (1, 2.5 and 5%) 5% TG1-doped agarose and potato starch biopolymer films were selected to be used for biofilm inhibition assays. Both biopolymer films demonstrated promising biofilm inhibition activity, however wider spectrum of antibiofilm activity was observed for biopolymers of agarose. Moreover, almost total biofilm inhibition was noticed for *E. coli* and *P. aeruginosa* in agarose and potato starch biopolymer films, respectively.

The present work showed to be very promising the application of these CPs/MOFs as an alternative to the use of antibiotics for the treatment and prevention of biofilms. It also contributes to an underexplored biofilm inhibition application of CPs/MOFs.

Further study may focus on assessing bioactive CPs and derived biopolymer films as candidates for treating bacterial infections as well as to assess their cytotoxicity, particularly in light of a known antiproliferative activity of various silver (I) derivatives including CPs. The ability of these materials to coat medical devices and even to treat skin infections must also be tested. In addition, it may be interesting to assess if the bacteria acquire resistance to the compounds through successive generations and even against polymicrobial biofilms.

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Appendix 1

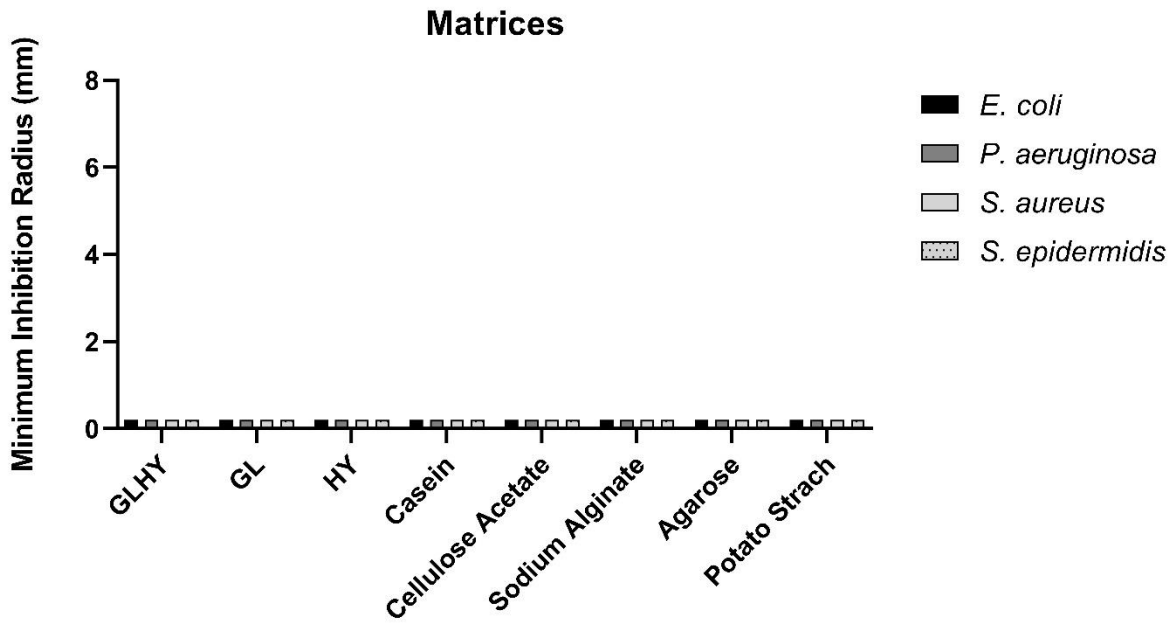

Figure 1. Qualitative antibacterial activity of the powdered matrices compounds against Gram-negative (*E. coli* and *P. aeruginosa*) and Gram-positive (*S. aureus* and *S. epidermidis*) bacteria, represented by their minimum inhibition radius (mean+-SD).