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**Solvants Eutectiques Profonds– de la Caractérisation Structurale
et à Haute Fréquence aux Applications Antimicrobiennes**

par

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Characterization to Antimicrobial Applications**

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Thank you, my love, for always being there for me.

Both, when sailing against and with the wind.

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"Messieurs, ce sont les microbes qui auront le dernier mot"

– **L. PASTEUR**

*"I have a feeling, that sometimes, the bacterial growth and experimental results
depend on the phase of the moon..."*

– **T. SWEBOCKI**

AVIS DE RÉVISIONS

Cette thèse a été évaluée par les membres du jury de la soutenance de doctorat. Suite à leurs remarques, les révisions suivantes ont été effectuées :

- Réorganisation des sections du manuscrit ;
- Réduction de la longueur de l'introduction ;
- Ajout d'une discussion sur la méthodologie appliquée, avec le soutien de la littérature ;
- Ajout d'informations sur l'utilisation des contrôles dans l'expérience (le cas échéant) ;
- Corrections mineures de style et de grammaire.

NOTICE OF REVISIONS

This thesis has been evaluated by the PhD defence committee. Based on their feedback, the following revisions have been made:

- Reordered sections of the manuscript;
- Reduced the length of the introduction;
- Expanded the discussion on the applied methodology, with supporting references from the literature;
- Included additional information on the use of controls in the experiment (where relevant);
- Made minor stylistic and grammatical corrections.

**CLAUSE MORALE VOLONTAIRE SUR L'UTILISATION
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Certaines parties de cette thèse ont été rédigées avec l'aide d'outils assistés par IA. Leur utilisation comprend :

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Résumé

Dans un monde dominé par des microbes, les humains ne représentent qu'une petite fraction des organismes vivants. Certains microbes sont bénéfiques, mais d'autres, comme les bactéries résistantes aux antibiotiques, posent une menace majeure. En 2019, environ 8,88 millions de décès ont été attribués à la septicémie bactérienne, dont 4,95 millions liés à la résistance bactérienne. Ce chiffre pourrait atteindre 10 millions de victimes d'ici 2050, soulignant la nécessité de stratégies alternatives pour lutter contre la résistance bactérienne.

Les travaux de cette thèse de doctorat concernent l'exploration des solvants eutectiques profonds (SEP) comme agents antimicrobiens. Ces matériaux ont des propriétés uniques grâce à des réseaux de liaisons hydrogène. Dans cette étude, neuf SEP ont été examinés : quatre conventionnels (reline, ethaline, glyceline, R-glyceline) et cinq avec des acides organiques (AO-SEP : glycoline, T-glycoline, maloline, maline, oxaline). Les travaux comprennent la synthèse, la caractérisation physico-chimique, le criblage antibactérien, l'évaluation des biofilms et cellules persistantes, et leur utilisation comme co-médicaments pour réduire la résistance bactérienne, et les effets antifongiques.

La thèse comprend cinq chapitres. Le premier décrit la synthèse et la caractérisation des SEP, confirmant la formation de réseaux de liaisons hydrogène et leur stabilité thermique. La caractérisation micro-ondes montre que les SEP conventionnels sont meilleurs pour le stockage et la dissipation d'énergie de haute fréquence.

Le deuxième chapitre concerne les résultats de l'activité antibactérienne des SEP. Les AO-SEP montrent une activité supérieure à celle des SEP conventionnels. Une étude sur douze souches révèle que les AO-SEP inhibent la croissance bactérienne à des concentrations inférieures à 1%, tandis que les SEP

conventionnels nécessitent des concentrations plus élevées. Les AO-SEP perturbent la paroi cellulaire, alors que les SEP conventionnels forment des pores.

Le troisième chapitre traite de l'utilisation des SEP contre les biofilms et les cellules persistantes. Les biofilms de SARM et *E. coli* montrent que certains AO-SEP sont efficaces à faibles concentrations. La coloration Live/Dead confirme ces résultats. Les résultats ont montré diverses cinétiques d'action pour tous les SEP.

Le quatrième chapitre explore les SEP comme co-médicaments. Les résultats de cette étude ont démontré que les valeurs des CMI de l'ampicilline et la carbénicilline ont été réduites en les associant avec des SEP. La colistine et d'autres peptides ont montré une efficacité augmentée en présence de SEP. Deux stratégies (utilisation de DES et de nisine ensemble ou de manière séquentielle) pouvant augmenter l'efficacité de la nisine contre les biofilms ont été vérifiées, le traitement séquentiel étant le plus efficace.

Le dernier chapitre explore les SEP comme agents antifongiques. Sur les trois souches fongiques (*Candida albicans*, *Candida auris* et *Aspergillus fumigatus*) investiguées, les résultats obtenus ont démontré une action nettement plus forte des SEP, réduisant leur virulence en perturbant les colonies fongiques.

En conclusion, dans cette thèse, j'ai exploré les SEP comme matériaux antimicrobiens, offrant des perspectives pour des stratégies innovantes contre la résistance bactérienne et les infections fongiques, répondant aux défis de santé mondiale du 21^{ème} siècle.

Abstract

In a world dominated by microbes, humans represent only a small fraction of living organisms. Some microbes are beneficial, but others, such as antibiotic-resistant bacteria, pose a major threat. In 2019, about 8.88 million deaths were attributed to bacterial sepsis, including 4.95 million related to bacterial resistance. This number could reach 10 million victims by 2050, highlighting the need for alternative strategies to combat bacterial resistance.

In this thesis, we explored deep eutectic solvents (DESs) as antimicrobial agents. These materials have unique properties due to hydrogen bonding networks. The study examines nine DESs: four conventional (reline, ethaline, glyceline, R-glyceline) and five with organic acids (OA-DESs: glycoline, T-glycoline, maloline, maline, oxaline). The research includes synthesis, physicochemical characterization, antibacterial screening, biofilm eradication evaluation, persister cell testing, use as co-drugs to reduce bacterial resistance, and antifungal effects.

The thesis comprises five chapters. The first describes the synthesis and characterization of DESs, confirming the formation of hydrogen bonding networks and thermal stability. Microwave characterization shows that conventional DESs are better for high frequency energy storage and dissipation.

The second chapter evaluates the antibacterial activity of DESs. OA-DESs exhibit superior activity compared to conventional DESs. A survey of twelve strains reveals that OA-DESs inhibit bacterial growth at concentrations below 1%, while conventional DESs require higher concentrations. OA-DESs disrupt the cell wall, while conventional DESs form pores.

The third chapter addresses the use of DESs against biofilms and persister cells. Biofilms of MRSA and *E. coli* reveal that some OA-DESs are effective at low concentrations. Live/Dead staining confirms these results. Additional tests show various action kinetics for all DESs.

The fourth chapter explores DESs as co-medications. The results of this study demonstrated that the MIC values of ampicillin and carbenicillin were reduced when combined with SEPs. Colistin and other peptides showed increased efficacy in the presence of SEPs. Two strategies (using DES and nisin together or sequentially) that could increase the efficacy of nisin against biofilms were tested, with the sequential treatment being the most effective.

The final chapter explores DESs as antifungal agents. Three fungal strains (*Candida albicans*, *Candida auris*, and *Aspergillus fumigatus*) show significantly stronger action from DES, reducing virulence by disrupting fungal colonies.

In conclusion, this thesis explores DESs as antimicrobial materials, offering perspectives for innovative strategies against bacterial resistance and fungal infections, addressing the global health challenges of the 21st century.

1. Introduction

1.1 Challenges and Opportunities in the Treatment of Bacteria

1.1.1 Antimicrobial Resistance, Bacterial Infection-Related Deaths, and Antibiotic Consumption

“Messieurs, ce sont les microbes qui auront le dernier mot” (en: Gentlemen, it is the microbes who have the last word) – is a quote often attributed to Louis Pasteur, the father of modern microbiology, who lived in the late 19th century. And it seems, almost two centuries later, that this quote has never been more relevant than now. In the world dominated by kingdoms of microbes, such as fungi, viruses and bacteria, other living organisms strive to adapt to a common pool of nutrients available on earth. Here, many of the microbes, in order to survive, exhibit pathogenicity, either intrinsic or acquired. This is well-reflected not only in many pandemics that ravaged human population, with the recent one sparked by a representative of coronaviruses, but also by the growing severity of the bacterial infections and antimicrobial resistance (AMR).

According to joint studies by the Institute for Health Metrics and Evaluation and University of Oxford^[1,2], in 2019 alone around 13.66 million people died from microbe-induced sepsis. More than 65% of them (8.88 million) were attributed to bacterial infections from which 4.95 million were associated with bacterial resistance and 1.27 million were directly attributed to infections induced by resistant species. Among the infamous leaders of annual death toll caused by bacteria, we find *Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Enterobacter* spp., and *Escherichia coli* – which form a group called ESKAPEE^[3,4]. This group is sometimes also called “escape” as they seem to, literally, escape many generations and classes of antibiotics, increasing the pace of the seemingly never-ending arms race between humans and microbes.

The recent antimicrobial resistance surveillance in Europe data for 2021-2023 published by the European Centre for Disease Prevention and Control^[5], indicated a growing antimicrobial resistance, among which, a third generation of carbapenems and cephalosporins are facing much higher resistance from *E. coli* and *K. pneumoniae*. What is more, based on data from 13 countries that took part in these studies, in 70% of isolates from 2021, *E. coli* and *S. aureus* were leading with 37.9% and 17.2%, and were higher than for 2020. In the same year, 25% of the countries reported percentages below 5% for *S. aureus* to exhibit methicillin resistance but in other 30% of the countries methicillin-resistant *S. aureus* (MRSA) contributed to 25% of *S. aureus* generation.

“What to expect in the future?” – is a question oftenly asked by scientists as well as medical personnel. In the well-known report, chaired by Jim O’Neill, predictions have been made that aimed to calculate the possible death toll associated with AMR. In this report, it is estimated that by 2050 this number will grow significantly and will reach around 10 million, surpassing such causes of death as cancer (8.2 million), diabetes (1.5 million) and diarrheal diseases (1.4 million). Despite AMR being a natural process, occurring via genetic mutations, many factors can enhance the pace of AMR development. The acceleration of its emergence and dissemination results largely from human activity, particularly the inappropriate and excessive use of antimicrobial substances for treating, preventing, or managing infections in humans, animals, and plants^[6-8].

According to a recent report by Browne et al.^[9], the global consumption of antibiotics is on the rise. Just between 2000 and 2018 almost all the global burden of disease (GBD) regions crossed 10 defined daily doses (DDD) per 1000 population (**Figure 1**), with high-income super-region (covering western Europe, southern Latin America, north America, Asia Pacific, and Australasia) leading in numbers. Authors of this report have found that as of 2018 a 14.3 DDD per 1000 population were used (corresponding to 40.2 billion DDD overall), which also corresponds to an increase of 46% of antibiotic use when compared to 2000. Particular regions

that are characterized by the highest number of antibiotic consumptions are south Asia, north Africa and Middle East, east Asia, western Europe, and high-income north America which cross more than 7% of global consumption (3000 million DDD).

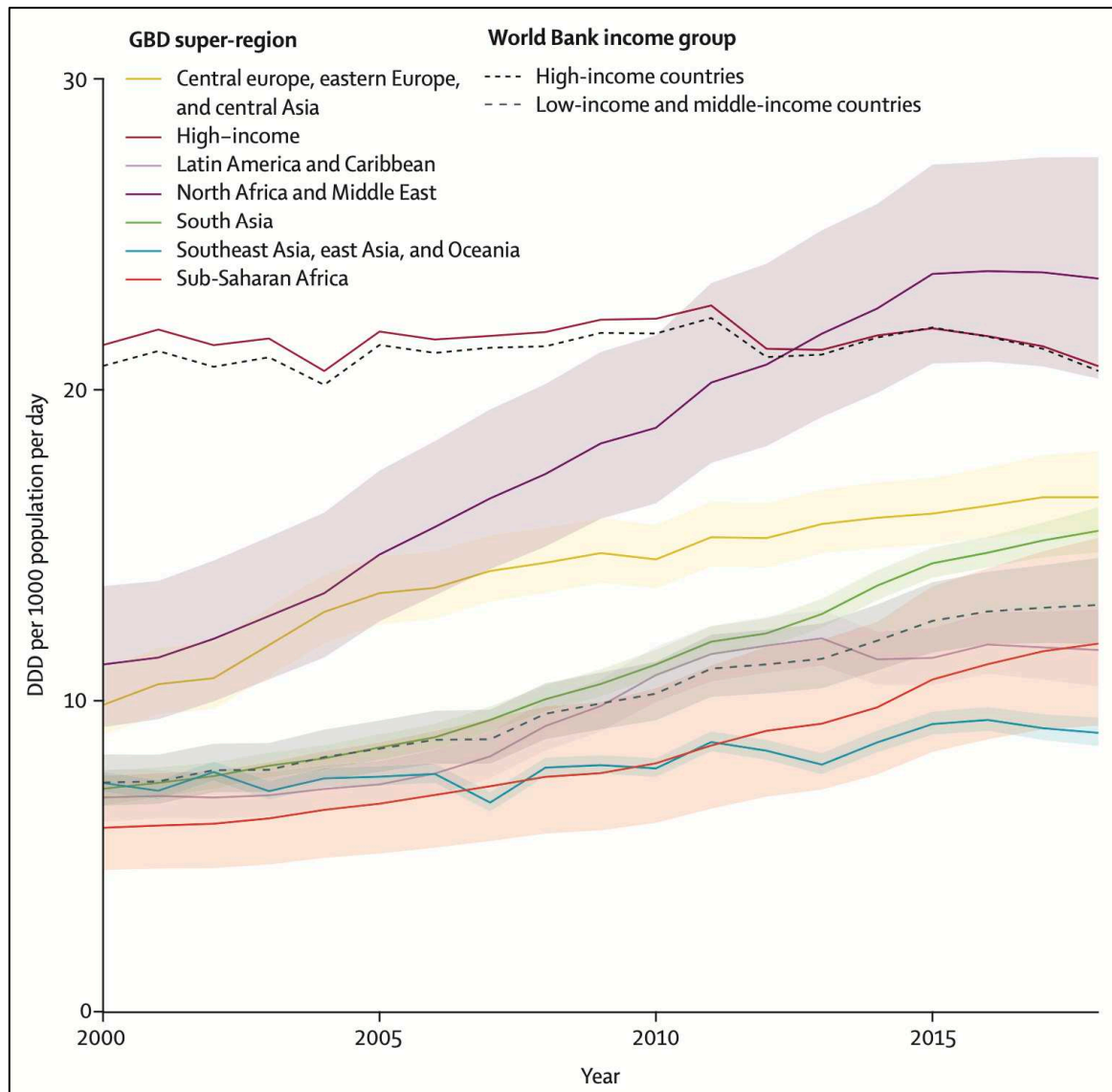


Figure 1. Changes over time in the total rates of antibiotic consumption across GBD super-regions and income groups classified by the World Bank (ribbons indicate 95% uncertainty intervals)^[9], © Elsevier.

Use of antibiotics for human treatment is only one of many aspects of a potential antibiotic overuse. According to an article published by Mulchandani et al.^[10], the global use of antibiotics in farming is predicted to rise significantly by 2030.

Countries that will likely most contribute to increased antibiotic use are Pakistan (+44%), Australia (+16%) Russia, Mexico, Brazil (each +13%), and Thailand (+12%), placing their cumulative antibiotic use close to China, a “leader” in this category (Figure 2).

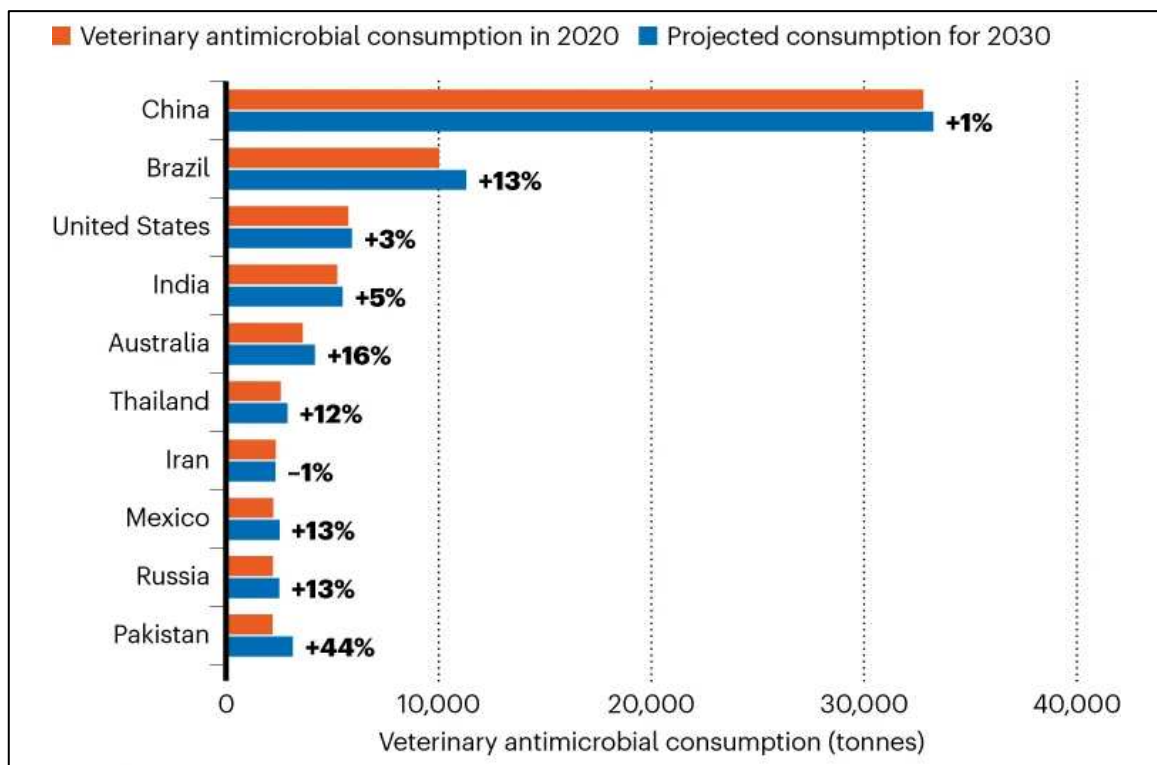


Figure 2. Antimicrobial consumption as of 2020 and its predicted values for 2030^[11], © Springer.

The overuse of antibiotics in farming poses significant dangers to both human health and the environment. Continuous administration of antibiotics to livestock will likely lead to the development of antibiotic-resistant bacteria, which can then be transmitted to humans through the food chain or environmental contamination^[12]. This “proliferation” of antibiotic resistance undermines the effectiveness of these drugs in treating bacterial infections in both animals and humans, jeopardizing public health by rendering once-effective treatments ineffective. Furthermore, the runoff from farms can contaminate water sources^[13], further disseminating antibiotic residues and resistant bacteria into the environment, exacerbating the problem on a larger scale. Thus, curtailing the

overuse of antibiotics in farming is crucial to safeguarding both human health and the environment.

1.1.2 Bacterial Infections around Us – from our Guts to our Hospitals

Bacteria, despite their small size and seemingly innocuous presence, are ubiquitous and could be aptly described as the invaders of our world. It is estimated that on the Earth there exist five million trillion trillion bacteria (5×10^{30} , around 2×10^{15} tones)^[14], being outnumbered only by phages, the bacteria viruses (10^{31}). It is then safe to assume, that bacteria colonized our world, and we should expect to find them everywhere. Indeed, bacteria colonized not only the “surface” of the world, but other organisms as well – including humans. According to Sender et al.^[15] a total number of bacteria in a 70 kg-human body is almost equal to that of the human cells and is equal to 3.8×10^{13} (around 2 kg).

Interestingly, bacteria fulfill several vital roles within the human body, encompassing both advantageous and detrimental functions. One of their primary contributions lies in digestion, particularly in the gut microbiota^[16]. Here, bacteria aid in breaking down food and facilitating digestion. Through fermentation of certain indigestible carbohydrates, they produce essential vitamins like B^[17] and K^[18], along with metabolizing bile acids. This digestive assistance is fundamental to nutrient absorption and overall digestive health. Moreover, bacteria play a pivotal role in regulating the immune system^[19]. The gut microbiota helps in the development and function of immune responses. These microbes regulate immune responses, train immune cells, and defend against pathogens by competing for resources and space. Emerging research also indicates a connection between gut bacteria and brain function, impacting mood and behavior through the gut-brain axis^[20]. This suggests implications for mental health conditions, such as depression and anxiety.

However, it is obvious that not all the bacteria are “good” for a human health or the environment. When assessing the nature of the bacteria, or rather their

pathogenicity, one has to consider aspects, such as virulence factors, invasiveness, toxicity, transmission, and resistance to host defense systems^[21]. Also, a pathogenicity depends strictly on the host – a certain strain can be beneficial to one species while being harmful to other, such as in a case of *Salmonella*, which is commonly found in gut microbiota of birds^[22]. But the presence of bacteria in the environment also plays a pivotal role in the spread of diseases and ecological balance.

Awareness of bacterial presence in food and agriculture is paramount to prevent foodborne illnesses and maintain animal health. Throughout the food supply chain, from production and processing to distribution and consumption, vigilance is necessary to mitigate bacterial contamination. Critical Control Points must be identified and managed effectively, employing measures such as stringent sanitation practices, temperature control, and proper handling procedures^[23]. Monitoring the microbial load of food products is essential to ensure compliance with safety standards, as high levels of bacterial contamination can pose significant risks to consumer health. In the realm of animal health, bacterial diseases pose substantial threats to livestock and agricultural productivity. Pathogens such as *Salmonella*, *E. coli* and *Campylobacter* can cause a spectrum of diseases in animals, ranging from gastrointestinal infections to reproductive issues and systemic illnesses^[24]. Veterinarians and farmers alike must implement robust biosecurity measures, vaccination programs, and appropriate antibiotic usage to prevent and control bacterial diseases in livestock. Regular monitoring and surveillance are crucial to promptly detect and address outbreaks, safeguarding both animal welfare and the integrity of the food supply. Understanding the abundance of bacteria around us, one could pose a question: "How is it possible that we are not sick all the time?".

The human body employs a sophisticated array of defense mechanisms to ward off bacterial infections. These defenses begin with physical barriers like the skin^[25] and mucous membranes^[26], which act as formidable shields against bacterial

invasion. The skin forms a protective barrier that prevents bacteria from entering the body, while mucous membranes secrete mucus, containing antimicrobial substances (like antimicrobial peptides), to trap and eliminate bacteria before they can penetrate deeper tissues. Additionally, various secretions, such as tears^[27,28], and saliva^[29], have antimicrobial properties, further fortifying the body's defenses.

Beyond physical barriers, the innate immune system swiftly responds to bacterial threats through nonspecific mechanisms. Neutrophils and macrophages, for instance, engulf and destroy bacteria through a process called phagocytosis^[30]. Natural killer cells patrol the body, identifying and eliminating infected cells^[31]. Complement proteins, part of the innate immune system, enhance the ability of antibodies and phagocytic cells to clear bacteria^[32]. These rapid and nonspecific responses provide crucial early defense against bacterial infections, buying time for the more specialized adaptive immune system to mount a targeted response.

However, certain factors can compromise the body's defenses, making individuals more susceptible to bacterial infections. Immunodeficiency disorders such as AIDS^[33], chronic diseases^[34], malnutrition^[35], age-related changes^[36], and certain medical treatments (use of immunosuppressants) can weaken the immune system, impairing its ability to fend off bacterial invaders effectively. Consequently, individuals with compromised immune function are at heightened risk of developing bacterial infections, which may manifest as localized or systemic diseases depending on the type of bacteria involved and the site of infection. Understanding these vulnerabilities underscores the importance of maintaining overall health and implementing preventive measures to reduce the risk of bacterial infections.

Quite often we can find bacteria in places least expected, such as in hospitals. Bacteria present in hospitals pose a significant concern due to the potential for healthcare-associated infections (HAIs) or nosocomial infections^[37-39]. Hospitals provide an environment where various bacterial pathogens can thrive, facilitated

by factors such as close proximity of patients, invasive medical procedures, frequent use of antibiotics, and compromised immune systems of patients. These factors create opportunities for the transmission of bacteria between patients, healthcare workers, and the hospital environment itself. Bacteria commonly implicated in HAIs include *S. aureus*, including MRSA^[40], *Clostridium difficile*^[41], *A. baumannii*^[42], and *P. aeruginosa*^[43], among others.

Preventing the spread of bacterial infections in hospitals and nursing homes requires stringent infection control measures, including hand hygiene, environmental cleaning and disinfection, appropriate use of personal protective equipment, and surveillance for HAIs^[44]. Additionally, antimicrobial stewardship programs are essential to promote judicious antibiotic use, minimize the development of antibiotic resistance, and prevent the emergence of multidrug-resistant bacteria^[45]. By implementing comprehensive infection prevention and control strategies, hospitals can mitigate the risk of bacterial infections and safeguard the health and safety of patients, healthcare workers, and visitors.

Taking all of this into account, we can say that bacteria, ubiquitous in our world, serve vital functions but also pose risks to human health. While they aid digestion and immune regulation, pathogenic strains can cause diseases, demanding careful consideration of factors like virulence and transmission. Vigilance is essential in environments, such as hospitals, where bacterial pathogens thrive, necessitating robust infection control measures to prevent healthcare-associated infections. Through strategies like hand hygiene, sanitation, and judicious antibiotic use, hospitals can mitigate risks and ensure a safe healthcare environment.

1.1.3 Adaptive Features of Bacteria

Formerly in Monera kingdom (until 1977), bacteria are now recognized as a separate domain (together with archaea, which was also a part of Monera kingdom, and now is a separate domain). Bacteria, are usually a single cell

organism, being in a size range of one up to ten micrometers. Different categorizations of bacteria provide insights into their survival strategies and ecological roles. Understanding these categories helps in predicting their behavior in various environments and their potential impacts on human health and ecosystems. For instance, morphology classification can give clues about how bacteria interact with their surroundings and other organisms. Gram staining allows to distinguish between different types of bacteria based on their cell wall compositions, which can influence their susceptibility to antibiotics and other treatments. Metabolic classification sheds light on how bacteria obtain energy and nutrients, which is crucial for their adaptation to diverse environmental conditions. By studying these categories, scientists can better comprehend bacterial diversity and develop strategies for controlling harmful bacteria while harnessing beneficial ones for various applications. Now, let's delve into the main categories:

1. **General morphology** – Bacteria exhibit various shapes and arrangements, influencing their ability to move, adhere to surfaces, and interact with other organisms. For example, cocci bacteria like *Staphylococcus* are often found in clusters and are associated with, while bacilli bacteria, such as *E. coli*, commonly occur singly and can form biofilms. Understanding these morphological characteristics aids in identifying bacterial species and predicting their ecological roles.
2. **Gram staining** – The Gram staining technique is a fundamental tool in bacterial classification, separating them into Gram-positive and Gram-negative groups based on differences in their cell wall structures. Gram-positive bacteria, like *S. aureus*, possess a thick layer of peptidoglycan, making them more resistant to certain environmental stresses and antimicrobial agents. In contrast, Gram-negative bacteria, such as *E. coli*, have an outer membrane that can confer resistance to antibiotics and facilitate interactions with their surroundings. This differentiation is crucial for diagnosing bacterial infections and selecting appropriate treatment strategies.

3. **Metabolism** – Bacteria exhibit diverse metabolic strategies, which influence their ecological niches and interactions with other organisms. Aerobic bacteria, like *P. aeruginosa*, thrive in oxygen-rich environments and play essential roles in nutrient cycling and decomposition. Anaerobic bacteria, such as *Clostridium* species, can survive in oxygen-deprived conditions and are implicated in various diseases and ecological processes. Autotrophic bacteria, like *Nitrosomonas*, utilize inorganic compounds as energy sources and contribute to nitrogen cycling in ecosystems. Heterotrophic bacteria, including many pathogens and soil microbes, rely on organic compounds for energy and growth, affecting nutrient dynamics and ecosystem stability.

Understanding the diverse metabolic capabilities of bacteria is crucial for various applications, including bioremediation, agriculture, and biotechnology. By harnessing the metabolic diversity of bacteria, scientists can develop innovative solutions for environmental challenges and improve human health and well-being.

1.1.4 Antimicrobial Resistance – Forms and Treatment Methods

The proliferation of resistant strains within the overall population, coupled with scientists' ongoing efforts to develop new generations of antibiotics, resembles an endless arms race in which humans are unlikely to emerge victorious. Furthermore, the term "resistance" commonly connotes solely antibiotic resistance, yet in reality, microbes exhibit various forms of resistance. Three primary types include:

1. **Typical Antibiotic Resistance:** This refers to the ability of bacteria to resist the effects of antibiotics through various mechanisms such as enzyme production to deactivate the antibiotic, changes in cell permeability to prevent antibiotic entry, or efflux pumps to expel the antibiotic from the cell.
2. **Biofilms:** Biofilms are communities of microbes encased within a self-produced extracellular matrix. This matrix provides protection and resistance

against antibiotics and host immune responses, making biofilm-associated infections challenging to treat.

3. **Persister Cells:** Persister cells are a subpopulation of bacteria that enter a dormant or slow-growing state, rendering them tolerant to antibiotic treatment. These cells can survive antibiotic exposure and, upon favorable conditions, resume growth, contributing to recurrent infections and treatment failure.

1.1.4.1 Antibiotic Resistance

The history of the antibiotics reaches out to early 20th century, when compounds from the group of organoarsenics were used. While they were indeed among the earliest substances recognized for their antibacterial properties, they are not typically considered the first antibiotics. Arsenic-based compounds like arsphenamine (Salvarsan) were used to treat syphilis, demonstrating antibacterial effects^[46]. However, they were later replaced by more effective and less toxic antibiotics such as penicillin and sulfonamides.

Penicillin, discovered by Alexander Fleming in 1928, is widely regarded as the first true antibiotic due to its effectiveness against a wide range of bacteria and its relatively low toxicity to humans. Since then, 38 classes of antibiotics have been discovered (**Figure 3, Table 1**). Each of the class has its “development window” – a time needed for an antibiotic to be used in the clinic for the first time; and “resistance window” – a time for the first resistance occurrence to be reported. Unfortunately, no antibiotic class is flawless, and the emergence of resistance is inevitable. The most recent class of antibiotics, known as diarylquinolines, was approved by the U.S. Food and Drug Administration (FDA) in 2012^[47]. This approval marks a lapse of more than a decade since the approval of any new antibiotic class. Additionally, this class has been issued a “black box warning” by the FDA, the most severe cautionary label indicating significant risks of preventable, serious, or potentially life-threatening adverse effects as suggested by medical studies.

However, it must be pointed out that the emergence of the resistance does not necessary mean that the antibiotic is not used anymore. Many of the antibiotics, from classes where the resistance has been observed are still commonly used, such as carbapenems, tetracyclines, penicillins, and cephalosporins. However, the previously mentioned data suggests, that we are in a dire need of new classes or generations of the currently existing classes if we want to effectively combat bacterial infections.

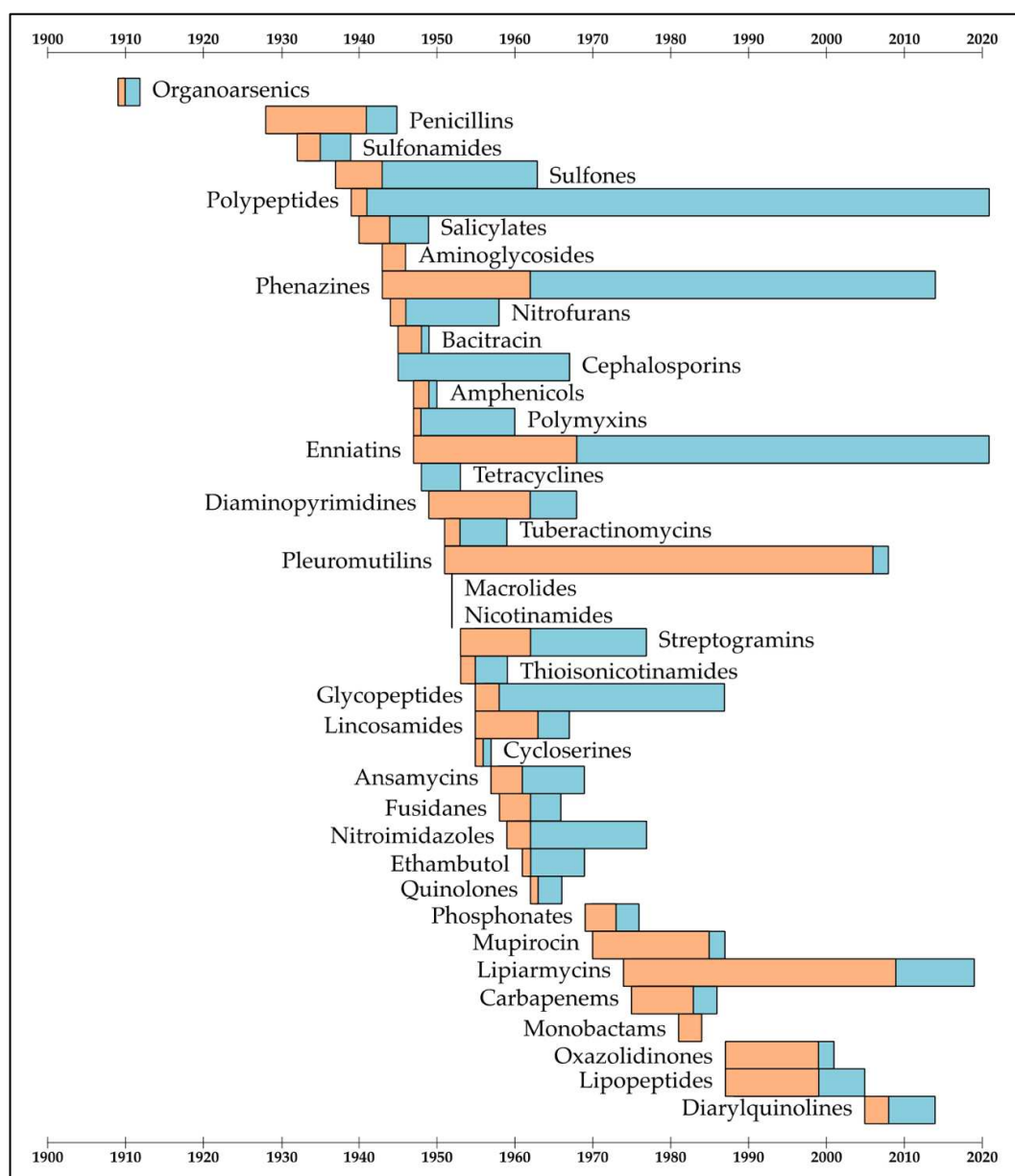


Figure 3. "Development" and "resistance" windows of 38 classes of antibiotics used since the beginning of 20th century^[48], © MDPI.

Table 1. List of 38 classes of antibiotics, with their targets in bacterial cell.

Antibiotic class	Target	Example	Ref.
organoarsenics	metabolism	arsenic trioxide	[49]
penicillins	cellular wall synthesis	amoxicillin	[50]
sulfonamides	folic acid synthesis	sulfamethoxazole	[51]
sulphones	folic acid synthesis	dapsone	[52]
polypeptides	varies (cellular membrane)	polymyxin b, colistin	[53]
salicylates (co-drug)	metabolism	salicylic acid	[54]
aminoglycosides	protein synthesis	gentamicin	[55]
phenazines	metabolism	pyocyanin	[56]
nitrofurans	metabolism	nitrofurantoin	[57]
bacitracin	cellular wall synthesis	bacitracin	[58]
cephalosporins	cellular wall synthesis	cephalexin	[59]
amphenicols	protein synthesis	chloramphenicol	[60]
polymyxins	cell membrane	colistin	[61]
enniatins	protein synthesis	enniatin	[62]
tetracyclines	protein synthesis	doxycycline	[63]
diaminopyrimidines	folic acid synthesis	trimethoprim	[64]
tuberactinomycins	cellular wall synthesis	tubercidin	[65]
pleuromutilins	protein synthesis	retapamulin	[66]
macrolides	protein synthesis	azithromycin	[67]
nicotinamides	metabolism	isoniazid	[68]
streptogramins	protein synthesis	quinupristin/dalfopristin	[69]
thioisonicotinamides	metabolism	thiacetazone	[70]
glycopeptides	cellular wall synthesis	vancomycin	[71]
lincosamides	protein synthesis	clindamycin	[72]
cycloserines	cellular wall synthesis	cycloserine	[73]
ansamycins	RNA polymerase	rifampin	[74]

fusidanes	protein synthesis	fusidic acid	[75]
nitroimidazoles	DNA synthesis	metronidazole	[76]
ethambutol	cellular wall synthesis	ethambutol	[77]
quinolones	dna gyrase/topoisomerase	ciprofloxacin	[78]
phosphonates	cellular wall synthesis	fosfomycin	[79]
mupirocin	protein synthesis	mupirocin	[80]
lipiarmycins	RNA polymerase	ripostatin	[81]
carbapenems	cellular wall synthesis	meropenem	[82]
monobactams	cellular wall synthesis	aztreonam	[83]
oxazolidinones	protein synthesis	linezolid	[84]
lipopeptides	cell membrane	daptomycin	[85]
diarylquinolines	varies	delamanid	[86]

1.1.4.2 Biofilms

Biofilms, often called the “cities of microbes”, are complex communities of microorganisms that adhere to surfaces and are enveloped in a matrix of extracellular polymeric substances (EPS). They can form on various biotic and abiotic surfaces, ranging from natural environments like rivers and rocks to human-made structures such as medical implants, pipes, and even teeth^[87]. Biofilms play significant roles in various ecological processes, industrial processes, and human health, but they also pose challenges, particularly in medical settings due to their inherent resistance to antimicrobial agents^[88].

One of the key properties of biofilms is their ability to provide protection and survival advantages to the microorganisms residing in their structure. The EPS matrix acts as a physical barrier that shields the microbial cells from external threats, including immune responses and antimicrobial agents. This protective environment fosters microbial growth and allows for the exchange of genetic material, contributing to the resilience and adaptability of the biofilm community. EPS itself is composed of various biomolecules, such as nucleic acids,

exopolysaccharides, proteins, and lipids^[89] (**Figure 4**). Such environment not only protects the EPS-enveloped bacteria heavy metals, pH changes, desiccation and so on, but also effectively limits the penetration of the antibiotics to bacterial cells.

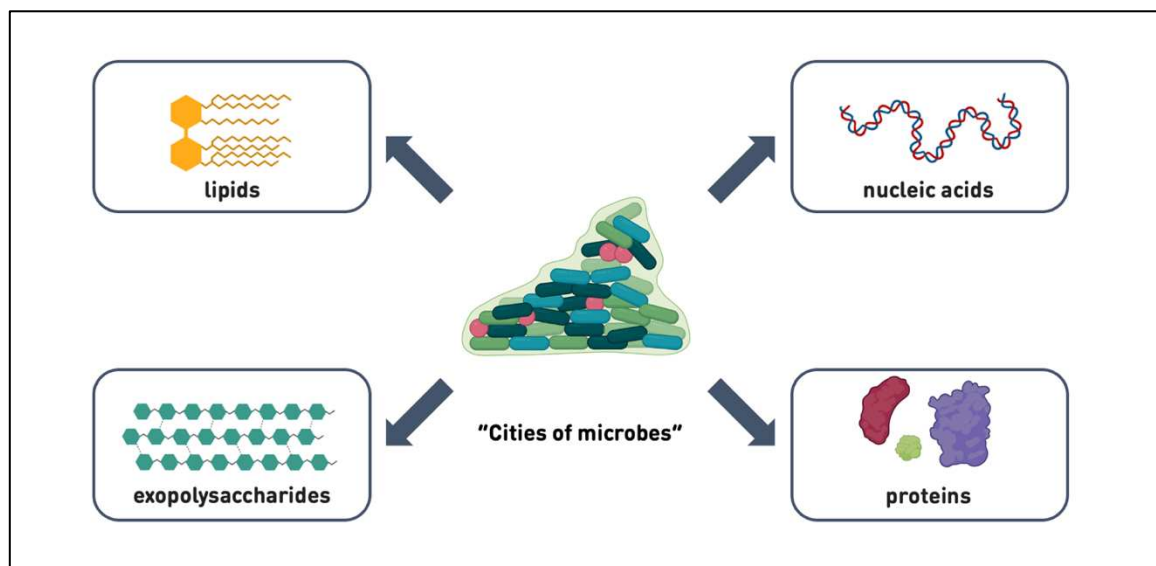


Figure 4. Typical composition of biofilms.

Moreover, biofilms exhibit inherent resistance mechanisms that make them highly resilient to antibiotics and other antimicrobial treatments. Within the biofilm structure, microbial cells can adopt dormant or slow-growing states, making them less susceptible to traditional antibiotic mechanisms that target actively dividing cells. Additionally, the EPS matrix can limit the penetration of antimicrobial agents, further reducing their effectiveness against biofilm-associated microorganisms. As a result, biofilm-associated infections are often more challenging to treat and may require prolonged or alternative therapeutic approaches. In fact, higher concentrations of antibiotics are usually needed to eradicate biofilms, which may result in the resistance and potential harm to the patient.

The resistance of biofilms poses significant challenges in various fields, including medicine, where biofilm-related infections are a leading cause of persistent and recurrent infections associated with medical devices, such as catheters, prosthetic implants, and wound dressings^[90]. In industrial settings, biofilms can lead to

biofouling and corrosion of surfaces, affecting the performance and lifespan of equipment and infrastructure^[91].

Efforts to combat biofilm-related issues involve understanding the complex dynamics of biofilm formation and developing strategies to disrupt or prevent biofilm formation and enhance the efficacy of antimicrobial treatments. These approaches may include the development of novel antimicrobial agents^[92], biofilm-disrupting compounds^[93], and surface modifications to inhibit biofilm adhesion^[94]. Additionally, interdisciplinary research efforts that combine microbiology, materials science, engineering, and medicine are crucial for addressing the challenges posed by biofilms and developing innovative solutions to combat biofilm-associated problems.

1.1.4.3 Persister Cells

Persister bacteria cells (also called persisters) represent a fascinating and, when it comes to treatment, challenging aspect of microbial biology, demonstrating a remarkable ability to withstand the onslaught of antimicrobial agents and environmental stresses^[95]. These elusive cells, typically constituting a small fraction of the overall microbial population, possess the unique capability to enter a state of dormancy or slow growth, rendering them temporarily impervious to conventional antibiotic treatments. This phenomenon of antibiotic tolerance exhibited by persister cells has profound implications for the management of biofilm-related infections, contributing significantly to treatment failures, chronicity, and recurrence^[96].

At the heart of persister biology lies a complex interplay of genetic, physiological, and environmental factors. Various molecular mechanisms underpin the formation and maintenance of persister cells, including the upregulation of stress response pathways, alterations in metabolism, and the activation of specific survival genes. These adaptive strategies enable persister cells to effectively shut

down essential cellular processes targeted by antibiotics while maintaining a dormant state that allows them to survive hostile conditions.

Persister cells play pivotal roles in a diverse array of microbial communities, ranging from acute infections to chronic biofilm-associated conditions. Within biofilms, persister cells serve as a reservoir of antibiotic-tolerant bacteria, facilitating the long-term survival and persistence of these complex microbial communities^[97]. As biofilms provide a protective niche for persister cells, they pose significant challenges in clinical settings, contributing to the chronicity and recurrence of biofilm-related infections, such as those associated with medical implants, wounds, and indwelling devices^[98,99].

Efforts to combat persister cells and mitigate their impact on public health require a multifaceted approach. Researchers are actively investigating the molecular mechanisms underlying persister formation and seeking innovative strategies to target and eradicate these resilient subpopulations. Novel antimicrobial agents, combination therapies, and approaches aimed at disrupting persister metabolism or resuscitation pathways show promise in enhancing the efficacy of existing antimicrobial treatments and combating persistent infections^[100].

Furthermore, advances in diagnostics and surveillance techniques are essential for identifying and monitoring persister populations in clinical settings, enabling early intervention and tailored therapeutic strategies. Collaborative efforts between microbiologists, clinicians, and drug developers are paramount in addressing the challenges posed by persister cells and safeguarding public health against the threat of antibiotic resistance and treatment failure.

1.1.5 Current Treatment Opportunities – State-of-the-Art

The data mentioned at the beginning of this thesis is a key reason for the search of alternative ways to combat bacteria and treat bacterial infections. Among alternative approaches adopted so far, we find bacteriophage-based therapies^[101],

use of the antimicrobial peptides^[102], nanotechnological approaches^[103], and immunotherapy^[104], with the first three being the most promising as of now.

1.1.5.1 Phage-Based Therapies

Phage-based therapies offer a promising alternative to conventional antibiotics in the fight against bacterial infections^[105]. Their mode of action typically involves a series of steps. First, the phage attaches itself to the surface of the bacterial cell using tail fibers or other surface proteins, recognizing specific receptors on the bacterial surface. Once attached, the phage injects its genetic material, either DNA or RNA, into the bacterial cell. This genetic material takes over the bacterial machinery, directing it to produce more phage components rather than the normal bacterial components. As a result, the bacterial cell becomes a factory for producing new phages. Eventually, the bacterial cell lyses, or bursts open, releasing the newly formed phages to infect other bacterial cells in the vicinity. This lytic cycle effectively leads to the destruction of the bacterial population (**Figure 5**). Among the biggest advantages of this approach is the high specificity and potential for personalized medicine. Specificity of this methods dictates the utilization of viruses that target and infect specific bacterial strains, may spare beneficial bacteria, and potentially reduce the risk of disrupting the microbiota. The same reason may lead this therapy to be tailored (unlike conventional broad-spectrum antibiotics), making phage-bases therapies personalized and thus reducing the unwanted side effect for a patient. However, the same issues seem to be the biggest disadvantage of this approach. Personalized medicine is burdened with economic challenges and the time until the market adapts to it may be very long. Specificity of the phage-bacteria interaction may also result in the emergence of resistance, making this therapy ineffective. Due to its novelty this approach may, and will likely, face regulatory issues, which makes it being still under evaluation for clinical applications^[106].

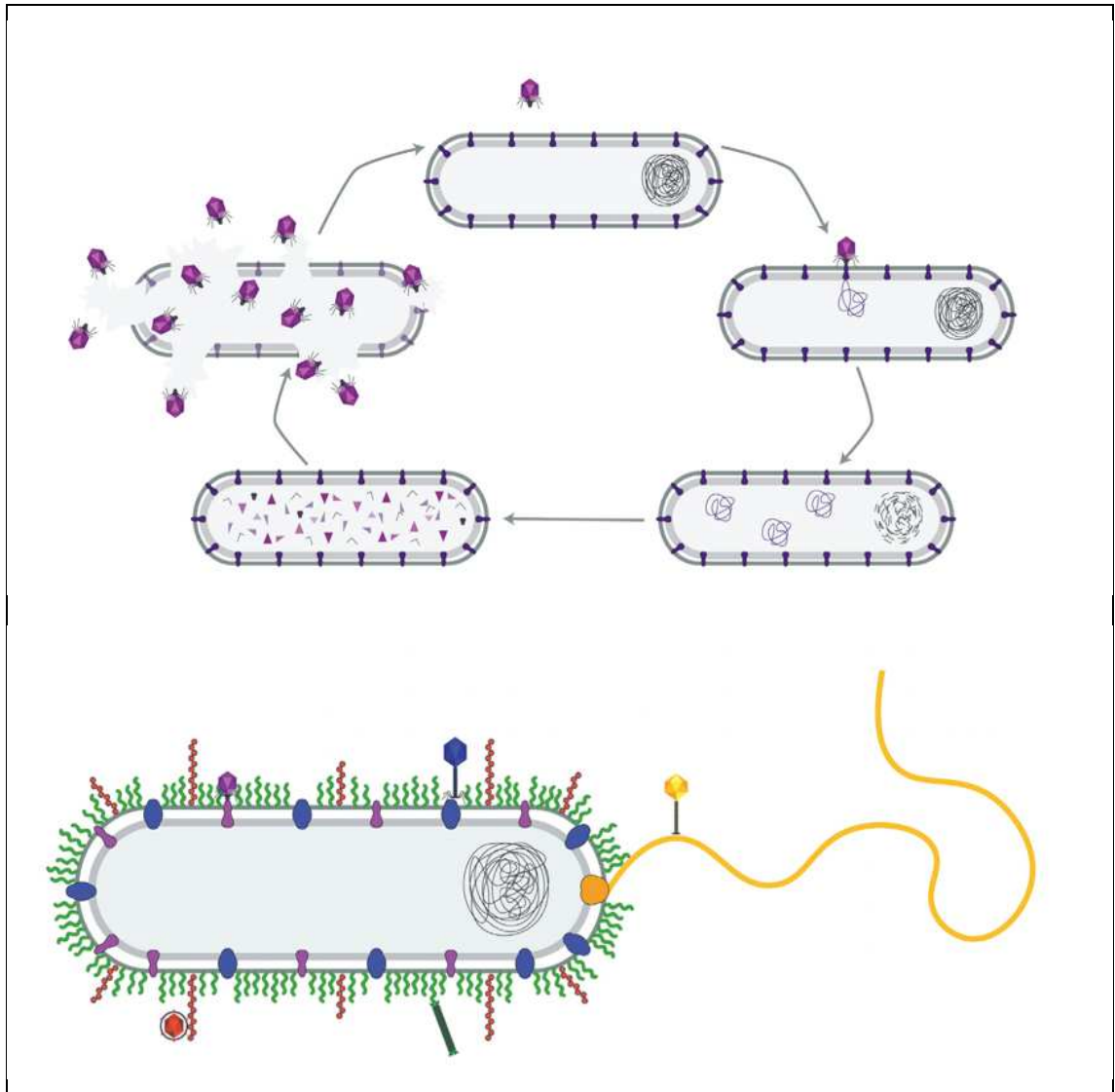


Figure 5. A simplified mechanism mode-of-action of phages (top), and possible cellular targets for phages, such as pili, flagella, porins, and efflux pumps (bottom)^[107].

On the implementation side, another issue arises. Would bacteriophages be compatible for treatment of humans? Should not we expect the antiviral response from our own bodies? In a very recent paper by Podlacha et al.^[108] authors have proved that phage therapy should be safe for use. Indeed, a cocktail of bacteriophages leads to a start of immunological response, however it is hindered just before the last phase. This means, that phage-based therapies can be considered as safe for use for animals and humans.

Use of page-based therapies still needs to be improved. Application of phages against AMR was presented in many case reports in emergency phage therapies against resistant *P. aeruginosa* and *A. baumannii*. In the report by LaVergne et al.^[109], an elderly patient overcome craniectomy complicated by postoperative infection. Isolates revealed the presence of multi drug-resistant *A. baumannii*, which exhibited resistance to azithromycin, chloramphenicol, and rifampin. Despite exhibiting susceptibility to colistin, and use of combinational therapies (colistin + rifampin + azithromycin) the condition of patient did not improve, and phage cocktail was administered. In another report by Duplessis et al.^[110], a pediatric patient experienced a bacteremia (sepsis) caused by *P. aeruginosa*. The administered antibiotics covered meropenem, tobramycin, aztreonam, colistin, and polymyxin B – to which isolated strain were susceptible. Sadly, the strains started to exhibit resistance very quickly. Because of the patient's allergies to certain antibiotics and the presence of antibiotic resistance, the medical team opted to administer a bacteriophage cocktail. Unfortunately, in both case reports, the patients passed away. However, their deaths were not attributed to the therapy itself but rather to the advanced stage of sepsis and other underlying illnesses. A successful implementation of phage therapy was reported by Schooley et al.^[111]. An elderly patient suffering from diabetes was admitted to a hospital and diagnosed with necrotizing pancreatic. An infection caused by MDR *A. baumannii* was also diagnosed. The administered antibiotics failed to control the infection, so phage therapy was implemented. A resistance to the initial bacteriophage cocktails emerged during treatment. An *A. baumannii* isolate which was obtained 8 days after the initiation of bacteriophage therapy, was found to be resistant to both of the initially used bacteriophage cocktails. This resistance prompted the rapid selection of additional bacteriophages capable of targeting this resistant isolate. Patient initially critical, unresponsive, with renal failure improved after systemic intravenous administration of bacteriophage therapy.

1.1.5.2 Antimicrobial Peptides

Antimicrobial peptides serve as recognized alternatives to conventional small molecule antibiotics^[112,113]. This diverse group contains both synthetic and naturally occurring peptides and finds application across various domains. It serves as a crucial last-resort treatment for bacterial infections caused by Gram-negative species. Additionally, nisin, a bacteriocin produced by *Lactococcus lactis*, merits attention for its widespread use as a food preservative^[114], often identified by its E-number, E234. Antimicrobial peptides are known to exhibit low potential for inducing antimicrobial resistance^[115]. This is due to their broad-spectrum activity, which also makes them a good choice (**Table 2**), for the treatment of diverse bacterial infections.

Table 2. Common antimicrobial peptides, and peptide families, and their origin and mechanism.

Name	Mechanism	Source	Ref.
colistin	disrupts bacterial cell membrane (Lipid A binding)	bacteria	[116]
nisin	binds to cell membrane, disrupts cell wall (Lipid II binding)	bacteria	[117]
LL-37	disrupts bacterial cell membrane (various targets)	human	[118]
magainin	forms pores in bacterial cell membrane	frogs	[119]
defensins	disrupts bacterial cell membrane (various targets)	various	[120]
cecropins	forms pores in bacterial cell membrane	moths, pigs	[121]
melittin	forms pores in bacterial cell membrane	honeybee	[122]
dermcidin	disrupts bacterial cell membrane	human	[123]
plantaricin	disrupts bacterial cell membrane	bacteria	[124]
daptomycin	binds to bacterial cell membrane, disrupts function (cell membrane)	bacteria	[125]
plectasin	inhibits cell wall synthesis (bacterial cell wall precursor)	fungi	[126]
bacitracin	inhibits cell wall synthesis (bacterial cell wall synthesis via divalent metal ions)	bacteria	[127]
polymyxin B	disrupts bacterial cell membrane (lipid A binding)	bacteria	[128]

Peptides' complex molecular structures underpin their therapeutic efficacy, making them favorable choice for treatment. However, this same complexity presents challenges in synthesizing them cost-effectively. Also, some antimicrobial peptides, may exhibit cytotoxic (such as in the case of colistin causing severe nephrotoxicity^[129]) effects against host cells at higher concentrations, raising concerns about their safety and potential side effects in clinical use. Furthermore, the susceptibility of peptide structures to degradation poses a significant challenge, potentially limiting their effectiveness in therapeutic applications. This vulnerability underscores the necessity for additional protective measures, such as employing nanocarriers, to enhance the stability and bioavailability of peptides.

1.1.5.3 Nanotechnology

Nanotechnology presents numerous opportunities for the treatment of bacterial infections. In this realm, nanomaterials (**Figure 6**) serve as carriers for drugs, employing methods like encapsulation (e.g., lipid nanocapsules (LNCs)) or sorption (e.g., alginate nanoparticles (Alg NPs)).

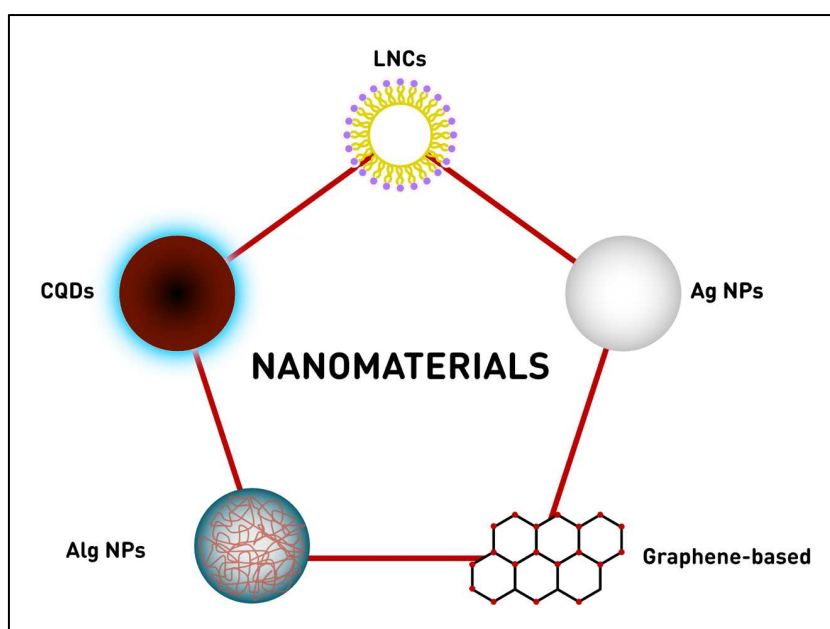


Figure 6. Types of nanomaterials that can be used in nanotechnologically-based therapies.

Additionally, they can demonstrate antibacterial effects through chemical alteration (e.g., graphene, carbon quantum dots (CQDs)) or inherent properties (e.g., silver nanoparticles (Ag NPs)). These materials can be studied separately, but a more interesting categorization would consider intrinsic (direct mode-of-action) and inherited (indirect mode-of-action) antibacterial properties. This would give us a better insight in various strategies in nanotechnological approach to combat AMR.

Probably, the most recognized nanomaterial when it comes to its antibacterial activity and a prime representative of direct mode-of-action are silver nanoparticles. Ag NPs exhibit potent antibacterial activity against a wide range of pathogens, making them promising candidates for various biomedical and environmental applications. Their small size and large surface area-to-volume ratio enable efficient interaction with bacterial cell membranes, leading to disruption of cellular functions and eventual cell death. Moreover, Ag NPs can inhibit bacterial growth through multiple mechanisms, including generation of reactive oxygen species and interference with cellular processes^[130].

A good example of their effectiveness was shown by Li et al.^[131,132]. In their study, authors have demonstrated that Alg NPs were able to inhibit the growth of both Gram-positive and Gram-negative species with concentrations as low as 10 µg/mL for *E. coli* and 20 µg/mL for *S. aureus*. Interestingly, in both cases, major disruption of the bacterial morphology was observed such as (but not limited to) – pore formation, major disruption of bacterial cell wall, and DNA condensation. On the other hand, in a review published in 2020 by Ferodus and Nemmar^[133], authors demonstrated that the majority of in vitro research has illustrated that the cellular uptake of Ag NPs depends on their size, dosage, and coating. Subsequent in vivo biodistribution analyses have revealed the accumulation of silver and its toxicity in both nearby and distant organs, following the exposure to these nanoparticles. Despite their cytotoxicity, Ag NPs have found their use as coatings in various

branches of industry, serving as a stable, and economically affordable solution^[134,135].

Laser-induced graphene (LIG) is an interesting antimicrobial material, which is being more and more often studied by many groups^[136–138]. Here a high power laser is used to synthesize multi-sheet, highly developed, 3D graphene-based materials from carbon-based sources. The material is highly tunable, and can exhibit either hydrophilicity or hydrophobicity depending on the gas used during its manufacturing process^[136]. Highly developed, negatively charged surface further promotes the inhibition of bacterial adherence to the surface of the material. In studies reported by Singh et al.^[138], a conductive LIG surface prevented the growth of *P. aeruginosa* biofilms. What is more, LIG powder also exhibited size-dependent antibacterial activity, with achieving around 50 and 80% bacterial inhibition for small-sized and big-sized powder, respectively. Due to its conductivity, the material displays voltage-dependent antibacterial activity as-well. A small voltage of 1.5 V resulted in less than half log unit-reduction, while increase of the voltage up to 2.5 V effectively eradicated the bacteria with around 4 log units (which corresponds to 99.99% of bacterial eradication).

Indirect approach usually covers either drug carriers or chemical modification of the surfaces of the material. In the first group, lipid nanocapsules and liposomes represent a promising strategy. These nanocarriers consist of a lipid bilayer surrounding a core, offering a versatile platform for encapsulating hydrophilic and hydrophobic drugs alike. Their tunable properties, including size, surface charge, and composition, enable precise control over drug release kinetics and targeting efficiency^[139]. Moreover, lipid nanocapsules exhibit exceptional biocompatibility and stability, minimizing potential cytotoxicity and ensuring prolonged circulation in the bloodstream. Their ability to bypass biological barriers and selectively accumulate in target tissues makes them particularly attractive for treating various diseases, ranging from cancer^[140] to neurological disorders^[141,142]. Example of excellent use of LNCs against Gram-positive strains was reported by Cé et al.^[143].

In their work, authors reported a synthesis of fusidic acid-loaded lipid-core nanocapsules. Fusidic acid is a steroid-based antibiotic, commonly used in topical applications. The biological behavior of the drug was altered by nanoencapsulation, leading to enhanced performance of the formulations against *S. aureus*, *Enterococcus faecalis*, and *Staphylococcus epidermidis* strains. Compared to the fusidic acid alone, nanoencapsulation resulted in a 64-fold increase in inhibitory activity against *S. aureus*, a 128-fold increase against *E. faecalis*, and approximately an 8-fold increase against *S. epidermidis*. Another approach was reported by Valcourt et al.^[144]. Authors combined essential oils with antibiotics within lipid nanocapsules. Three major components were encapsulated: carvacrol from oregano oil, eugenol from clove oil, and cinnamaldehyde from cinnamon oil. In vitro studies evaluated interactions between essential oil-loaded LNCs and doxycycline against five Gram-negative strains (*E. coli*, *K. pneumoniae*, *A. baumannii* (RCH and SAN), and *P. aeruginosa*) showcasing no inhibitory interactions, but revealing a synergistic bactericidal effect. Time-kill kinetic assays confirmed this synergy, with at least a 3-log difference observed between the combination and the most active agent alone. Scanning electron microscopy demonstrated alterations in bacterial membranes, including the presence of holes and leakage of cellular contents upon exposure to the combination of essential oil-loaded LNCs and doxycycline.

Another interesting example is the use of surface-modified CQDs. Carbon quantum dots are a fascinating class of nanomaterials with a wide range of applications in areas such as bioimaging, sensing, drug delivery, and optoelectronics. These nanoscale carbon-based structures typically have sizes less than 10 nanometers and possess unique optical, electronic, and chemical properties. CQDs are typically synthesized through low-cost, scalable methods from various carbon sources, including organic precursors like citric acid, sugars, or waste materials like carbon black. Their exceptional photoluminescence, high

surface area, and biocompatibility make them highly attractive for biomedical applications, including bioimaging and biosensing.

Due to their versatile surface chemistry and customizable composition, CQDs are employed for direct modification to combat bacteria. Notably, as reported by Jijie et al.^[145], amine-terminated carbon dots functionalized with ampicillin offer a dual-action antibacterial approach, acting as carriers and visible light-triggered agents. This innovative platform synergistically combines ampicillin's antibacterial properties with CQDs' inherent capabilities. Immobilizing the antibiotic onto the CQDs' surface, along with the generation of moderate levels of reactive oxygen species under visible light exposure, proves highly effective in inhibiting bacterial growth. Under light, CQDs alone reduce the bacterial population by 4 log units (at 400 µg/mL). With ampicillin grafted onto the surface, total bacterial eradication occurs at 100 µg/mL, which further drops to 50 µg/mL with the addition of a light stimulus, demonstrating the system's efficacy. In another work, by Sviridova et al.^[146], quaternary ammonium salts with different alkyl chain lengths have been grafted onto the surface of CQDs. Modified CQDs proved to be effective in removal of *S. aureus* bacterial biofilm at concentration as low as 64 µg/mL and what is also important exhibited a very low cytotoxicity towards human cells.

Apart from the above mentioned examples in the realm of nanotechnology there exist many different and promising materials and approaches, such as colistin loaded Alg NPs^[147], "few-layer" graphene used as an irritant for photodynamic therapy^[148], and nanowall structured carbon-based materials for mechanical distortion of bacterial morphology^[149]. These innovative advancements signify the vast potential of nanotechnology in revolutionizing antimicrobial strategies, offering tailored solutions at the nano-scale that hold promise for combating infectious diseases and enhancing antimicrobial efficacy across various domains, from medicine to materials science. However, considering the arguments presented and the data outlined earlier in this chapter, it becomes imperative to explore alternatives. There is a pressing need to pursue antimicrobial drugs that

exhibit both high activity and stability, possess a low potential for inducing antimicrobial resistance, and are easy to synthesize and cost-effective.

1.2 Deep Eutectic Solvents

Deep eutectic solvents (DESs) are a group of biomaterials, that comprise various hydrogen bond donors and acceptors. These mixtures, first reported by Abbott et al. in 2003^[150], are characterized by the presence of an eutectic point – a temperature at which the components of the mixture undergo a phase transition, resulting in a unique, low-melting-point mixture, inheriting some of the properties of their parent substances while possibly emerging new ones.

Since their discovery, DESs have brought more and more interest with observable boom over the recent years (**Figure 7**). Their medical-related applications contribute to 25–50% of the papers published in the last ten years.

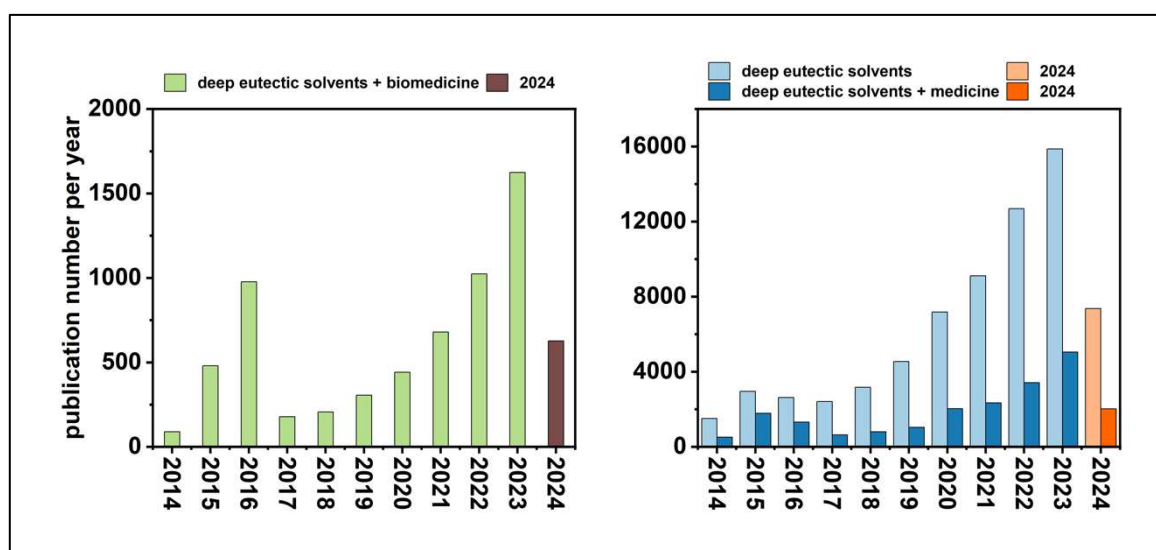


Figure 7. Number of publications as of June 2024 (keywords: deep eutectic solvents, deep eutectic solvents + medicine, deep eutectic solvents + biomedicine) according to Digital Science & Research Solutions, Inc., London, UK via app.dimensions.ai (accessed June 6th, 2024).

A similar trend was also observed in the number of works published in DESs within a topic of biomedicine, which focuses more on the molecular aspect of diseases. Due to their chemical composition, negligible adverse effect on the environment,

and low cytotoxicity, DESs are considered as green solvents^[151]. DESs have found their use in many fields of chemistry, such as catalysis^[152], metal processing^[153], nanotechnology^[154], electrochemistry^[155,156], and many others. Their versatility of application arises from the number of possible combinations of parent substances (PSs) used for their formulation. Unsurprisingly, they have also been investigated in various aspects of biomedicine, such as antibacterial treatment, drug delivery and drug solubilization.

The physicochemical nature and application of DESs has already been covered in many review articles^[157–161], including two crucial applications in (bio)medicine – drug delivery enhancement and treatment of bacterial infections. El Achkar et al.^[161] summarized the different routes of formulation of DESs as well as, the most relevant parameters controlling their formation and physicochemical properties. In a recent, extended, review article, Hansen et al.^[159], gave a deep and detailed discussion on the microscopic mechanisms governing the structure-property relationships. In the same review, they have also mentioned various applications of DESs, ranging from metallurgy to pharmaceutical and biomedical fields. In the review by Liu et al.^[160], the importance of the presence of natural deep eutectic solvents (NADESs) in natural products and biological systems was highlighted through various applications, such as extraction and chromatographic media. Zainal-Abidin et al.^[162] and Huang et al.^[163] described, respectively, the application of DES in drug discovery and delivery systems, and as active pharmaceutical ingredient (API) delivery systems in the treatment of metabolic diseases such as cancer, diabetes, and atherosclerosis.

DESs are quite commonly misclassified as ionic liquids (ILs). They do, in fact, share some properties with them, such as low volatility, non-flammability, and high chemical stability. However, their structural nature differs significantly. ILs rely solely on ionic interactions between PSs, while DESs rely almost exclusively on hydrogen bond interactions between PSs. While some ionic interaction may occur in the intricate network of hydrogen bonds of DESs (mainly due to presence of

halogen ions) this does not qualify them as ILs, and thus, it is important to emphasize that DESs and ILs belong to two distinct families of solvents.

1.2.1 In the Intricate Lattice of Hydrogen Bonds – Physicochemical Basis of the DESs

As mentioned before, deep eutectic solvents are mixtures of hydrogen bond donors (HBDs) and acceptors (HBAs) (Lewis and Brønsted acids and bases), which are characterized by the presence of the eutectic point. Eutecticity is a physicochemical occurrence, when two or more compounds can completely dissolve in each other, and their phase transition is described by thermal equilibrium ($\Delta G = 0$) (**Figure 8**). For a binary eutectic system such equilibrium is also described by the following phase diagram:

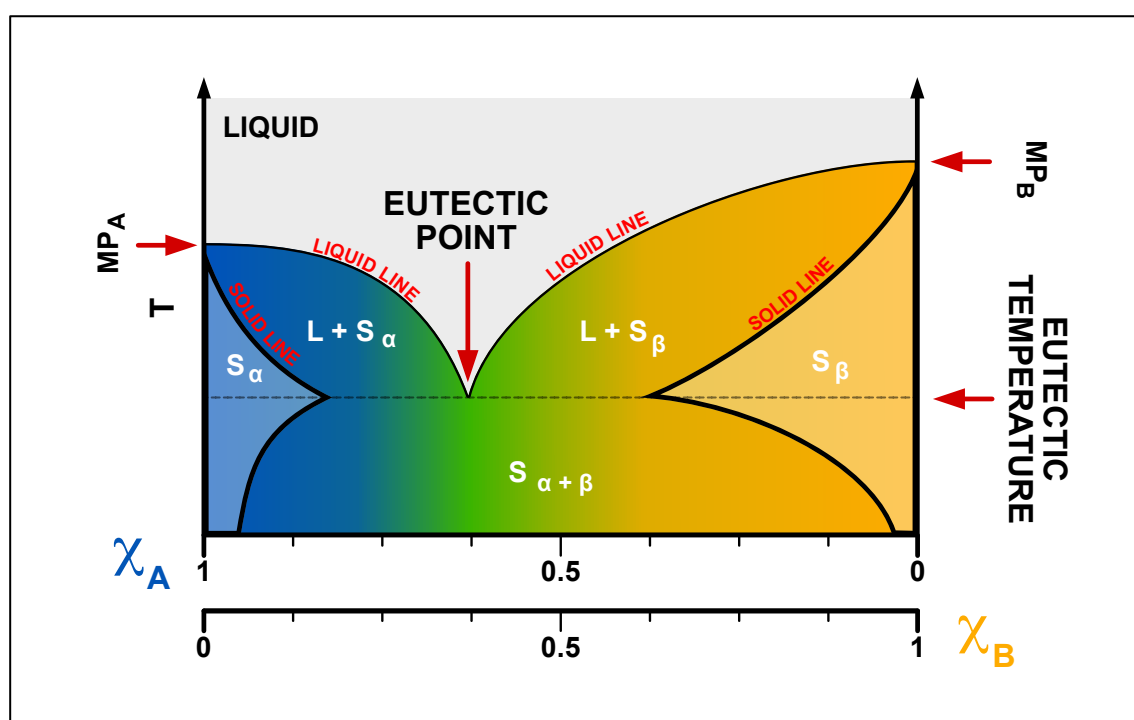


Figure 8. Phase diagram of binary eutectic system: MP – melting point, T – temperature, χ – molar fraction, A and B – parent substance, S – solid phase, L – liquid phase, α/β – phase names.

In order to understand the phase diagram above, one has to first imagine a liquified mixture of substances A and B. If our mixture corresponds to either left or right side of the coincidental of the top lines (above the liquid line) it is first in its fully

liquified state (single, liquid A+B phase). Then, when the mixture cools down, and starts overlapping with the liquid (L) + solid (S) α or β phase it is still partially liquified with some of the substrate from the mixture crystallized. Such mixture can then transition into a homogenous, solid α phase or a mixture of solid α and β phases. Composition of both, liquid and solid phases, depends on the ratio of substances A and B as well as the temperature. However, at some ratio of A and B another phase transition can be observed. At this ratio a fully liquified mixture and transition directly into a mixed solid phase α and β , omitting the liquid + solid α or β as well as solid α or β state. Such phenomenon can only occur at strict molar ratio of parent substances. A point at which transition from liquid $\alpha + \beta$ to solid $\alpha + \beta$ occurs is called a eutectic point and the temperature at which it occurs is called a eutectic temperature.

Putting it simply, eutectic mixture is a mixture which is characterized by melting point significantly lower than these of parent substances. This is well reflected in the prime example of DES – reline, a mixture of urea (U) and choline chloride (ChCl) in a molar ratio 2:1. Both exhibit high melting points (MPs) ($MP_U = 132-135^\circ\text{C}^{[164]}$; $MP_{ChCl} = 305^\circ\text{C}^{[165]}$), but when mixed a resulting mixture liquefies at $12^\circ\text{C}^{[150]}$.

Even though, ideal DESs should rely solely on non-covalent bond interactions, some sterical changes may occur. The mechanism of the association of DESs has been investigated by Araujo et al. on the example of reline, using inelastic neutron scattering^[166]. In their study, authors found that both choline chloride and urea undergo sterical changes. The planar sp^2 structure of urea (in crystal form) becomes tetragonal (sp^3), and the chloride anion of choline chloride is slightly displaced, which decreases the steric hindrance of the torsional movement of methyl groups. These two combined effects lead to the formation of the hydrogen bond lattice in the structure of reline. Therefore, it can be presumed that similar behavior is present in other organic DESs (**Figure 9**).

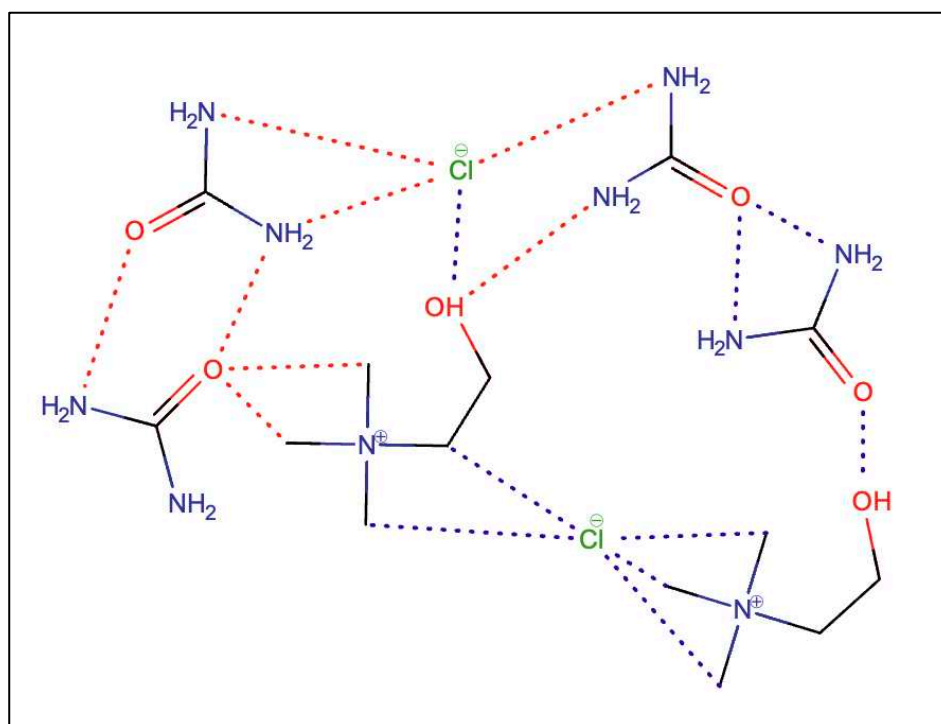


Figure 9. Schematic illustration of the interaction lattice between the molecules of reline (blue lines indicate bonds present in solid PSs; red lines indicate bonds present also in reline clusters) (adapted)^[166].

What must be considered in the structural nature of DES is the way we describe the molecule of the DES itself. In 2021, Zhang et al. raised an interesting question – “Should deep eutectic solvents be treated as a mixture of two components or as a pseudo-component?”^[167]. In the above-referenced work, the authors discussed how we should describe the molecular character of DESs. On the molecular level, are DESs mixtures of two components or should we perceive the molecules that build the DESs (and interact in the given molar ratio) like a single molecule, despite the lack of covalent bonds between parent substances? This factor is essential as it allows us to describe the molecular weight (M_w) as either $M_{w(DES)} = f_{(PS1)}M_{w(PS1)} + f_{(PS2)}M_{w(PS2)}$ (where f is a molar fraction, $\sum f = 1$) or as $M_{w(DES)} = y M_{w(PS1)} + x M_{w(PS2)}$ (where x and y are molar coefficients). Based on the viscosity, density, and spectral data of diluted choline chloride-based DESs, they concluded that DESs exhibit features that reflect their pseudo-component nature. This is important for the proper description of the DESs interaction and concentration.

The liquefaction of DESs mixture, is a direct result of the emergence of the hydrogen bond lattice. Hydrogen bonds are a weak type of bonds, which rarely reaches more than 40 kJ/mol. When compared to other types of bonds, such as ionic or covalent, hydrogen bonds are one of the weakest bonds, tied only with London dispersion forces (one of the van der Waals interactions). However, due to a possibility of their occurrence in a great number at the same time between the molecules, their presence can pronounce in a macro scale. Also, since no covalent bonds should form in ideal DESs, the formation of DESs, should not be described as a reaction, but rather an association.

Major phenomena accounted to the formation of hydrogen bonds are the existence of the liquid state of the matter (intermolecular hydrogen bond) and molecular stabilization (via intramolecular coupled with electron resonance). Interestingly, though, the sole presence of hydrogen bond does not guarantee that a given substance will be a liquid in the close-to-room temperatures. There are couple of factors that influence liquid formation and/or melting point. Apart from the presence and number of hydrogen bonds, these are the local geometry and molecular mass of the molecules. These factors are the main limiters when it comes to the association of DES. Even though, the number of possible combinations may seem limited only by the number of HBAs/HBDs, the formation of DESs is in fact limited and sometimes hard to predict.

There are many advantages associated with DESs. Apart from their application versatility, they are also considered to be green solvents. This is, however, partially true. Indeed, many DESs include environmentally friendly components, resulting in a considerably less harmful impact on the environment in comparison to other substances like chlorinated organic solvents. However, due to a multitude of the combinations of PSs, one cannot generalize and must analyze environmental impact of every single DES separately. Then, DES are a cost-effective substitute and are much easier to synthesize than commonly used solvents. That seems to be, in fact, true. A lot of substances used as PSs are widely available compounds,

such as sugars, amino acids, and alcohols (including polyols). There are also a lot of different cost-effective synthesis methods that can be used for the formulation of DESs. The most common one is a heating of parent substances. If one avoids crossing the decomposition temperature, then a synthesis has the overall efficiency close to 100% as no side products should appear. However, as there are in fact some evidence reporting a reaction between PSs other techniques might be used as an alternative^[168]. Here, one of the most common ones is the solubilization of PSs in water, followed by vacuum evaporation. That way, one can avoid increasing the temperature too much, which may prevent the unwanted reaction. Another, mechanochemistry approach, might be grinding and/or milling. Violent mixing increases the ratio of contact between the molecules of PSs, resulting in local formation of DESs which slowly progresses over time. Here, though, many factors must be considered. Slower grinding will result in less quantity of the DESs obtained in each time, however, it will also generate less heat. Increased milling speed will save time and is a very good approach if we expect no reaction or decomposition. Last technique adapted for the synthesis of DESs is sonication, during which cavitation forces locally heat up the sample present in a water/sonic bath. All the techniques except water dissolution can be also performed without the access of the water in the atmosphere of inert gas. This is a useful advantage if the presence of water is highly unwanted.

1.2.2 Current Categorization of DESs

DESs can be categorized in various ways, depending on the principles adopted. In one of the first classifications, based on the work by Abbott et al.^[169], four categories of DESs were defined:

- TYPE I: quaternary ammonium salt + metal chloride
- TYPE II: quaternary ammonium salt + metal chloride hydrate
- TYPE III: quaternary ammonium salt + HBD
- TYPE IV: metal chloride hydrate + HBD

Alongside this classification, other terms describing families of DESs, were coined, based on the origin of the PSs used and/or applications of DESs.

For example, therapeutic deep eutectic solvents (THEDESs) are DESs containing APIs. The formation of DESs either improves the efficacy of certain drugs and/or allows the material to be applied in other forms of therapy. THEDES formulation has many great benefits. One of them is the possibility of the penetration of the biological barriers. However, the most important one is API liquefaction, which helps overcome solubility limitations and polymorphism and improves drug bioavailability.

Some bright examples of THEDESs are mixtures of menthol with either ibuprofen (improvement of transdermal delivery)^[170] and benzoic acid or phenylacetic acid (antimicrobial agents)^[171,172]. Another example of THEDES studied in-depth is a choline chloride and geranic acid mixture. This DES worked effectively as an antimicrobial agent^[173] and improved the transdermal delivery of insulin^[174,175].

Another group of DESs is the previously motioned, NADESs (natural deep eutectic solvents). NADESs may share some applications with THEDESs, but the criteria on which the family of these solvents emerged rely strictly on the PSs used. NADESs are synthesized using naturally occurring biomolecules – carbohydrates, amines, carboxylic acids etc. These molecules are common metabolites; therefore, NADESs are considered the greenest family of DESs. Good examples of this type of DESs are mixtures of glucose and sucrose as well as ChCl and maleic acid, which were, for example, used to improve the solubility of curcumin in photodynamic therapy^[176]. NADESs can also be very good solubilizers. For example, NADES comprising malic acid, water, proline, and lactic acid enhanced the solubility of berberine (anti-inflammatory and antibacterial drug) almost ten times (25 mg/mL) when compared to water (2.10 mg/mL) or ethanol (2.75 mg/mL).

Deep eutectic solvent derivatives (DESDs) are another class of DESs. The definition of this group is not quite clear; however, a common feature of such mixtures is the

lack of sharp eutectic points. Usually, such mixtures are ternary systems – like a mixture of choline chloride, glycolic acid, and oxalic acid^[177]. However, it is worth noting that not much data is available about this group – especially regarding the proper physicochemical measurements that would allow us to verify the existence of a eutectic point.

As the above-mentioned classification systems are quite general, many DESs may (and usually do) overlap some of the definitions. Moreover, based on these systems, we cannot predict the biological and physical properties of DESs. This is where the categorization and analysis of DESs based on the type of PS used for their formulation might be helpful, as the properties of DESs depend on the PSs used.

The study, application and categorization of DES is a very active field, as witnessed by the increasing number of reports over the last few years. Interestingly, many of the proposed DESs are composed of terpenes, alcohols, polyols, amines, carbohydrates, and carboxylic acids, with the latter being particularly interesting due to their biological, chemical, and physical properties (**Figure 10**).

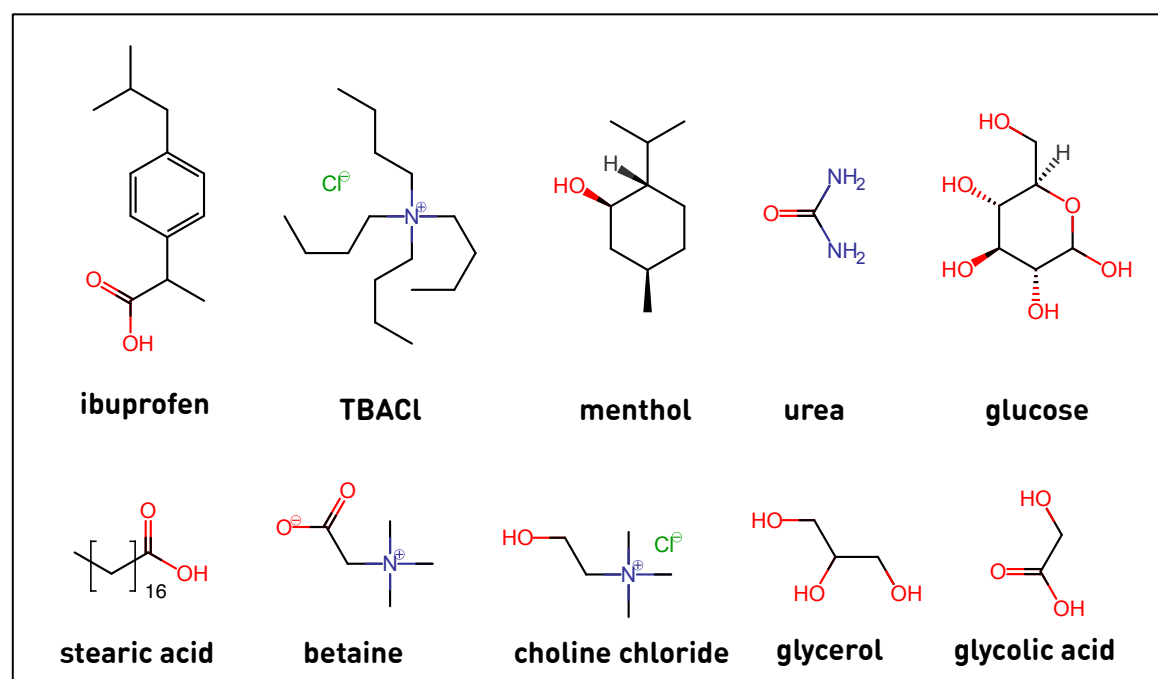


Figure 10. Examples of PSs used for the synthesis of DESs (TBACl – tetrabutylammonium chloride).

This covers either carboxylic, (poly)carboxylic and/or fatty acids. The use of organic acids seems to be a very promising strategy, as carboxylic groups are easily involved in formation of hydrogen bonds, which lies at the root of the association of DESs. In addition, organic acids are already applied in medicine as drugs or antibacterial agents. Thus, it seems appealing to formulate them in the form of DESs and further investigate their properties.

1.2.3 Applications of Organic Acid-Based DESs in Biomedicine

1.2.3.1 *Organic Acids and Their Biomedical Properties*

Carboxylic acids are a group of substances that exhibit many interesting biochemical and physicochemical properties. The presence of a carboxylic group endows the molecule with hydrophilicity. The pK_a of carboxylic groups of most of the organic acids is between 3.5 to 4.5; this means that the majority of these molecules will be in their deprotonated (ionized) form in biological media^[178].

They usually bind to specific proteins/receptors, such as dopamine beta-hydroxylase (ascorbic acid^[179]) or NimA-related protein (lactic acid^[180]). Still, they are also involved in metabolic processes such as the Krebs cycle. A prime example of the interaction of organic acids with proteins is isobutylphenylpropionic acid, also known as ibuprofen (IBU). Its mechanism of action relies mainly on the reversible inhibition of COX-1 and COX-2 enzymes^[181]. This widely used drug can migrate into the cell thanks to its partial solubility in lipids, facilitating its intake. It helps to relieve the pain while counteracting fever and inflammation. Ibuprofen is almost entirely metabolized with minimal to no presence in the urine.

Antibacterial properties of organic acids, such as acetic or benzoic acid, are also very intriguing (**Figure 11**). Some organic acids (mainly phenolic) can interact with the lipid layer of bacteria by dissolving in them and changing their charge and permeability^[182,183]. This is due to other indirect effects, such as a change of polarity and/or disruption of the integrity of the cell membrane by forming pores. Also, an indirect interaction with specific proteins occurs on the cell membrane. The cell

membrane disruption leads to ion transport destabilization, resulting in the impairment of adenosine triphosphate generating systems^[184,185].

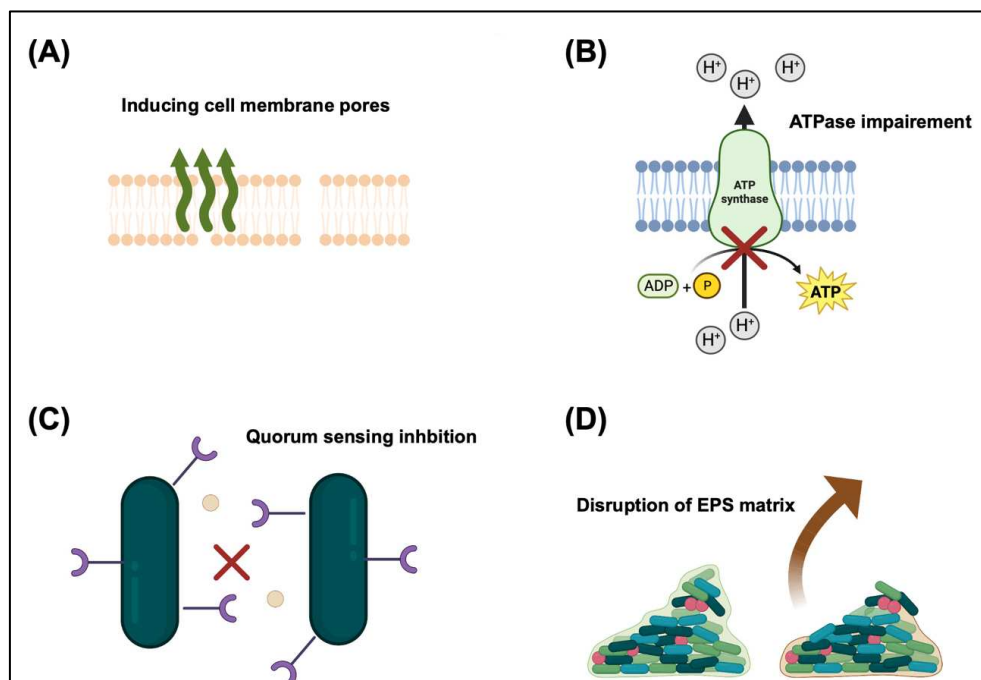


Figure 11. Mechanism of action of carboxylic acids on bacteria: **(A)** disruption of cell membrane structure, **(B)** ionic imbalance resulting in malfunction of various enzymes, **(C)** inhibition of QS pathways, **(D)** biofilm degradation by affecting extracellular polymeric matrix.

Quorum sensing (QS) – a mechanism of bacteria communication during which information about cellular density is exchanged, is of great importance for bacteria's virulence and biofilms' formation. Various organic acids can counteract the growth of bacteria through interaction with QS either by inhibiting the synthesis, functionality, and/or transport of autoinducers or by antagonizing autoinducer receptors^[186,187]. Thanks to this mechanism, adhesion and maturation of biofilm are also hindered^[188].

Due to their structure, which allows hydrogen bond network formation, organic acids can be used as HBDs/HBAs to form organic acid-based deep eutectic solvents (OA-DESS). Such formulations are expected to be, usually, water soluble and combine desired features of organic acids while increasing their permeability

– placing OA-DESs as a class I drug, according to the biopharmaceutical characterization system of drugs (**Figure 12**).

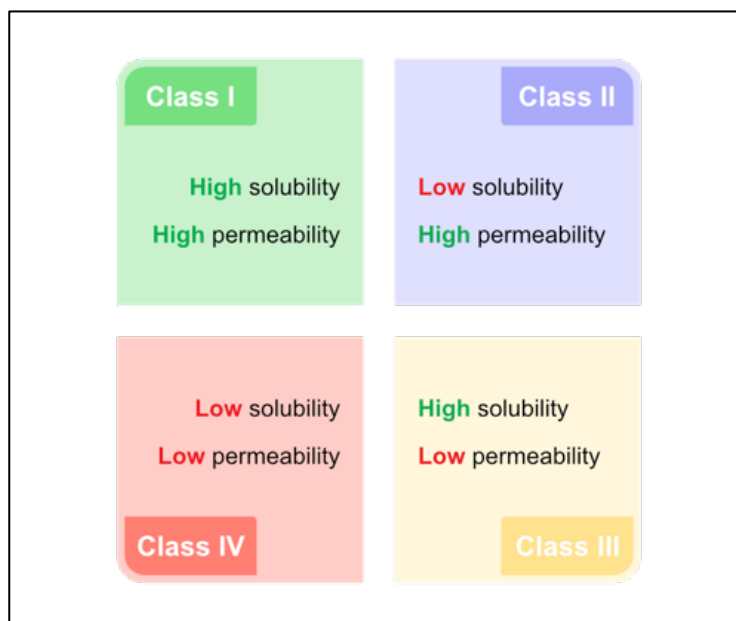


Figure 12. Biopharmaceutical classification system of drugs.

1.2.3.2 *Antibacterial Properties of OA-DESs*

As many organic acids exhibit antibacterial properties, it seems rational to look for ways of improvement of their efficacy through their formulation as DESs. The physical properties of DESs, like their high viscosity and low vapor pressure, could potentially extend their interaction with tissues. DES formulation should also allow OA-DESs, for example, to exhibit a synergistic effect between PSs on bacteria, including biofilms, and the possibility of controlled release of APIs if needed.

A prime example of the antimicrobial properties of OA-DESs was reported in 2016 by Zakrewsky et al.^[173], who investigated a mixture of geranic acid and choline bicarbonate (CAGE) in a 2:1 molar ratio. CAGE has been tested on 47 pathogens, including bacteria, fungi, and viruses (**Figure 13**). It displayed excellent antimicrobial properties while being benign to human cells and tissues. Geranic acid does not mix with water. However, after heating together with water-soluble choline bicarbonate, it could form a stable mixture. CAGE has been shown to have

extremely low MBC of around 0.2–0.5% for *M. tuberculosis*, MRSA, *Propionibacterium acnes*, *Candida albicans*, and *Aspergillus niger*.

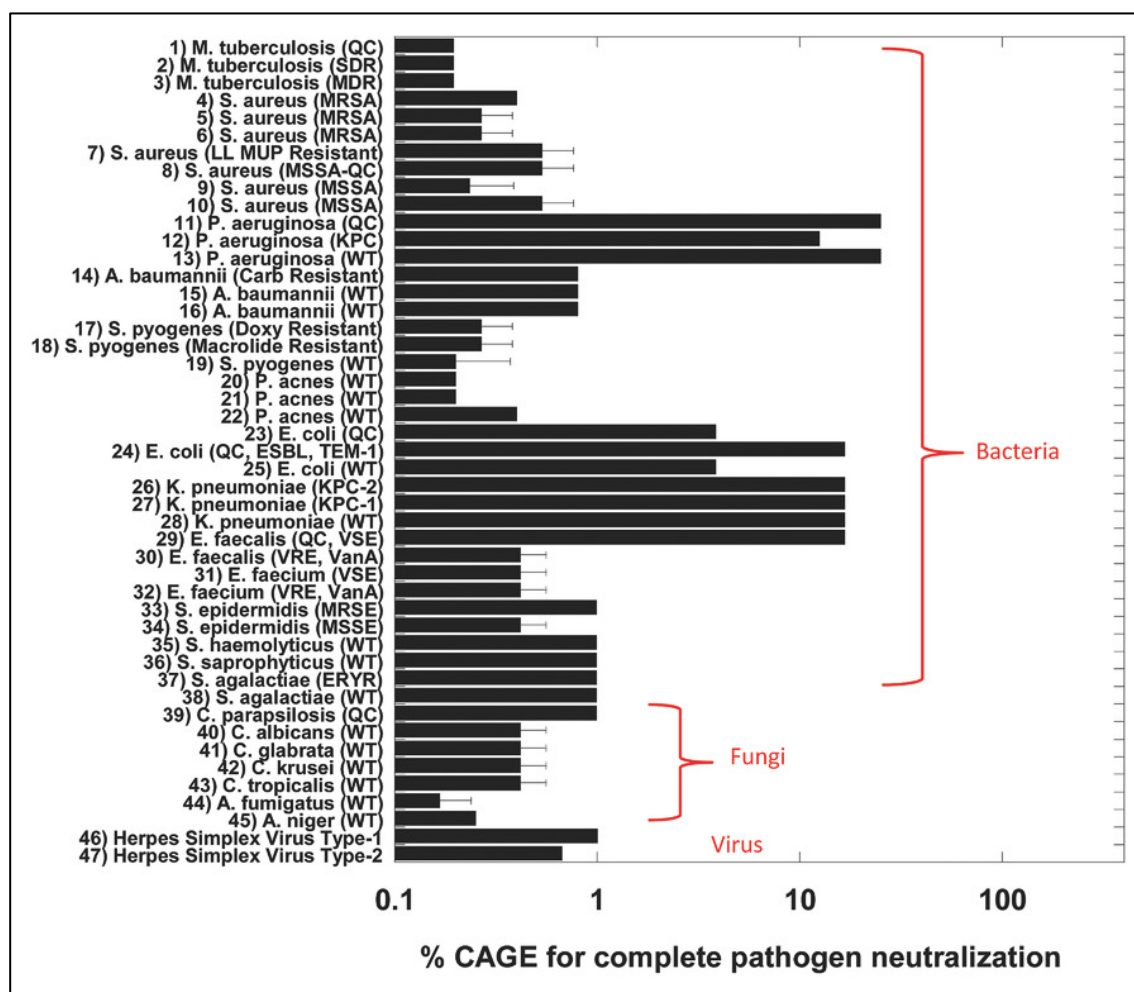


Figure 13. The effectiveness of CAGE OA-DES against 47 pathogens, including bacteria, viruses, and fungi^[173].

In addition, it was also very effective against Herpes simplex virus (HSV) Type-1 and 2. Based on dynamic light scattering analysis, it was suggested that the possible mechanism of bacterial inhibition occurs through their inactivation rather than destruction by OA-DES. CAGE disinfection potential has been also compared with benzalkonium chloride, povidone iodine and chlorhexidine acetate (presurgical sanitizer) for its cytotoxicity against keratinocytes. CAGE maintained almost 100% cell viability up to 10 mM, while referral substances killed practically 100% of the cells at concentrations as low as 0.01 mM. This result highlights that

this OA-DES is an excellent candidate for a new generation of topical partially hydrophobic disinfectants^[173].

Regarding limited hydrophilicity, a fascinating group of organic acids are saturated fatty acids. They share some of the properties with simple carboxylic acids. Still, the presence of saturated carbon chains makes them partially dissolve in lipids, which might be a key to the penetration of the biological membranes. Saturated fatty acids can be used to synthesize DESs; however, during microbiological tests, the factor of diffusion of DESs in the medium must be considered. DESs are liquids, and due to diffusion-related phenomena when compared to solid substances like many antibiotics and disinfectants, they should be investigated by using disk-diffusion assays as well as assessing their minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values. In addition, in the case of fatty acids, which do not dissolve in water, a small amount of dimethyl sulfoxide has to be used to optimize the measurements and improve their solubility while limiting the cytotoxicity towards healthy (human) cells^[188].

Combining fatty acids with APIs is possible and may also result in the formation of THEDESs. As an example, menthol (M), which is a water insoluble, terpene alcohol (0.4 mg/L), is capable of forming stable DESs with stearic (SteA), lauric (LauA) and myristic (MyrA) acids^[189]. Further investigation revealed that a mixture of M:SteA exhibited the lowest cytotoxicity towards HaCaT cells. Stearic acid displayed no MIC/MBC due to a poor water solubility, while MIC and MBC values of menthol were between 4–8 and 8–16 mM, respectively (depending on the strain investigated (*S. aureus*, MRSA, methicillin-resistant *S. epidermidis* (MRSE))). After solubilization in the form of DESs, the MIC decreased to around 3.52–6.52 mM and MBC to 6.52–13.03 mM. Interestingly, DESs of M: SteA have been proven to boost the wound healing process by increasing the recovery of wound area by around 20% compared to control.

The same fatty acids, mixed as binary OA-DESs with capric acid (CapA), have been tested for bacterial eradication and biofilm removal potential^[190]. While none of the OA-DESs inhibited the growth of Gram-negative *E. coli* and *P. aeruginosa*, they were effective against Gram-positive *S. aureus*, MRSE and yeast strain of *C. albicans*.

Fatty acids seem to be very effective against Gram-positive bacteria. However, most dermal infections are caused by the formation of biofilms composed of Gram-negative and Gram-positive bacteria. Therefore, to increase the effectiveness of OA-DESs, alternative approaches have been studied. Following the idea of CAGE, where much smaller molecules are used, formulations that comprised menthol, mixed with phenylacetic (PhaA), acetylsalicylic (AcA) or benzoic (BenzA) acids have been studied as well^[172]. These substances have been proven effective antimicrobial agents^[191–196]; thus, their DESs formulations have been assessed against *E. coli*, *S. aureus* and *B. subtilis*. In their approach, Aroso et al.^[172] used the above-mentioned APIs in the formulation of OA-DESs. The effectiveness of these APIs has been boosted by the addition of menthol. All the formulations have been effective against all the tested strains with MIC as low as 1 mg/mL for *B. subtilis* and as high as 4 mg/mL for Gram-negative *E. coli*.

In the case of DESDs, ternary systems comprising organic acids have also been tested. One of the examples is the OA-DES of betaine (B), malic acid (MaA) and glucose (G) or proline (P) in a 1:1:1 ratio^[197] (**Table 3**). Betaine acts as an HBA, while malic acid and glucose or proline are HBDs. This mixture seems to be an excellent formulation due to the use of small metabolites expected to exhibit low cytotoxicity and good biocompatibility. The formulations were effective against *E. coli*, *P. mirabilis*, *S. typhimurium*, *P. aeruginosa* and *S. aureus*. In the same study, the authors demonstrated that OA-DESs that comprised citric acid (together with glucose or fructose and glycerol) were even more effective towards the same strains of bacteria. It also seems that using small metabolites as PSs affected the EC₅₀ values towards HeLa, HEK293T, and MCF-7 cells with values over 2 mg/mL.

The joint effect of high antibacterial potential with low cytotoxicity makes such combinations even more promising materials, which may be investigated in the near future.

Table 3. Antibacterial activity (and minimum inhibitory values (MIC)) of chosen OA-DESs against selected bacteria and yeast.

	Gram-negative				Gram-positive					Yeast	Ref.
	<i>S. typhimurium</i>	<i>P. aeruginosa</i>	<i>P. mirabilis</i>	<i>E. coli</i>	<i>B. subtilis</i>	<i>S. aureus</i>	MRSA	MRSE	<i>P. acnes</i>	<i>C. albicans</i>	
CapA:LauA	N/I	-	N/I	-	N/I	+	+	+	N/I	+	[190]
CapA:MyrA	N/I	-	N/I	-	N/I	+	+	+	N/I	+	[190]
CapA:SteA	N/I	-	N/I	-	N/I	+	+	+	N/I	+	[190]
M:SteA	N/I	N/I	N/I	N/I	N/I	+	+	+	N/I	N/I	[189]
CAGE	N/I	+	+	+	N/I	N/I	+	+	+	+	[173]
B:MaIA:P	+	+	+	+	N/I	+	N/I	N/I	N/I	N/I	[197]
B:MaIA:G	+	+	+	+	N/I	+	N/I	N/I	N/I	N/I	[197]
M:Benza	N/I	N/I	N/I	+	+	+	N/I	N/I	N/I	N/I	[172]
M:PhaA	N/I	N/I	N/I	+	+	+	N/I	N/I	N/I	N/I	[172]
M:AcA	N/I	N/I	N/I	+	+	+	N/I	N/I	N/I	N/I	[172]
ChCl:OxA	+	+	+	+	N/I	+	N/I	N/I	N/I	+	[172]
reline	+	+	+	+	N/I	+	N/I	N/I	N/I	N/I	[172]
CitA:P	+	+	+	+	N/I	+	N/I	N/I	N/I	N/I	[172]
CitA:Gly:G	+	+	+	+	N/I	+	N/I	N/I	N/I	N/I	[172]
CitA:F:Gly	+	+	+	+	N/I	+	N/I	N/I	N/I	N/I	[172]
CapA:M:solutol	N/I	-	N/I	+	+	+	N/I	N/I	N/I	+	[198]

CapA:M	N/I	N/I	N/I	+	N/I	+	N/I	N/I	N/I	N/I	[199]
CapA:Thymol	N/I	N/I	N/I	+	N/I	+	N/I	N/I	N/I	N/I	[199]

“+” indicates strain susceptible to OA-DES; “-” indicates strain non-susceptible to OA-DES; “N/I” indicates strain not investigated for susceptibility; *S. typhimurium* stands for *Salmonella typhimurium*, *P. mirabilis* stands for *Proteus mirabilis*.

Based on the examples above, we can state that OA-DESs own great potential as active antimicrobial agents. Not only do they manage to kill Gram-positive effectively but can also target Gram-negative bacteria. The latter is particularly interesting due to another double-lipid membrane and periplasmic space, which inhibit most substances from penetrating their cytoplasm. Also, as evidenced for fatty acid-based OA-DESs, particular specificity may be reached based on the presence of the Gram layer. OA-DESs have proven to be also effective against MRSA, MRSE and HSV – pathogens being hard to neutralize. This makes OA-DESs a promising alternative to small-molecule antibiotics. Even though not every reported OA-DES is soluble in water (i.e., ones that comprised menthol) – they can also be applied topically for the potential removal of biofilms, which should enhance the process of wound healing. Overall, OA-DESs are a group of materials which will be developed further in this direction, even though more testing for their cytotoxicity towards healthy human cells is required. They act as antibiotics while also having protein extraction potential, which is rarely tested ex vivo.

1.2.4 Drug Delivery Using OA-DESs

Another valuable aspect of (bio)medicine is drug delivery. However, drug delivery often encounters the problem of low bioavailability, which could be due to several factors:

- route of administration,
- permeability,
- solubility,
- stability.

There are different ways of tackling these challenges. In general, one can either change the route of administration (if possible), chemically modify the drug to enhance its solubility, and/or encapsulate it to improve its stability. Different strategies have been proposed over the recent years, but the general target is the same, i.e., achieve the lowest possible final drug concentration, which still ensures its effectiveness. That is why technologies targeted at improving drug permeability while increasing solubility and maintaining stability are crucial to resolving this hurdle. Here, OA-DESs might be a handy tool to ensure all these aspects. As OA-DESs function as microenvironments, they provide an elastic hydrogen bond lattice able to stabilize most of the molecules. Furthermore, depending on the composition of OA-DESs, one can alter the hydrophilicity of the solvent, enabling more significant dissolution of drugs that poorly dissolve in water. The high viscosity of OA-DESs also limits the contact of the API with oxygen, which prevents it from atmospheric oxidation.

The first ever work on the use of DESs in the delivery of API was reported as early as 1998 by Stott et al. The authors proposed OA-DESs, composed of ibuprofen and terpenes: LD-menthol, L-menthol, thymol, and 1,8-cineole^[170]. By measuring the flux of ibuprofen, they found that all the investigated terpenes enhanced the flux of ibuprofen, with thymol-driven enhancement by 11 times. This work sparked interest in transdermal delivery enhancement by DESs – mainly, that organic acids seemed to be excellent HBDs.

Following that, transdermal delivery of ibuprofen using OA-DES was reported also in 2015 by Park and Prausnitz^[200]. Their study demonstrated the effectiveness of ibuprofen/lidocaine DES in vivo as an anesthetic. In addition, they found out that the solubilization of lidocaine with ibuprofen affected its permeability and increased the absorption of lidocaine in rat models. Another example of the application of ibuprofen as PS of OA-DES was described in 2019 by Pereira et al.^[201]. They have investigated four formulations of limonene with menthol, capric acid or ibuprofen. All the OA-DESs were effective antiproliferative agents, even though

only OA-DES of ibuprofen and limonene (in 1:4 ratio) inhibited HT29 proliferation without comprising cell viability, inferring that this formulation is a potential anti-cancer drug and similar formulations may be developed soon, either as a direct use as a drug or as a medium for synthesis of other drugs^[202].

In the case of drug delivery, non-steroidal anti-inflammatory drugs (NSAIDs) are also of particular interest. Such drugs as ibuprofen, paracetamol or naproxen have poor solubility in water, and thus their effect may be inhibited when administered in aqueous solutions. Here, OA-DESs may be a viable means, as they can improve the solubility of different substances. Formulations proposed by Lu et al.^[203] seem to be promising. Mixtures of different HBAs/HBDs proved very effective when solubilizing NSAIDs. Among the 18 tested formulations, 10 of them comprised organic acids. For HBAs, choline chloride, choline bitartrate (ChBT), betaine, tetrapropylammonium bromide (TPAB) and ethylammonium chloride (EACl) were investigated. At the same time, malonic (MaloA), oxalic (OxA), levulinic (LevuA), lactic (LacA), glutaric (GluA), and glycolic acid (GlycA) have been tested. Stable OA-DESs have been assessed as solvents for five NSAIDs: aspirin (ASP), naproxen (NAP), ibuprofen, ketoprofen (KET) and paracetamol (PAR) (**Table 4**). The results were impressive – thanks to the use of OA-DESs, the solubility of aspirin and paracetamol was increased 5 to 20 times. As for the poorly soluble drugs (ibuprofen/naproxen/ketoprofen), OA-DESs improved their solubility by 4000 times. As the type of HBD seems to play a significant role in the solubility of API, HBA will also affect this. A comparison of OA-DESs comprising levulinic acid revealed that switching ChCl for other HBA improved the solubility of all the drugs, especially those poorly dissolving in water.

It is worth noting here that OA-DESs have also been applied as effective antibiotic carriers. In their original work, Zakrewsky et al.^[173] used CAGE to treat ear infections in vivo on a rat model. When compared with saline, CAGE itself was much more effective than 1% solution of clindamycin. However, combining the two reduced rat ear thickness by ca. 70% after four days, compared to the eight days

required for pure CAGE. This indicates that CAGE successfully boosted the antibacterial effect of the antibiotic by improving its transdermal delivery.

Table 4. Solubility of chosen NSAIDs in water and examined OA-DESS^[203].

Solvent	NSAID									
	f	IBU	f	NAP	f	KET	f	PAR	f	ASP
Water	-	0.07 ± 0.00	-	0.06 ± 0.00	-	0.34 ± 0.00	-	19.95 ± 0.12	-	7.03 ± 0.03
ChCl:GluA	363	25.38 ± 0.20	351	21.04 ± 0.33	177	60.32 ± 0.42	12	238.10 ± 3.73	11	76.28 ± 0.61
ChCl:GlycA	136	9.49 ± 0.05	139	8.37 ± 0.09	65	22.09 ± 0.19	11	224.80 ± 3.02	10	67.43 ± 1.42
ChCl:MaloA	370	25.87 ± 0.13	366	21.98 ± 0.21	180	60.99 ± 0.31	13	266.3 ± 2.44	18	126.30 ± 2.06
ChCl:OxA	450	31.51 ± 0.20	232	13.90 ± 0.13	25	8.53 ± 0.21	9	186.70 ± 1.49	12	86.32 ± 1.16
ChCl:LevuA	112 4	78.69 ± 0.93	528	31.67 ± 0.40	567	192.90 ± 3.62	14	279.00 ± 3.20	19	135.00 ± 0.19
ChCl:LacA	256	17.95 ± 0.2	216	12.95 ± 0.07	114	38.88 ± 0.53	13	254.00 ± 1.84	13	91.30 ± 1.83
EACl:LacA	295	20.67 ± 0.47	60	3.58 ± 0.04	95	32.24 ± 0.18	7	141.20 ± 0.73	6	44.57 ± 0.71
TPAB:LevuA	278 3	194.80 ± 1.57	215 2	129.10 ± 0.83	882	299.80 ± 3.33	16	316.50 ± 1.94	23	163.70 ± 2.30
B:LevuA	400 3	280.20 ± 2.68	127 7	76.64 ± 0.72	968	329.10 ± 4.42	11	212.10 ± 1.92	21	149.80 ± 0.96
ChBT:LevuA	501	35.05 ± 0.42	157	9.45 ± 0.11	150	51.02 ± 0.64	4	89.07 ± 1.04	5	37.06 ± 0.51

"f" indicates dissolution improvement factor (f-times increase, rounded up/down) of a drug in OA-DESSs compared to water.

However, the inner dissolution of substances is also an important criterion when it comes to the synthesis of DESSs. Based on the work of Aroso et al., the dissolution

efficiency of three APIs – PhaA, AcA and BenzA increased when combined with menthol to form OA-DESs. PhaA, AcA and BenzA have been previously investigated for their antimicrobial properties and proved to be effective agents against fungi and bacteria^[204–206]. Interestingly, MIC and MBC values of the tested DESs were higher than those of pure PSs. This indicates that, even though the dissolution of the APIs has increased, their effect has been slightly hindered. In contrast, similar studies by Duarte et al.^[171] showed that OA-DES formulations of ibuprofen, benzoic acid and phenylacetic acid with menthol had much better permeability and diffusion coefficient when compared to neat parent substances (**Table 5**).

Table 5. Dissolution efficiency of AcA, PhaA and BenzA in phosphate buffered saline (PBS) and formulated in OA-DESs^[171,172].

PS/OA-DES	Dissolution Efficiency (%)	Permeability (10^5 cm s^{-1})	Diffusion Coefficient ($10^6 \text{ cm}^2 \text{ s}^{-1}$)	Time* (h)
AcA	11	N/I	N/I	N/I
M:AcA (3:1)	72	N/I	N/I	N/I
BenzA	70	0.9 ± 0.01	0.73 ± 0.09	3.5
M:BenzA (3:1)	87	6.8 ± 0.63	6.43 ± 1.42	0.5
PhaA	37	16 ± 2.3	3.14 ± 0.18	2
M:PhaA (3:1)	81	18 ± 0.38	4.66 ± 0.13	3
M:PhaA (2:1)	78	13 ± 0.59	5.73 ± 0.43	2
IBU	N/I	4.6 ± 0.14	2.39 ± 0.43	>8
M:IBU (3:1)	N/I	14 ± 1.53	4.32 ± 0.34	3

“*” indicates time needed for 50% release of the API through polyethersulfone membrane.

High permeability and a high diffusion coefficient resulted in much lower diffusion times through polyethersulfone membranes, making this type of OA-DESs a very promising delivery system. As the influence of the structure of HBA/HBD was not so often discussed, the authors used NMR to assess how the structure of organic acid affects diffusion and found that the size of the API molecule does not influence its diffusion^[171].

OA-DESs and DESs are good solvents for the dissolution of small molecules and for much bigger, high-molecular-weight biomolecules. Here, one of the first studies conducted in 2011 by Choi et al.^[207] demonstrated that DNA (from male salmon), albumin, and amylase displayed good solubility in certain NADESs, with DNA having 46% higher solubility in OA-DESs consisting of malic acid and proline when compared to water. Following this work, Dai et al.^[208] assessed more than 70 NADESs for solubilizing small biomacromolecules. In the final group of 5 tested NADESs, OA-DES of lactic acid, glucose, and water seemed particularly effective in dissolving gluten (88 times more effective) and DNA (34 times more effective). Moreover, the same OA-DESs enabled the dissolution of starch up to a concentration of 7.5 mM.

Proteins themselves are interesting components in the aspect of their delivery. However, their structure-dependent function relies on many weak interactions, namely hydrogen bonds, hydrophobic interactions, $\pi - \pi$ stacking, and electrostatic interactions (including van der Waals forces)^[209,210]. This makes their function susceptible to environmental changes, often limiting their availability and solubility. Here, OA-DESs, characterized by highly developed hydrogen bond lattice and elasticity of molecules with high coordination potential, might be interesting. Choi et al.^[207] first raised awareness of the role of NADESs, naturally found in living organisms, on the stability of proteins. This hypothesis was re-evaluated in 2013 by Esquembre et al.^[211], in 2015 by Su and Klibanov^[212], and in 2017 by Sanchez-Fernandes et al.^[213] using model proteins and demonstrated that DESs are well-structured and activity preserving solvents.

Parallel to the studies mentioned above, applications of choline-based OA-DESs in the dissolution and transport of certain proteins have been conducted (**Table 6**). In 2018, Tanner et al.^[175] delivered transdermal insulin using CAGE and its variants. Formulations of 1:1, 1:2, 1:4 and 2:1 of CAGE have been investigated for their conductivity and viscosity, thermal stability as well as by NMR to characterize the internal interactions of solvents. In their research, the authors first studied the

interactions of CAGE formulations on the stratum corneum (outermost layer of skin) to characterize the possible transport mechanism.

Table 6. Selected OA-DESs as protein carriers.

OA-DES	Protein	Ref.
CAGE	BVA	[174]
CAGE	OVA	[174]
CGLY	TNF α	[174]
CAGE	INS	[174,175]
CAGE	GLP-1	[214]

“BVA” indicates bovine serum albumin; “OVA” indicates ovalbumin; “INS” indicates insulin “GLP-1” indicates glucagon-like peptide-1.

As revealed by FTIR spectroscopy, due to its partial hydrophobicity, CAGE acts as a solvent for lipids, disrupting lipid bilayers. Moreover, the extraction potential strictly depends on the amount of geranic acid in CAGE, with a 1:4 ratio being as effective as the neat geranic acid itself. Interestingly, though the 1:2 and 1:4 mixtures were the most effective in transporting insulin (as verified by confocal microscopy using diffusion Franz cell), the effectiveness of other ratios was comparable to PBS.

Furthermore, OA-DESs have also been used to transport biomolecules such as monoclonal antibodies. These specific antibodies are currently used in the therapy of (among others) cancer^[215–217] and arthritis^[218,219]. They are usually administered via subcutaneous or intravenous injection, but OA-DESs might open a possibility for their delivery via the gastrointestinal tract. In their recent work, Angsantikul et al.^[214] studied the effectiveness of a mixture of choline chloride and glycolic acid (CGLY, in 2:1, 1:1 and 1:2 molar ratios) in the transport of TNF α antibody, focusing on the stability of the antibody in OA-DESs, in vitro transport and in vivo uptake. Interestingly, CGLY in 2:1 and 1:1 ratio, had a negligible effect on the stability of the antibodies, which have retained their high binding activity even at 20% v/v of

saline. At the same time, the 1:2 mixture inhibited the binding almost completely. CGLY 2:1 and 1:1 did not affect the functionality of TNF α when stored at 4°C; however, the 2:1 ratio inhibited the binding ability of the antibody when stored at room temperature. Cell viability on the Caco-2 cell line resulted in ratio-dependent IC₅₀ – the smaller the fraction of choline bicarbonate, the lower the IC₅₀. The ratio of 2:1 turned out to be the best candidate and was tested further. The results have underlined that CGLY 2:1 was an excellent transporter of the antibody, exerting no adverse effect on the rat model. Interestingly, thanks to its micro-extraction potential, this OA-DES decreased the viscosity of mucus, which enhanced the antibody uptake.

Looking at all the examples above, we can clearly state that OA-DESs significantly impact the dissolution of drugs. Not only do they improve the solubility of small molecular drugs, but they can also act as APIs themselves. Owing to their physicochemical properties, they seem to be an excellent environment for the dissolution of high molecular weight molecules such as proteins and peptides – a challenge we still face today.

It must be pointed out that even though using OA-DESs increases the solubility of certain APIs, reaching therapeutic concentrations is still a challenge. Here again, we must look at the main factors limiting drug bioavailability. If by implementing OA-DESs, we can increase the solubility and stability of the API for certain routes of administration. More drugs will reach the target. If more drugs will be delivered using OA-DESs compared to other administration strategies, then adaptation of OA-DESs is beneficial and a frog leap in this field. Hopefully, in the following years, we will see a clear development in this topic and, finally, a successful application in vivo.

1.2.5 Drug Solubilization Using Conventional DESs

DESs have already been implemented in many fields of chemistry^[159,163]. Interestingly, DESs have also found their use in (bio)medicine as-well. They have

been proved to exhibit antimicrobial properties^[189,190,197]. Due to their capability of forming vast hydrogen networks, DESs' potential use as cosolvents for proteins was also studied. A really good insight in this field was reported in the review published in 2022 by Yadav and Venkatesu^[220]. In fact, since the introduction of DESs, numerous studies have highlighted their significance as solvent media for preserving macromolecules and promoting protein stabilization due to their distinctive characteristics. DESs can steer the microenvironment of biomolecules, and a set of good examples has been provided by Juneidi et al.^[221], Sanchez-Fernandez et al.^[213], and Toledo et al.^[222]. In the first one, a glyceline was able to enhance the water-enzyme interactions, which resulted in improved esterification reactions. Toledos' group proved that the increase in hydrogen bonding enhanced the enzyme activation. Fernandez et al. proposed that pure DES replaces water around amino acids in the active site, fostering a partially folded conformation as the addition of water leads to a hydration shell formation, facilitating protein folding. Sun et al.^[223] noted that DES-induced hydrogen bond networks can serve as a protective layer, stabilizing proteins against ion interference. Building on these findings, recent research showcased a protein stabilization mechanism through the formation of a hydration shell around the active site, enhancing structural and thermal stability. An interesting work in this topic was also published in 2020 by Yadav et al.^[224]. In their work, the authors have investigated the influence of glyceline, a DES composed of choline chloride and glycerol and reline, a DES composed of choline chloride and urea, on the biological and physical properties of alfa-chymotrypsin, a key digestive enzyme produced by the pancreas. Not only both the DES improved the thermal stability of the protein, but also enabled it to maintain its activity and structure. In the latter scenario, the enzyme's activity was effectively maintained despite the presence of urea, which typically has a negative impact. This was achieved through the formulation as DES, which successfully altered the properties of urea. DESs influence on the antibiotic stability was also investigated. In a recent work of Olivarez et al.^[225], a classical DES

composed by betaine and urea was tested as a cosolvent for two antibiotics from the family of beta-lactams. It was found that the antibiotics maintained their structure and activity and were able to prolongate the activity of imipenem, when compared to the case where water was used as a solvent.

1.3 Use of Microwave Sensing as a Novel Approach in Medicine

DEs represent a neoteric class of materials that is still in its early stages of integration into (bio)medicine. Embracing interdisciplinary approaches, particularly in diagnostics, therapy, and theranostics, is prudent. While X-ray imaging stands as a cornerstone in medical diagnostics thanks to its spatial resolution directly related to the irradiation wavelength, employing high-energy radiation to penetrate the human body and generate images through interactions with tissues, its ionizing nature, with frequencies averaging at 10^{18} Hz and energies up to 10 keV^[226], necessitates strict control to mitigate potential health risks, especially in cancer radiotherapy. To address these concerns, alternative forms of radiation, such as those utilized in magnetic resonance imaging (MRI), gain traction. MRI employs a fluctuating magnetic field to penetrate the body, typically in the frequency range of tens to hundreds of Hz, providing a safer imaging alternative^[227]. In an MRI machine, the strong magnetic field aligns the spins of nuclei (mainly protons). When radiofrequency pulses are applied, they can temporarily disturb this alignment. When the pulses stop, the nuclei return to their aligned state, emitting radiofrequency signals as they do so^[227]. By measuring these signals, detailed images of the internal structures of the body can be determined. MRI, while invaluable in medical diagnostics, presents several drawbacks. Its high cost and limited availability can restrict patient access, and the time required for scans can result in scheduling challenges. Additionally, patient discomfort, especially for claustrophobic individuals, and potential risks associated with contrast agents pose concerns. MRI may not be suitable for patients with metallic implants, and its loud scanning noise can be unsettling.

For some time now, another type of irradiation is studied for its direct implementation in medicine – that is microwave irradiation. This type of non-ionizing irradiation, usually in 10^6 - 10^{10} Hz range also presents an energy below 10-4 eV seems to be a perfect candidate for a novel technique for medical imaging and therapy^[228].

Microwaves, exhibit unique interactions with various materials, characterized by measured features such as *S*-parameters from which complex permittivity of the material under investigation can be deduced^[229]. These interactions play a crucial role in numerous fields, ranging from telecommunications^[230] to material processing^[231] and biomedicine^[232].

S-parameters, also known as scattering parameters, are fundamental quantities for describing how microwave signals interact with devices or materials. These parameters encompass both magnitude and phase information, providing a comprehensive understanding of signal behavior. In the context of material interaction, *S*-parameters offer insights into how microwaves are absorbed, reflected, or transmitted by a given material. By analyzing changes in *S*-parameter values after a calibration procedure, one can deduce material properties, such as dielectric constant and dielectric loss, crucial for applications like non-destructive testing and microwave imaging. State-of-the-art techniques for determining material dielectric properties, especially in biology and chemistry, include vector network analyzers (VNAs), time-domain reflectometry (TDR), resonant cavity methods, and open-ended coaxial probes^[233–235]. VNAs are widely used for their precision in measuring *S*-parameters over a broad frequency range, allowing for detailed characterization of complex permittivity. TDR provides high-resolution temporal and spatial information, making it suitable for heterogeneous and layered materials. Resonant cavity methods offer high sensitivity and accuracy for small sample volumes, making them ideal for biological samples and chemical compounds. Open-ended coaxial probes are particularly useful for in vivo and in vitro measurements due to their non-destructive nature and ease of use in various

environments such as liquids. These advanced techniques are essential for advancing research and applications in fields such as biomedical diagnostics, materials science, and chemical analysis, ensuring accurate and reliable material characterization. Two-port S -parameters, also known as scattering parameters, are fundamental in microwave engineering and play a pivotal role in characterizing the behavior of microwave devices and circuits. They describe how microwave signals propagate between two ports of a device or circuit under test. Each port represents a distinct input or output connection, and the S -parameters quantify the amplitude and phase of the signals at these ports.

For a two-port network (quadrupole), there are four primary S -parameters: S_{11} , S_{12} , S_{21} , and S_{22} ^[236].

- **Reflection coefficients S_{11} (port 1) and S_{22} (port 2).**

These parameters represent the ratio of the reflected wave at port to the incident wave at it. It quantifies how much of the signal is reflected back into the source at a given port.

- **Transmission coefficients S_{21} (from port 1 to port 2) and S_{12} (from port 2 to port 1).**

These parameters describe the ratio of the transmitted wave at one port to the incident wave at the other port. It quantifies how efficiently the signal passes between ports.

These parameters are typically represented in matrix form, allowing for easy analysis of microwave networks using linear algebra techniques. Moreover, S -parameters are often measured using VNAs in experimental setups, providing invaluable insights into the performance and characteristics of microwave devices and circuits.

Complex permittivity, another key concept in microwave-material interaction, characterizes a material's ability to store and dissipate electromagnetic energy under the influence of an electric field. It comprises both real (ϵ' , dielectric

constant) and imaginary (ϵ'' , dielectric loss) components, representing the material's ability to store energy and its inherent lossiness, respectively. Understanding the complex permittivity of a material enables prediction of its microwave behavior, including absorption, reflection, and transmission characteristics. For instance, materials with high dielectric constant and low dielectric loss are more prone to microwave absorption, making them suitable for applications like microwave heating in medical treatments.

In comparing dielectric properties (**Table 7**)^[237], vacuum serves as the baseline with a relative dielectric constant of 1.0 and no dielectric loss, indicating no interaction with electric fields. Air, closely resembling vacuum, has a dielectric constant of about 1.0006 and negligible loss, making it nearly lossless. Water, with a high dielectric constant of 80.1, demonstrates a strong ability to store electric energy, though it also has a relatively high dielectric loss of 0.157, indicating substantial energy dissipation. Glass, with a relative dielectric constant ranging from 3.7 to 10, exhibits very low dielectric losses, highlighting its effectiveness as an insulating material. Polyethylene, used extensively in cable insulation, has a dielectric constant between 2.25 and 2.35 and a very low dielectric loss of about 0.0003, making it an excellent insulator with minimal energy loss. This comparison illustrates the wide range of dielectric properties among these materials, aiding in selecting the right material for specific insulating and electronic applications.

Table 7. Dielectric constant and dielectric loss values of different materials.

Material	Dielectric constant	Dielectric loss
vacuum	1.0	0.0
air	1.0006	~0
water (at 20°C, 1 MHz)	80.1	0.157
glass	3.7-10	~0.005

The interaction between microwaves and materials is influenced by various factors, including frequency, material composition, and geometry^[238]. At certain

frequencies, materials exhibit resonance phenomena, absorbing microwaves more efficiently due to matching their “natural” frequencies. Additionally, the composition of materials, such as dielectric properties and conductivity, significantly impacts microwave interactions. For instance, polar molecules within a material may align with an oscillating electric field, leading to dielectric heating effects observed in microwave ovens^[239]. Furthermore, the geometry and thickness of a material also affect microwave interactions. Thin films or coatings may exhibit different microwave behavior compared to bulk materials due to surface effects, while complex geometries can lead to scattering and multiple reflections, altering the overall interaction profile^[240].

Measurement tools such as VNAs are indispensable in characterizing microwave devices and circuits^[236]. VNAs provide precise measurements of *S*-parameters, enabling engineers to assess the performance and behavior of components across a wide frequency range. Calibration is a critical aspect of VNA measurements, ensuring accuracy by compensating for systematic errors introduced by cables, connectors, and other components in the measurement setup. Various calibration techniques, such as Short-Open-Load-Thru^[241,242] and Automatic Calibration Module^[242], are employed to achieve accurate and reliable measurements.

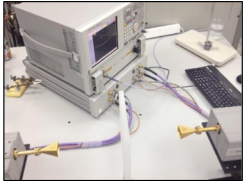
Measurement systems such as VNAs are interfaced with microwave sensors to interact with the material under investigation. VNAs when combined with appropriate sensors, enable detailed characterization and analysis of various materials. A variety of sensors have been introduced, each tailored to the specific requirements of the application. The choice of sensor depends significantly on the form of the material measured—whether it is solid, liquid, or gas—as well as the volume of the material under investigation. Additionally, the level of measurement accuracy and sensitivity required plays a crucial role in sensor selection. For instance, certain applications may demand high precision and sensitivity, necessitating the use of advanced sensors with specialized designs. In contrast, other applications might prioritize larger volume measurements or different

material forms, requiring sensors optimized for those conditions. This versatility in sensor design ensures that VNAs can provide accurate and reliable data across a wide range of applications, making them indispensable tools in both industrial and biomedical fields.

Here, microwave sensors (**Table 8**) play a vital role in a myriad of applications, from industrial processes to biomedical diagnostics. Coaxial probes are commonly used for non-destructive testing of materials, offering the advantage of measuring material properties without damaging the sample. However, their precision and spatial resolution are influenced by the probe's aperture size, which determines the area of interaction with the material. This makes them suitable for applications where large-scale or averaged measurements are sufficient, rather than requiring high spatial resolution. For example, in industrial settings, coaxial probes are effective for monitoring moisture content, detecting impurities, and evaluating dielectric properties of bulk materials. In biomedical diagnostics, they are utilized to assess tissue properties, detect anomalies, and monitor physiological changes non-invasively.

The sensitivity and accuracy of coaxial probes depend on the design and operating frequency, which can be optimized for specific applications. They provide reliable data when used within their design parameters, but for applications requiring fine spatial resolution or high precision, alternative microwave sensor designs, or additional techniques might be necessary. Understanding the limitations and appropriate use cases of coaxial probes ensures that they are effectively integrated into various diagnostic and monitoring systems.

Table 8. Different types of microwave sensor solutions; ©AIP Publishing, Smiths Interconnect, IEEE, Princeton Microwave.

Sensor Type	Broadband Operation	Suited for Low-Loss Materials	Suited for High-Loss Materials	Typical Frequency Range	Ref.
 STRIPLINE	Yes	Yes	Yes	1–50 GHz	[243]
 COAXIAL PROBE	Yes	Yes	Yes	0.1–20 GHz	[244]
 DIELECTRIC RESONATOR	No	Yes	No	2–110 GHz	[245]
 FREE-SPACE MEASUREMENT SYSTEM	Yes	Yes	Yes	8–110 GHz	[246]

Transmission line sensors, such as waveguides and microstrips, offer versatile solutions for monitoring critical parameters like impedance and dielectric properties. These sensors constitute one of the primary methods for material characterization, largely relying on the use of metallic waveguides. The technique is broadband, enabling it to cover a wide range of frequencies, which makes it

highly effective for various applications. However, this method can be somewhat destructive because the material under test must be shaped to fit the dimensions of the waveguide, which can sometimes alter its properties or render it unusable for further testing. Despite their effectiveness, the requirement for the material to conform to the waveguide's dimensions limits their use in some scenarios, particularly when dealing with irregularly shaped samples or delicate materials that cannot be easily modified.

To address these limitations, alternative approaches involving planar transmission lines have been developed^[243,247]. These planar transmission lines, such as microstrip or coplanar waveguides, are loaded with the material under test in a non-destructive manner. This method allows the material to be placed on or integrated with the sensor, preserving its integrity and making the measurement process more flexible and adaptable to different sample forms and sizes. Planar transmission line sensors can be easily integrated into a variety of setups, including on-chip implementations for high-frequency measurements in integrated circuits and other compact electronic systems. These advancements have broadened the applicability of transmission line sensors, making them suitable for a wider range of materials, including liquids, powders, and biological tissues. The development of these non-destructive planar transmission line sensors has significantly enhanced the ability to measure and monitor material properties accurately without compromising the sample, which is particularly important in applications like biomedical diagnostics, food quality control, and advanced material research.

Dielectric resonator sensors exploit the resonance phenomenon to detect changes in material properties, offering high sensitivity and stability in applications such as moisture sensing and chemical detection. Suited for low-loss applications and relatively small volumes of materials, these sensors are particularly effective where precision and minimal signal loss are critical. Technologies from companies like QWED Systems in Poland have significantly advanced the field by

providing innovative solutions that enhance the accuracy and efficiency of material characterization. QWED's products, such as the Split Post Dielectric Resonator are equipped with specialized software for precise measurement of complex permittivity and dielectric loss tangent, making them highly regarded in both industrial and research settings^[248]. These diverse sensor technologies cater to a wide range of industrial, medical, and scientific needs, driving continuous innovation in sensing and measurement techniques.

Microwaves have already found their use in medicine, serving as an effective platform for the detection of breast cancer. Unlike conventional mammography, which employs ionizing radiation, microwave imaging relies on low-power microwave signals, offering a safer alternative. This approach typically comprises a radar-based system, where the radar functions similarly to a VNA. The radar sends out a microwave signal and measures the reflected signal to create an image. The reflected signals vary based on the different dielectric properties of normal and cancerous tissues, allowing for the detection of anomalies. Such systems are exemplified by the work of Wang^[249] and Smith et al.^[250], who have developed radar-based microwave imaging techniques that enhance the early detection and characterization of breast cancer, making this technology a promising tool in medical diagnostics.

These signals interact with breast tissue based on its dielectric properties, which vary between healthy tissue and tumors. Malignant tumors, for instance, often exhibit higher water content and altered cellular structures, leading to distinct dielectric properties. As microwave signals propagate through the breast tissue, they are reflected and scattered by these internal structures, including tumors. By analyzing the amplitude and phase of the reflected signals, sophisticated algorithms can reconstruct a 3D image of the breast, highlighting regions of interest. This approach offers several potential advantages, including improved tissue contrast and the absence of ionizing radiation. Moreover, microwave imaging shows promise in overcoming limitations associated with dense breast

tissue, where traditional mammography may be less effective. While still in the developmental stage, microwave imaging holds the potential to revolutionize breast cancer screening and diagnosis, offering a non-invasive, radiation-free alternative with the potential for early detection and improved patient outcomes.

In the paper of Bulyshev et al.^[251], the authors leverage the differences in dielectric properties between normal and malignant tissues to produce high-resolution, three-dimensional images of the breast. The study utilizes a cylindrical multiview configuration and a modified gradient method for image reconstruction at frequencies of 2, 3.5, and 5 GHz, with 3.5 GHz providing the best balance of image quality and resolution. The enhanced contrast at this frequency is critical for identifying small tumors. These dielectric contrasts are due to the higher water content and cellular density in malignant tissues compared to normal tissues, which affect their permittivity and conductivity. Similarly, in work of Meaney et al.^[252], the authors combine microwave tomography with magnetic resonance imaging (MRI) to exploit MRI's high spatial resolution and MT's dielectric property contrasts. This hybrid approach improves tumor localization and characterization, particularly in dense breast tissues where traditional mammography is less effective. Clinical trials confirm the integrated method's improved diagnostic accuracy by providing comprehensive data on both dielectric properties and anatomical structures.

Further advancements in the field are highlighted in the study of Grzegorzczak et al.^[253]. This study addresses practical challenges like long acquisition and reconstruction times by developing an optimized hardware system capable of acquiring data in under 2 minutes and reconstructing images in less than 20 minutes. The high-resolution images produced by this system, validated through over 400 clinical exams, demonstrate its potential for both screening and monitoring tumor response to chemotherapy. The system measures low-power signals and provides detailed images, crucial for identifying the heterogeneous structure of tumors, which typically have higher water content and cellular density.

Additionally, Meaney et al.^[254], provide the first clinical 3-D microwave tomographic images of the breast, demonstrating the method's ability to distinguish between malignant and benign tissues, track tumor progression, and assess treatment efficacy. These studies collectively highlight the significant strides made in microwave imaging, emphasizing its potential as a non-invasive, accurate, and practical tool for breast cancer detection and monitoring, driven by the biological properties of tumor tissues such as higher water content and increased cellular density.

Microwaves, with their unique interactions characterized by measured S -parameters and complex permittivity, hold immense promise for medical applications, offering non-ionizing and low-energy alternatives for imaging and therapy. Understanding microwave-material interactions, influenced by factors, such as frequency, composition, and geometry, is vital for leveraging their potential in diverse fields. Measurement tools like VNAs, complemented by precise calibration techniques, facilitate accurate characterization of microwave systems, propelling advancements in medical diagnostics and treatments.

2. The Aim of the Research

The research outlined in this thesis encompasses a multifaceted approach towards understanding and harnessing the potential of Deep Eutectic Solvents (DESs) across various domains. The overarching aims of the study are outlined as follows:

- 1. Synthesis, Structural and High Frequency Characterization:** An exploration into the synthesis and physicochemical properties of a selected DESs, including four conventional DESs and five OA-DESs. This involves characterization using polarized optical microscopy (POM), thermogravimetric analysis (TG), Fourier-Transform infrared spectroscopy (FTIR) and microwave spectroscopy (MW).
- 2. Evaluation of Antibacterial Potential:** Conducting thorough assessments using modified disk diffusion test (DD), minimum inhibitory and bactericidal assays (MIC/MBC), time-kill kinetics assay (TK) to gauge the antibacterial efficacy of the DESs under investigation. This involves subjecting them to rigorous testing against a spectrum of bacterial strains to ascertain their potential as antimicrobial agents.
- 3. Investigation of Biofilm Eradication Potential:** Delving into the capacity of DESs to eradicate bacterial biofilms formed by MRSA and *E. coli*, which pose significant challenges in antimicrobial therapy due to their inherent resilience. This aspect involves experimentation using colony forming assay (CF).
- 4. Assessment of Persister Cell Viability:** Exploring the impact of DESs on persister cells, a subpopulation of bacteria known for their heightened tolerance to antibiotics. This involves assessing the viability of persister cells in the presence of DESs to determine their efficacy in targeting these elusive bacterial subgroups.

- 5. Investigation of Mechanisms of Action:** Investigation of possible mechanisms underlying the antibacterial activity of both conventional and OA-DESs using transmission electron microscopy (TEM).
- 6. Adaptation as Co-Drugs:** Exploring the potential of DESs as co-drugs, particularly in combination with antibiotics and antimicrobial peptides, to combat antimicrobial resistance. This involves strategies aimed at synergistically enhancing the efficacy of existing antimicrobial agents through DES-based formulations.
- 7. Sneak-Peek into Antifungal Properties:** A preliminary exploration into the antifungal properties of DESs, thereby broadening the scope of their potential applications beyond antibacterial therapy. This aspect entails initial investigations aimed at assessing the efficacy of DESs against fungal pathogens, laying the groundwork for further exploration in this domain.

Through these comprehensive investigations, this research seeks to not only deepen our understanding of DESs' antimicrobial potential, but also paves the way for their practical applications in combating microbial infections and addressing the challenges posed by antimicrobial resistance.

3. Methodology

3.1 Microbial Strains and their Maintenance

Following bacterial and fungal strains have been investigated during research:

	Strain	Gram	Strain identifier
BACTERIA	<i>Acinetobacter baumannii</i>	-	RUH 134
	<i>Acinetobacter baumannii</i> (carbapenem-resistant, CRAB)	-	clinical isolate
		-	ATCC 25404
		-	ANSES* 289
	<i>Escherichia coli</i>	-	Résapath** 184
		-	KPD 217
	<i>Klebsiella pneumoniae</i>	-	ATCC 13883
	<i>Pseudomonas aeruginosa</i>	-	ATCC 15442
	<i>Pseudomonas fluorescens</i>	-	DSM 50090
	<i>Bacillus cereus</i>	+	ATCC 13061
	<i>Bacillus subtilis</i>	+	DSM 23778
	<i>Enterococcus faecalis</i>	+	ATCC 19433
	<i>Staphylococcus aureus</i> (methicillin-resistant, MRSA)	+	ATCC 43300
	<i>Staphylococcus aureus</i> (multidrug-resistant, MDRSA)	+	clinical isolate
FUNGI	<i>Staphylococcus epidermidis</i>	+	DSM 20044
	<i>Staphylococcus hominis</i>	+	KPD 910
	<i>Aspergillus fumigatus</i>		ATCC 204305
	<i>Candida albicans</i>		ATCC SC5314
	<i>Candida auris</i>		CHU 1.4.4.1.69

All the bacteria have been acquired either directly from the supplier or from the collaborators from the University of Gdansk, Polytech Lille, and University Ferhat Abbes Setif1, and were not subjected to further transfection. All the strains have been kept either on the Mueller-Hinton agar (MHA) or Luria-Bertani agar (Miller). The initial plates have been inoculated directly from glycerol stock colonies and have been prolonged sequentially onto fresh agar plates. Every freshly inoculated plate has been incubated at 35°C until discrete colonies were observed. Such plates were then placed in fridge set to 4°C and stored for a maximum duration of 4 weeks.

Fungal strains have been cultured on Sabouraud agar medium and then in Sabouraud dextrose broth on a rotary shaker for 18 h at 37°C. The fungal cultures obtained were then centrifuged at 600 × g for 3 minutes and washed in PBS.

All the culture media were supplied by both Sigma-Aldrich® and Beckton Dickinson® and were used interchangeably after assessing their effectiveness. Liquid media cover Muller-Hinton broth (MHB), Luria-Bertani broth (LBB), and Tryptic soy broth (TSB).

All the manipulations were done in BSL2 class laboratory in the microbiological cabinet.

3.2 Chemicals

All the chemicals were supplied by either Sigma-Aldrich® (Merck), Fischer Thermo Scientific® or Honeywell Fluka™ and have been used without any further purification. When needed, ultrapure water (type I) was used directly from the Sartorius Arium® Pro Ultrapure Water Systems.

3.3 Techniques

3.3.1 Synthesis of DESs

The process for synthesizing all the DESs involved heating premixed parent substances PSs in test tubes in a given molar ratio. This was performed in a

heating block without stirring. The temperature was gradually increased from 40 to 120°C, with occasional stirring using a vortex stirrer. Once the test tube content completely melted at a certain temperature, the heating was ceased, and the sample was allowed to cool down to room temperature in the heating block. The DESs samples were stored in glass test tubes with a specially designed 3D-printed insert containing a neutral drying agent (silica gel beads with an indicator, Merck®) to ensure the absence of water.

3.3.2 Density Assessment

Densities of all the DESs were assessed using direct weighing method using XPR6UD5 MettlerToledo® microbalance. First 900 µL of ultrapure water was added to a sterile Eppendorf® tube to ensure good solubilization of DES and put onto balance and tarred. Then, 100 µL of DES was added and stirred with pipette tip until reaching homogeneity and no visible traces of DES were visible inside the tip. The tube was weighed again, and the obtained mass was multiplied by ten in order to determine the density.

3.3.3 Polarized Optical Microscopy (POM)

Optical characterization of different DESs was carried out at room temperature using VHX-970F Keyence® microscope, equipped with a polarized light ring. The pure samples were deposited on a glass slide. All the micrographs were recorded at room temperature.

3.3.4 Fourier-Transform Infrared Spectroscopy (FTIR)

FTIR measurements were performed using Fischer Thermo Scientific® Nicolet® 8700 spectrometer, equipped with MCT-A detector and VariGATR™ grazing angle ATR module mounted Ge ATR crystal. Each spectrum consisted of 32 scans with a resolution of 6 cm⁻¹, in the range of 650-4000 cm⁻¹.

3.3.5 Thermogravimetric Analysis (TG)

Thermogravimetric analyses were performed using a TGA Q50 by heating the samples under nitrogen from room temperature up to maximum of 500°C. The samples were deposited onto platinum pans and heated with a temperature ramp of 20°C/min. and 60 cm³/min. gas flow.

3.3.6 Microwave Spectroscopy (MW)

The dielectric properties of DESs were measured using a calibrated vector network analyzer. Keysight Streamline P5008A with a software provided by the supplier. The measurement frequency range was set from 50 MHz to 10 GHz, with a power level of -15 dBm. The VNA was configured to capture 801 data points across the frequency range, with an intermediate frequency bandwidth (IF Bandwidth) of 100 Hz and an averaging factor of 5 to ensure accurate readings. The N1501A-104 coaxial probe (**Figure 14, Figure 15, Table 9**) was used, and calibrated using short, air and water.

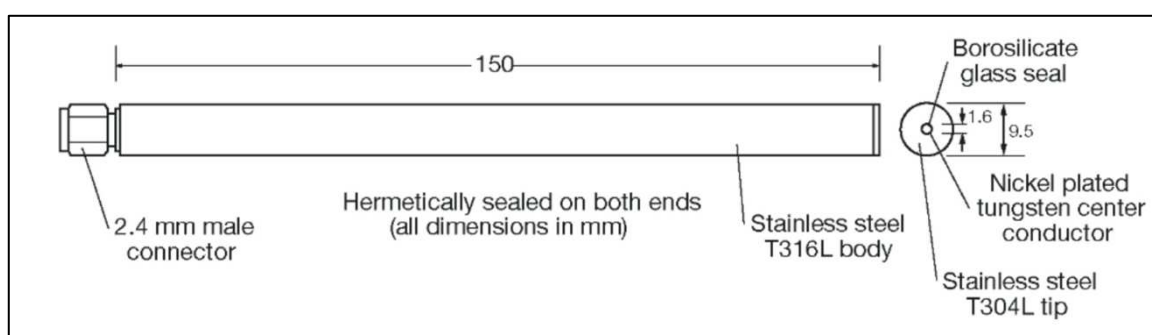


Figure 14. N1501A-104 coaxial probe dimensions, ©Keyence.

The temperature of the water used for calibration was maintained at 22.5°C. Calibration was performed using pure water at the specified temperature, open space and short. Measurements were then conducted on the first sample, followed by two mechanical reproducibility tests. This procedure was repeated for up to the eighth sample, each followed by one mechanical reproducibility test.

Table 9. Characteristics of N1501A-104 coaxial probe.

Characteristic	Value
	500 MHz to 50 GHz
Frequency Range (nominal)	Maximum limited by MUT properties: $f_{max} < \frac{ 285-125j }{\sqrt{\epsilon_r'} V} \text{ GHz}$ where j indicates a complex value
Temperature range	-40 to +200 °C
Temperature slew rate	< 10 °C/min.
Immersable length (approximate)	140 mm
Connector	2.4 mm male
Repeatability and resolution	Two to four times better than accuracy
Material Under Test (assumptions)	Material is “infinite” in size, non-magnetic ($\mu_r^* = 1$), isotropic (uniform orientation), and homogeneous (uniform composition). Solids have a single, smooth, flat surface with gap-free contact at the probe face.
Sample size (requirements)	Minimum 5 mm insertion and 1 mm around tip of probe
Expected Value (requirements)	Maximum recommended $\epsilon_r' < 100$ Minimum recommended $\tan\delta > 0.05$ Not recommended for low loss ($\tan\delta < 0.5$) materials with $\epsilon_r' > 5$
Accuracy (typical)	Dielectric constant: $\epsilon_r' = \epsilon_r' \pm 0.05 \left \epsilon_r' \right \times \epsilon_r^{\text{sub}} \times \epsilon_r^{\text{sup}} \pm 0.05 \left \epsilon_r' \right $

During the measurements, the probe was immersed 1 cm into the liquids. The ambient air temperature was recorded before and after the measurements, showing an increase from 22.2°C to 22.8°C. The temperature of the liquids was measured before and after the process, showing a slight decrease from 22.5°C to 22.2°C. The total measurement time was 1 hour, ensuring consistency and reliability in the data collected.

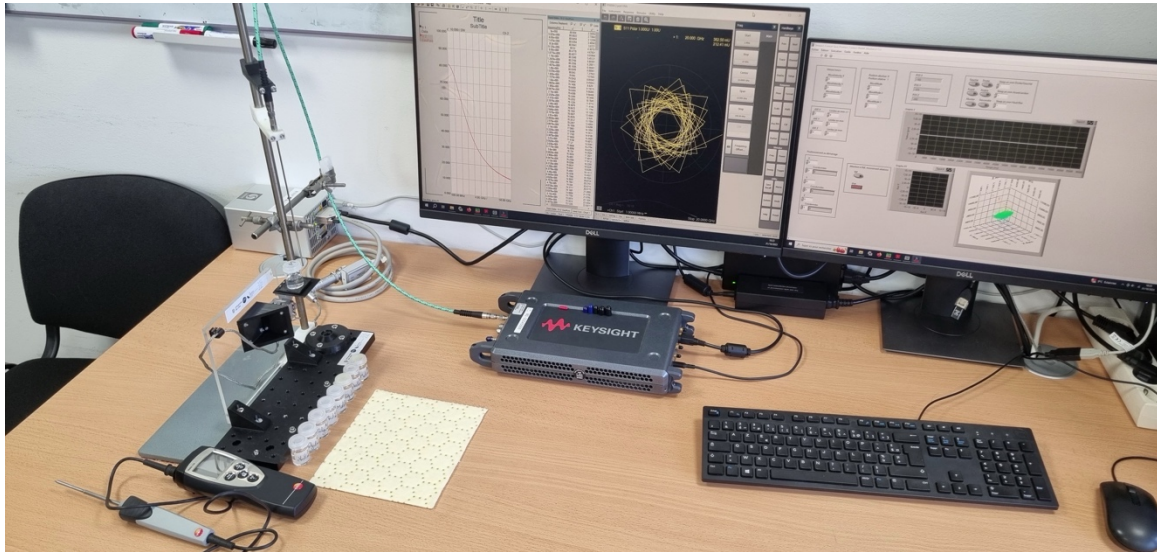


Figure 15. The setup used for microwave measurements (from the left: the nano-positioner, standard glass vials, VNA, computer).

3.3.7 Disk Diffusion Test (DD)

Disk diffusion tests were performed based on the CLSI guidelines (M100) with slight modifications. In short, investigated bacterial cultures were prepared from the stock culture that was thawed and plated from the ultra-low-temperature freezer onto the suitable medium, according to the supplier of the strain. Then, a single bacterial colony was transferred using sterile loop into 3 mL of MHB and was incubated (dynamically) at 35°C until reaching early-log phase (OD_{600} from 0.1 to 0.2). If the OD was greater than 0.1, it was adjusted to 0.1 by adding fresh MHB. Petri dishes ($\phi = 10$ cm) were prepared with 6 mm of MHA. Every batch was tested for possible contamination. Prepared plates were kept in the 4°C fridge and were used in less than 24 h after the preparation. Then, special paper disks ($\phi = 6$ mm),

supplied by Dominique Dutscher®, were disinfected by dipping in 70% ethanol and dried at room temperature for around 15 min. Then, 20 µL of non-diluted DESs or an aqueous solution of an antibiotic (control) containing 10 µg of AMP, 30 µg of CIP, or 5 µg of TET was pipetted onto a disk and dried for another 15 min. Meanwhile, adjusted liquid bacterial culture was spread onto a plate using sterile swab. Then, dried paper disks containing investigated substances were transferred onto an inoculated plate using sterile tweezers. Petri dishes were then sealed and incubated at 35°C overnight. Every plate was examined next morning, and inhibition halos (if visible) were measured in their narrowest point using a ruler.

3.3.8 Minimum Inhibitory and Bactericidal Concentration (MIC/MBC)

MIC values were assessed using broth microdilution method according to CLSI guidelines (M07-A13) and previously reported protocol^[255]. Briefly, investigated DESs were diluted with MHB by half. Then 100 µL of diluted DES were transferred into three wells in the sterile 96-well microplate and then diluted serially with MHB. Diluted standardized inoculum was prepared by incubation MHB bacterial cultures until reaching OD₆₀₀=0.1, then the culture was diluted 100 times with MHB. Afterwards, 50 µL of diluted standardized inoculum was added to the wells with growth control and tested substance. The prepared plates were sealed with a protective film and incubated overnight at 35°C. Then, the OD₆₀₀ of the wells' content was measured using PerkinElmer® VICTOR® Nivo™ Multimode Microplate Reader. The obtained data was plotted and modelled using Gompertz model. MBC values were determined directly after MIC values, according to CLSI guidelines (M26-A). In brief, the content of the wells where no visible growth was observed (MIC assessment) was transferred into a new, sterile 96-well microplate and diluted serially 10 times with NaCl (9 g/L). Then, 20 µL of the liquid content of each well was transferred onto a square petri dish containing MHA, which was then incubated at 35°C overnight. The MBC was assessed, where no visible bacterial growth was spotted. The non-treated bacterial cells were used as control.

3.3.9 Time-Kill Kinetics Assay (TK)

Time-kill kinetics assay was performed according to CLSI guidelines (M26-A). Briefly, all the investigated substances were dissolved in MHB to a total volume of 10 mL (in 15 mL test tubes) in concentrations equal to 1×MIC, 2×MIC, 4×MIC, and 8×MIC. Then, 33 µL of bacterial culture, adjusted to OD₆₀₀ of 0.1 were added to each tube, followed by vortex-shaking and immediate transfer of the tubes to an incubator set at 35°C. Aliquots (200 µL) were sampled from each tube at time intervals of 1, 2, 4, 8, and 24 h, and were further diluted on the microplate using NaCl (9 g/L) and subsequently plated onto BHI or MHA agar plates. The plates were then incubated for 24 h. The three-fold decrease was set as 0.01% of the number of the initial starting colonies (that is 500 CFU/mL). No control was used for TK measurements (internal comparison).

3.3.10 Transmission Electron Microscopy (TEM)

Prior to the TEM analysis, bacterial specimens were prepared. Herein, bacterial strains were grown until reaching OD₆₀₀=0.1 (in a total volume of 10 mL of MHB medium). Then, a substance of interest was added to obtain 8×MIC and the strain was incubated overnight at 35°C. Next day, bacterial suspensions were taken out of the incubator, and centrifuged at 1000 × g for 5 min. The supernatant was removed, and the obtained bacterial pellet was resuspended in 3 mL of 0.1 M PBS. This step was repeated two more times. After last centrifugation, the pellet was resuspended in 1 mL of 2.5% solution of glutaraldehyde, which was prepared by the dilution of stock 25% water solution with PBS. To fix the cells, samples were attached to formvar-coated EM grids for 10 min., quickly washed with water, then contrasted with 2% uranyl acetate for 30 s before soaking up the liquid and drying. Samples were observed at 80 kV with a Hitachi H7500 TEM, and images were acquired with a 1 Mpixel digital camera from AMT. Non-treated bacterial cells were used as control.

3.3.11 Optical Microscopy (OM)

All the fungal specimens were observed using Utilizing the Leica DMI8 microscope, with $\times 100$ magnification. Non-treated fungal cells were used as control.

3.3.12 Biofilm Culture

A single colony of MRSA or *E. coli* was transferred to 3 mL of TSB and incubated at 35°C until reaching OD₆₀₀ of 0.5-0.6. The culture was centrifuged (5 000 $\times$ g) for 5 min., followed by removal of supernatant. Then, bacteria were washed twice by resuspension of the formed pellet in 3 mL of potassium phosphate Buffer (PPB), followed by centrifugation. The suspended bacteria were then diluted to OD₆₀₀=0.01. Exactly, 1 mL of that prepared bacteria solution was transferred to a 24-well plate and left to adhere at room temperature for 2 h. After that time, liquid content of the wells was removed, and 1 mL of TSB was added to start the biofilm growth. Plate was covered with semi-permeable film prior to incubation, after which plates were put in the incubator at 35°C. The maturation of the biofilm lasted 24 h for MRSA (no medium changes) and 72 h for *E. coli* (medium change after each 24 h). After maturation, biofilms were washed twice with 1 mL of PPB, and then treated with 1 mL of nisin or DES, dissolved in PBS for 4 h at 35°C. Then, biofilms were washed three times with 1 mL of PPB and bacterial count was quantified using CF and CV.

3.3.13 Colony Forming Assay (CF)

The matured and treated biofilms were homogenized with 1 mL of PPB and scratched from the bottom surface of the well using pipette tip. The contents of the wells were transferred into 96-well microplate and diluted serially using PPB. Next, the contents of the plate were plated onto appropriate agar plates and incubated at 35°C overnight, after which colony counting was performed. The results were expressed as log(CFU/cm²). Non-treated biofilms were used as control.

3.3.14 Crystal Violet Staining (CV)

The matured and treated biofilms were fixed by adding 100 μ L of methanol and letting them sit for 5 min. Then, methanol was removed, and the fixed biofilms were stained with 200 μ L of CV solution (2 wt%) for 2 min. After that time, biofilm stain was removed and stained biofilms were washed with PBS to remove the unbound dye, until no staining of the solution was observed. Next, the biofilms were treated with 200 μ L of 10% acetic acid and mixed gently on the plate shaker for 5 min. to remove all the bound dye. The biomass of the biofilm in each well was quantified by measuring the absorbance at 570 nm (A_{570}) using the Perkin Elmer, VICTOR® Nivo™ microplate reader. The mass (m) of removed biomass was calculated using the following equation^[146]:

$$m_{removedbiomass} = \frac{A_{570(sample)}}{A_{570(control)}}$$

Non-treated biofilms were used as control.

3.3.15 Live/Dead Staining (LD)

Live/Dead Staining was performed using commercially available LIVE/DEAD™ BacLight™ Bacterial Viability Kit, supplied by Fischer Thermo Scientific®. The staining procedure starts with preparation of biofilm as described in point **3.3.12** of this section. Not treated (control) or treated (sample) biofilms, have been treated with 200 μ L of aqueous solution containing 1 μ L/mL of SYTO™ 9 dye and 1 μ L/mL of PI. The biofilms were left in contact with staining solution for 5 min. with the lights turned off. After that time, the liquid was removed and the biofilms were investigated using BioTek Cytation 5 Cell Imaging Multimode Reader equipped with 2.5 $\times$, 4 $\times$ and 20 $\times$ fold magnification lens system from Olympus with the corresponding excitation and emission filters (469-35/525-39 nm for SYTO™ 9 and 586-15/647-57 nm for PI). Non-treated biofilms were used as control.

3.3.16 Brightfield Microscopy (BFM)

Brightfield microscopy was performed using BioTek Cytation 5 Cell Imaging Multimode Reader equipped with 2.5×, 4× and 20× fold magnification lens system from Olympus. Non-treated biofilms were used as control.

3.3.17 Antifungal Effect Assay (AE)

The yeast cells underwent centrifugation for 3 minutes at $600 \times g$, followed by PBS washing, and were subsequently adjusted to a 0.5 McFarland standard. Twenty microliters of this culture were then introduced into 11 mL of RPMI medium. A hundred microliters were dispensed into a 96-well plate, supplemented with appropriate volume of the substance under investigation. The plate was then placed in an incubator at 37°C for 24 hours. OD₆₀₀ was measured using a FLUOstar Omega spectrophotometer. Non-treated fungal cells were used as control.

3.3.18 Software

All the calculations and graphs were prepared using OriginPro 2024 or Prism. Brightfield micrographs were recorded using Gen5 software, supplied by the manufacturer of multimode reader. TEM images were recorded using software supplied by the manufacturer.

4. Results

4.1 Synthesis and characterization of DESs

4.1.1 Polarized Optical Microscopy (POM)

A total of nine DESs were prepared (**Figure 16, Table 10**) by heating together parent substances mixed in a strict molar ratio under the dry atmosphere. After cooling down, DESs were stored at room temperature in a sealed glass test tubes with a 3D printed insert, suspended above the liquid, containing neutral drying agent.

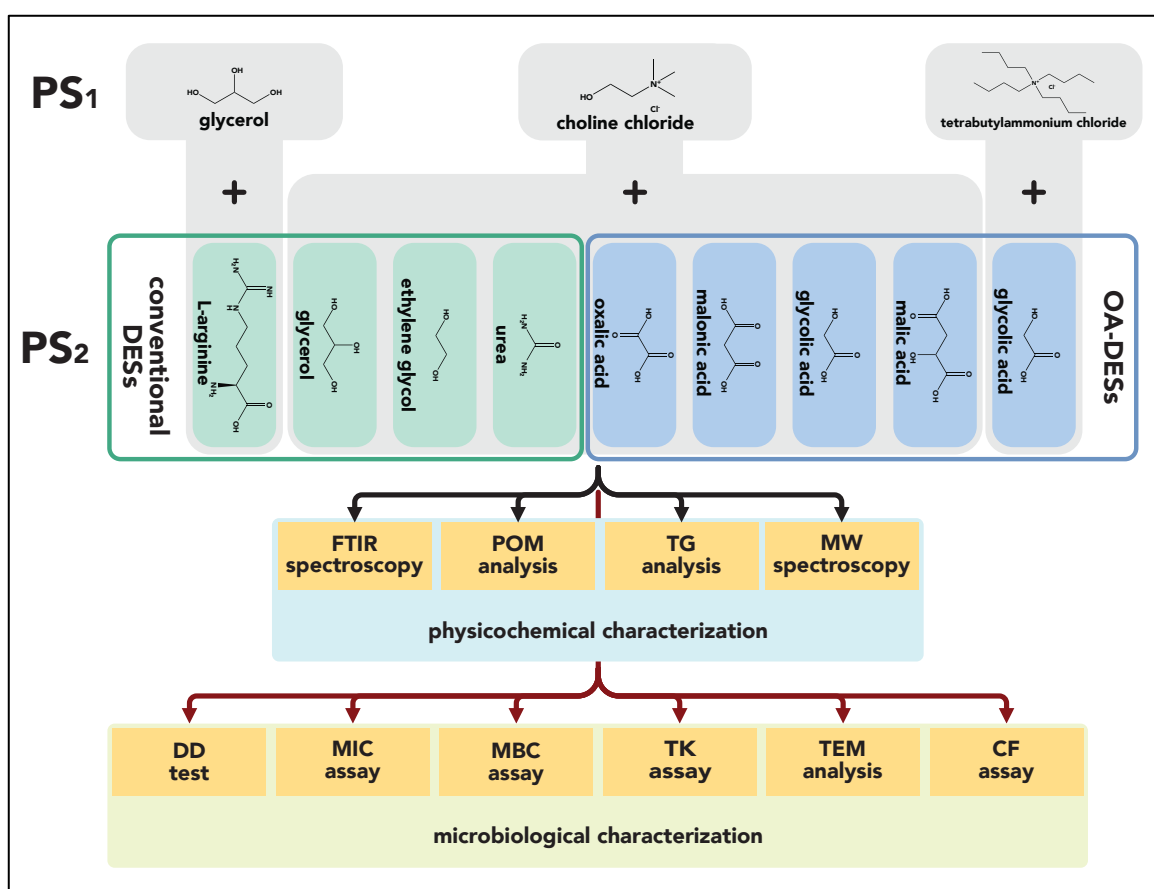


Figure 16. Parent substances used for the formulation of OA-DES and conventional DESs, together with a methodology used for physicochemical and microbiological characterization.

The stability of the obtained DESs was monitored after 72 h of storage, using POM (**Figure A1***). POM serves as a technique that enables to monitor the metastability of the material.

Table 10. List of prepared DESs with the molar ratio of the parent substances (n=3).

	DES	PS ₁	PS ₂	n _{PS1}	n _{PS2}	d (g/mL)	Ref.
OA-DES	glycoline	ChCl	GlycA	1	2	1.21 ± 0.04	[177]
	T-glycoline	TBACl	GlycA	1	2	1.14 ± 0.01	–
	maloline	ChCl	MaloA	1	1	1.12 ± 0.01	[256]
	maline	ChCl	MalA	1	1	1.14 ± 0.02	[257]
	oxaline	ChCl	OxA	1	1	1.13 ± 0.01	[257,258]
Conventional DES	reline	ChCl	U	1	2	1.17 ± 0.01	[150]
	ethaline	ChCl	EG	1	2	1.12 ± 0.03	[259]
	glyceline	ChCl	GLY	1	2	1.18 ± 0.01	[260]
	R-glyceline	Arg	GLY	1	4	1.09 ± 0.03	[261]

“DES” indicates deep eutectic solvent; “OA-DES” indicates organic acid-based deep eutectic solvent; “PS” indicates parent substance; “n” indicates molarity index of parent substances in the formulation; “d” indicates density; “ChCl” indicates choline chloride, “TBACl” indicates tetrabutylammonium chloride; “MaloA” indicates malonic acid; “MalA” indicates malic acid; “OxA” indicates oxalic acid; “GlycA” indicates glycolic acid; “GLY” indicates glycerol; “Arg” indicates L-arginine; “EG” indicates ethylene glycol; “U” indicates urea.

By implementation of polarized light, even smallest crystals of the substance can be observed. In the case of DESs, a metastable material should be fully liquefied^[262]. All the images were completely black showing no crystal formation, which indicated total liquefaction of all of the investigated DESs at room temperature.

* All the figures and tables which numbers start with letter “A” can be found in the appendix at the end of this thesis.

In parallel, the density of DESs was determined using direct weighing method, which allowed us to further compare the PSs to DESs during MIC assay evaluation.

4.1.2 Fourier-Transform Infrared Spectroscopy (FTIR)

To confirm the formation of the DESs on the chemical level (formation of hydrogen bonds), all the DESs were characterized by means of FTIR. FTIR allows to determine the presence of various covalent bonds based on their vibrational frequencies, which are presented in the form of bands – characterized by their intensity and position. However, if in the close proximity of the bond, there exist a hydrogen bond, shifts in the position and intensity of the band can be observed^[263-265]. The obtained spectra of DESs are a result of the overlap of the spectra of their PSs. The identified FTIR spectra bands correspond to the literature ones (deposited in SDBS: sdb.sdb.aist.go.jp). However, as the formation of DESs is a direct result of intramolecular hydrogen interactions, changes in the position and the intensity of the bands of the atoms involved in hydrogen bonds' formation were observed.

In the FTIR spectrum of glycoline (**Figure A2**), a broad but low intensity-band of O-H was observed with its maximum located at 3301 cm^{-1} , followed by the region of C-H stretching from 2379 to 3077 cm^{-1} . Then at 1739 cm^{-1} , a slightly doubled peak of C=O was observed (compared to 1697 cm^{-1} for pure glycolic acid). The band at 1484 cm^{-1} was due to C-O or O-H stretching. At 1087 cm^{-1} , a sharp possible overlapped band of C-O and C-N stretching was evident. Another C-N stretching band was present at 982 cm^{-1} . The stretching vibration of C-O was observed at 1191 cm^{-1} . The C-N stretching bands are located at 1083 and 956 cm^{-1} .

The FTIR spectrum of T-glycoline (**Figure A3**) revealed characteristic signals visible between 2769 to 3035 cm^{-1} of alkyl chains. The bands at 1730 cm^{-1} (strong) and 1380 cm^{-1} were assigned respectively to C=O and C-N vibrations. In the low frequency region, two bands at 1199 and 1096 cm^{-1} attributed to C-O stretchings were identified. Finally, the stretching of C-N was spotted at 883 cm^{-1} .

In the spectrum of maline (**Figure A4**), two overlapping bands were identified in the high frequency region and were attributed to O-H (3293 cm^{-1}) and C-H (2925 cm^{-1}) stretchings. A strong C=O stretching band was also found at 1727 cm^{-1} , followed by C-H scissor stretching at 1482 cm^{-1} and C-O stretching at 1172 cm^{-1} . C-N stretching bands were located at 1100 and 955 cm^{-1} .

The spectrum of maloline (**Figure A5**) comprised a broad signal ranging from 2345 to 3652 cm^{-1} which is a result of an overlap of the C-H and O-H stretchings. At 1742 cm^{-1} , a sharp signal of C=O was obvious, followed by the C-H scissoring and C-O stretching bands at respectively 1481 and 1180 cm^{-1} .

For the spectrum of oxaline (**Figure A6**), two overlapped groups of bands were identified and attributed to O-H (3329 cm^{-1}) and C-H ($2763\text{--}3099\text{ cm}^{-1}$) stretchings. A strong band of C=O stretching was located at 1728 cm^{-1} , along with C-H scissor stretching band at 1484 cm^{-1} and C-O stretching band at 1177 cm^{-1} (second one overlapped with C-N). Another C-N stretching band was located at 948 cm^{-1} .

For the reline (**Figure A7**), in the high frequency region, an overlapped amide A band and hydroxyl bonds were observed with two separated maxima at 3363 and 3197 cm^{-1} . In the low frequency region, a band of C=O stretching (1666 cm^{-1}) and amide I band (1616 cm^{-1}) could be easily distinguished. From 1384 to 1511 cm^{-1} , another band was visible, which may be an overlap of C-O and C-N bands. C-N stretching band was observed at 1160 cm^{-1} and C-O at 1083 cm^{-1} . Based on the data above, it was clear that all the groups present in the PSs were involved in the formation of a hydrogen bond network. This was confirmed by either a redshift of the stretching frequencies directly related to O-H, N-H, C=O, C(=O)-OH groups or by a decrease in the intensity of the adjacent bonds, such as C-O or C-N, C=N, and C=O. The FTIR analysis alone does not allow for a total quantification of the hydrogen bonds, but clearly confirmed their formation.

In the spectrum of ethaline (**Figure A8**), an O-H band was identified at 3307 cm^{-1} along with C-H stretching bands at 2931 and 2876 cm^{-1} . The C-H scissor stretching

band was located at 1483 cm^{-1} . A strong band at 1044 cm^{-1} was attributed to C-N/C-O overlapping.

The spectrum of glyceline (**Figure A9**) revealed signals attributed to O-H (3320 cm^{-1}) and C-H (2873 and 2935 cm^{-1}) stretchings. The C-H scissor stretching band (or O-H bending) was observed at 1481 cm^{-1} along with C-O stretching band at 1037 cm^{-1} . Another band was obvious at 1087 cm^{-1} and could be attributed to overlapped C-N and C-O stretchings. The C-N stretching was also found at 952 cm^{-1} . C-H rocking was confirmed by the presence of two signals between 840 and 914 cm^{-1} .

In the spectrum of R-glyceline (**Figure A10**), an overlapped band of O-H and guanidine group was located between 3004 and 3610 cm^{-1} , followed by a weak signal of C-H stretching between 2823 and 3000 cm^{-1} . In the region between 1525 and 1743 cm^{-1} , three weak signals attributed to N-H (1558 cm^{-1}), C=N (1619 cm^{-1}) and C=O (1673 cm^{-1}) were evident. Lastly, in the far-red region from 829 to 1160 cm^{-1} , three signals ascribed to glycerol C-H rocking (860 cm^{-1}), O-H bending (925 cm^{-1}), and C-O stretchings (1037 and 1100 cm^{-1}) were visible.

4.1.3 Thermogravimetric Analysis (TG)

The pivotal feature that any material, especially in biomedicine, should exhibit is its thermal stability in the operating temperature. The decomposition of the substance and or material can lead either to partial or total loss of its properties and/or generation of byproducts that can be harmful^[266,266]. Therefore, it is crucial to investigate DESs for their thermal stability. To that end, all the investigated DESs were studied by TG under nitrogen, following a heating rate from room temperature up to 400°C . All the DESs undergo progressive decomposition upon increasing the temperature. From the TG curves (**Figure A11**), decomposition of DESs appears to be divided in several steps which are overlapping over a large range of temperature above 140°C , making them difficult to identify clearly. Thus, for the sake of comparison, the temperatures at which weight loss of the samples are 5 wt.% ($T_{5\%}$), 33 wt.% ($T_{33\%}$) and 67 wt.% ($T_{67\%}$) were more specifically examined.

The temperature at which the weight loss of the sample is 5 wt.% can be considered representative of the initial degradation temperature. For ethaline, maloline and oxaline, this takes place at relatively low temperature of around 100°C, whereas it happens at much higher temperature for the other DESs. In the case of these three DESs, such a low value is attributed to the evaporation of absorbed water during DES preparation and handling, resulting from the high hygroscopicity of their components.

The DESs investigated herein are composed of hydrogen bond acceptor (i.e. ChCl, TBACl or Arg) linked to hydrogen bond donor (i.e. GlycA, MaloA, MaIA, OxA, GLY, EG, U) via hydrogen bonding, which is a type of weak intermolecular force that can be broken when temperature increases and reaches a certain level. Above 130-140°C, the degradation steps observed in TG curves could be representative of the beginning of the decomposition or volatilization of DES component with the lowest thermal stability, resulting from the dissociation of hydrogen bonding. As hydrogen bond donors present lower thermal stability than hydrogen bond acceptor counterparts, they can decompose firstly at their characteristic decomposition temperatures range, followed by the hydrogen bond acceptor at higher temperatures range. The temperatures at which weight loss of the samples are 33 and 67 wt.% (**Table 11**) highlight the temperatures magnitude at which these phenomena take place and allow to rank the DESs in the study in descending order of thermal stability as follows: reline>glyceline>maline, T-glycoline, glycoline>R-glyceline>oxaline>maloline, ethaline. Overall, TG results revealed that the thermal stability and/or the boiling point of hydrogen bond donors appear to have a crucial impact on the maximum operating temperature of DESs.

Table 11. Temperatures, as obtained by TG, at which weight losses of the samples are 5, 33 and 67 wt.%.

DES		T _{5%} (°C)	T _{33%} (°C)	T _{67%} (°C)
OA-DESS	glycoline	142	230	249
	T-glycoline	132	254	277
	maloline	98	164	294
	maline	174	228	279
	oxaline	109	211	283
Conventional DESS	reline	187	256	287
	ethaline	91	169	304
	glyceline	160	251	290
	R-glyceline	149	205	229

4.1.4 High Frequency Characterization of DESs – Microwave Spectroscopy (MW)

4.1.4.1 System Validation using Liquid of Known Dielectric Properties

Before measuring deep eutectic solvents (DESs), the microwave measurement setup was tested for accuracy and repeatability. Water, serving as a calibration medium, and isopropyl alcohol (iPrOH) were measured for their dielectric constant (ϵ') and dielectric loss (ϵ''). As shown in the **Figure 17**, the microwave spectra of isopropyl alcohol and water exhibit characteristic changes in both ϵ' and ϵ'' with increasing frequency. For isopropyl alcohol, the dielectric constant generally decreases with increasing frequency due to its polar nature, which allows its dipole moment to reorient with the applied electromagnetic field. However, as frequency increases, this reorientation ability diminishes, leading to a reduction in the dielectric constant. Water similarly shows a decrease in dielectric constant with increasing frequency. Known for its strong hydrogen bonding and high dipole moment, water has a very high dielectric constant at low frequencies. At higher

frequencies, similar to iPrOH, the dipoles in water molecules cannot keep up with the oscillating field, resulting in a lower dielectric constant.

These results are consistent with previous reports and known data^[267,268]. Compared to isopropyl alcohol, water typically has a much higher dielectric constant at lower frequencies, attributed to its stronger hydrogen bonding and higher dipole moment^[269], but both substances exhibit similar trends of decreasing dielectric constant with increasing frequency.

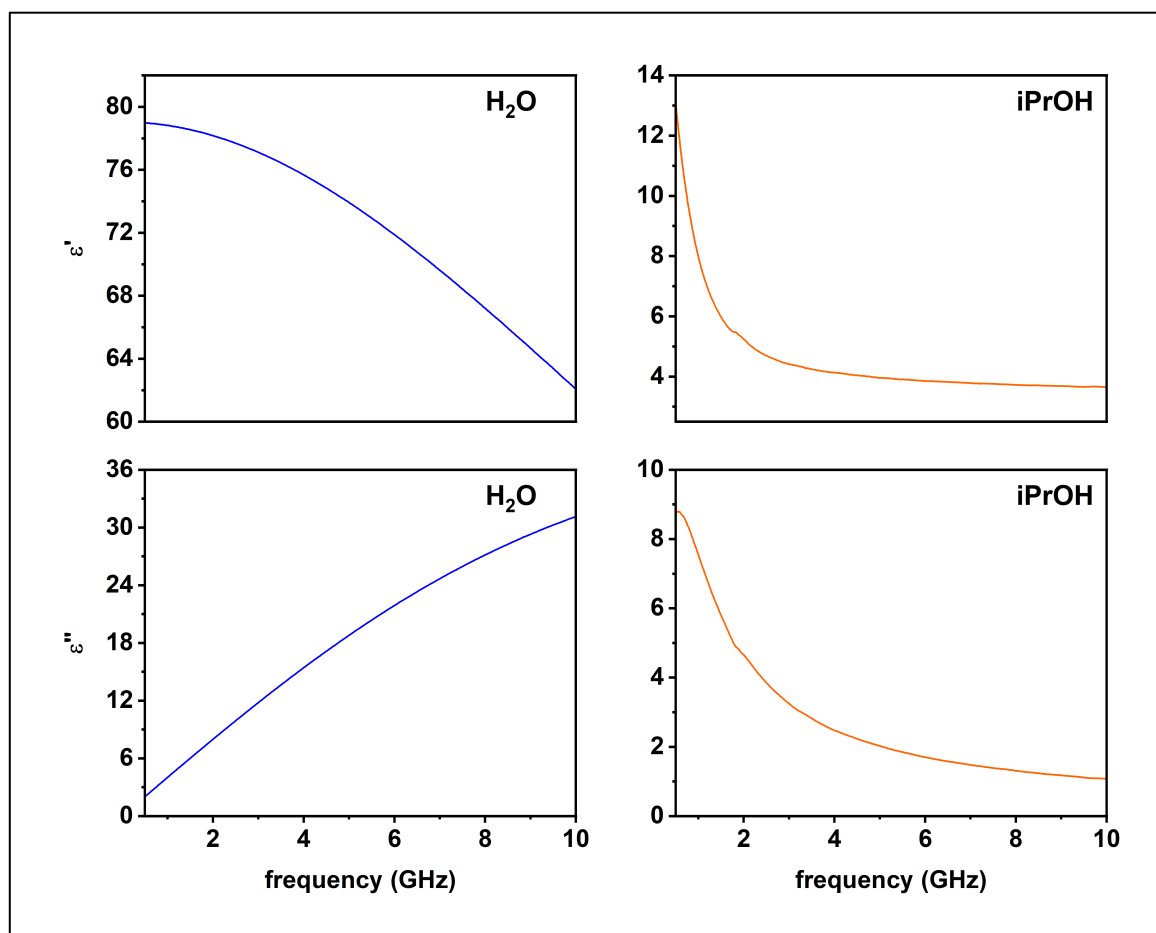


Figure 17. Dielectric constant (top) and loss (bottom) of water and iPrOH.

The dielectric loss (ϵ'') behaviors of iPrOH and water also show distinct differences across the frequency range. For isopropyl alcohol, ϵ'' starts relatively low and decreases steadily as the frequency increases, indicating that energy dissipation is higher at lower frequencies and reduces as the dipoles become less able to follow the rapidly oscillating electric field. In contrast, water exhibits a higher

initial ϵ'' at lower frequencies due to its strong dipole interactions and significant hydrogen bonding. Unlike isopropyl alcohol, the ϵ'' for water remains relatively constant across the frequency range, suggesting that water maintains a high level of energy dissipation due to its molecular structure and persistent hydrogen bonding, which continue to interact effectively with the electric field even at higher frequencies^[270].

The dielectric loss for isopropyl alcohol showed a maximum peak around 580 MHz, corresponding to the frequency at which molecular dipoles can most effectively follow the oscillating field, leading to maximum energy dissipation. Structurally, isopropyl alcohol ($\text{CH}_3\text{CHOHCH}_3$) is a polar molecule with a hydroxyl group (-OH) attached to a carbon chain, significantly contributing to its dipole moment. The presence of hydrogen bonding in iPrOH influences its dielectric properties. At lower frequencies, the -OH group can reorient to follow the electric field, resulting in a high dielectric constant. As frequency increases, the reorientation becomes less efficient, and energy dissipation (dielectric loss) shows a peak at the relaxation frequency. Water, with its bent H-O-H structure and strong hydrogen bonding, has a high dipole moment due to the large electronegativity difference between hydrogen and oxygen. Water's high dielectric constant at low frequencies is due to its strong dipole interactions and hydrogen bonding. As the frequency increases, the ability of water molecules to reorient decreases, leading to a drop in dielectric constant.

4.1.4.2 Systems Sensitivity Studies using Nanosystems

Encouraged by the previous results we have investigated the probes' sensitivity to nanoscopic phenomena was studied. Here, solutions of alginate nanoparticles at different concentrations have been studied. Alg NPs are a type of nanomaterials that are extremely rich in the -OH groups, which contribute to local dipole moment of the nanoparticles as well as solubility of the material. However, despite high

theoretical solubility alginate nanoparticles tend to irreversibly aggregate above certain concentration.

Alg NPs have been measured in six different concentrations, ranging from 0.1 to 5 mg/mL. As seen in the **Figure 18**, the spectrum of dielectric constant of Alg NPs exhibits a peak, located at 1.48 GHz, followed by stable decrease with occasional small increases of ϵ' value at 3.25 and 4.27 GHz.

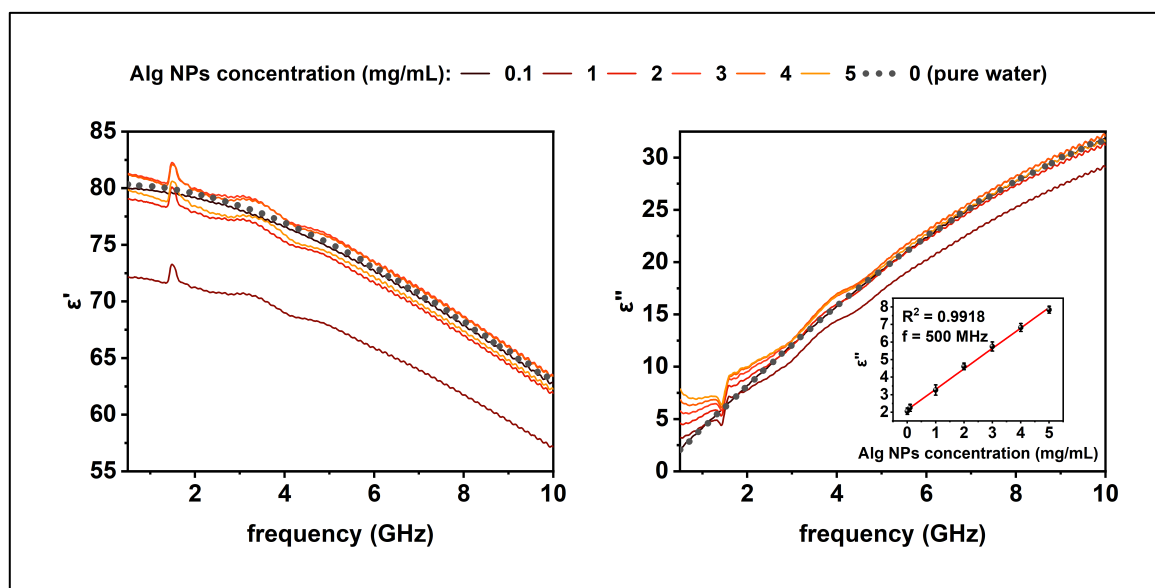


Figure 18. Dielectric constant (left) and loss (right) of Alg NPs in various concentrations; inset – linearity test for ϵ'' at $f = 500$ MHz.

The visible peak likely correspond to shifting polarity of hydroxyl groups of the nanomaterial, which are responsible for the storage of the energy, which is then dissipated in the form of heat, as reflected by ϵ'' , with its mirrored minimum and rapidly increase after it. However, it is clear that the overall changes of both ϵ' and ϵ'' values occur with changing concentration. In the case of dielectric constant, lowest concentration of Alg NPs was characterized with sudden drop of ϵ' value. Drop of relative permittivity arises from great initial rise of conductivity (mainly due to the presence of Na^+ and OH^-). As Na-disassociation is purely ionic phenomenon (salt-like) $-\text{OH}$ ionization is constant-dependent (pK_a/pK_b). This is why the permittivity increases after passing a somewhat “critical” aggregation

concentration. At one point, the aggregates will start dropping out of solution and observable permittivity is much closer to water. However, the Na-disassociation is clearly observed in the dielectric loss graph. Here we observe a stable, and linear increase of the ϵ'' value with its most visible changes in low frequency region.

4.1.4.3 Influence of the Coaxial Probes' Position on the Measurements

Following this experiment, it was decided to verify the probes' spatial sensitivity. For this the measurements were done in a standard 5 mL glass vial with a diameter of 2 cm. The probe was immersed into the recipient and then moved in the cubic lattice among twenty seven points with 1 mm of distance.

The probe was positioned exactly at the center of the vial, and the relative permittivity was measured according to the lattice, by vertical sections (**Figure 19**). The general section analysis of the lattice, reveal that the middle section was characterized by the greatest overall changes decrease of ϵ' value, when the probe deviated from its starting point. However, in all the sections major disturbances were observed, especially in the region from 500 MHz to 4 GHz. These disturbances were characterized by the wave-like form, with its major local maxima around 2, 3, and 3.5 GHz, and minima at 2.1 and 2.7 GHz. The occurrence of the disturbances is a result of the geometrical distortions of the electric field, when approaching the glass walls of the vial. The points of the highest deviation from the starting point (0, 0, 0) were: (-1, 0, 0), (-1, 0, -1), (1, 0, 0) for mid-plane; (0, 1, 0), (1, 1, 0), (-1, 1, -1) for the back-plane; and (1, -1, 0), (0, -1, 0), (-1, -1, -1) for the front-plane. These observations show that the biggest disturbances were spotted along the X axis at Z=0. And Z=-1 (with Y=-1). However, any deviation from the starting point resulted in significant disturbances in the measurements, suggesting that an absolute positioning at the center of the glass vial is crucial for stable measurements. For this any subsequent measurements in this recipient have to be done using nano positioner to assure being in the center of the vial.

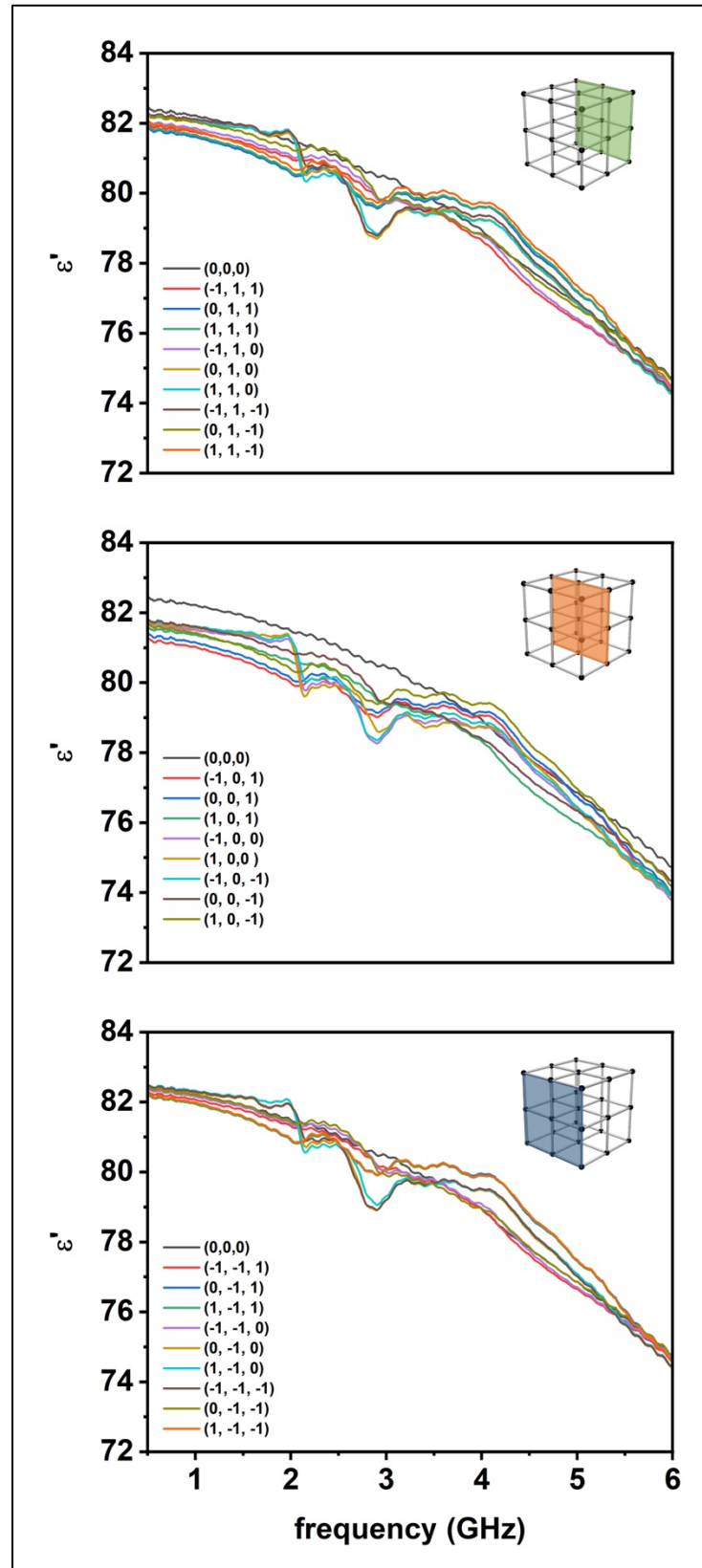


Figure 19. Dielectric constant disturbances among the 27-point lattice (1 mm step) in a standard glass vial.

4.1.4.4 Measurements of Deep Eutectic Solvents

Finally, the DESs were investigated for their microwave-based properties using a VNA and coaxial probe setup. The samples were analyzed for their dielectric constant and dielectric loss. The plateau region of the dielectric constant ($\epsilon_{\text{plateau}}$) was indicated with a red dotted line, while the point where a significant increase occurs (above 25% ($\epsilon_{75\%}$)) was marked with a blue dotted line.

In the spectrum of reline (**Figure 20**), the dielectric constant starts at around 10.5 at low frequencies and peaks at 125 MHz with a value of 11.99. This indicates a significant ability to store electric energy through polarization. As the frequency increases, the dielectric constant decreases, crossing the $\epsilon_{75\%}$ line at 2.25 GHz with a value of 8.52. The $\epsilon_{\text{plateau}}$ represents the minimum value of the dielectric constant, which is around 7.36. The frequency range where ϵ is above the $\epsilon_{75\%}$ line is 50 MHz to 2.25 GHz, suggesting that reline maintains its polarization capabilities over a broad frequency range. The dielectric loss at the same frequency as the peak of the dielectric constant (125 MHz) has a value of 18.52, indicating significant dielectric losses. As the frequency increases, the dielectric loss decreases, reaching a value of 2.62 at 2.25 GHz, indicating that reline becomes less lossy at higher frequencies.

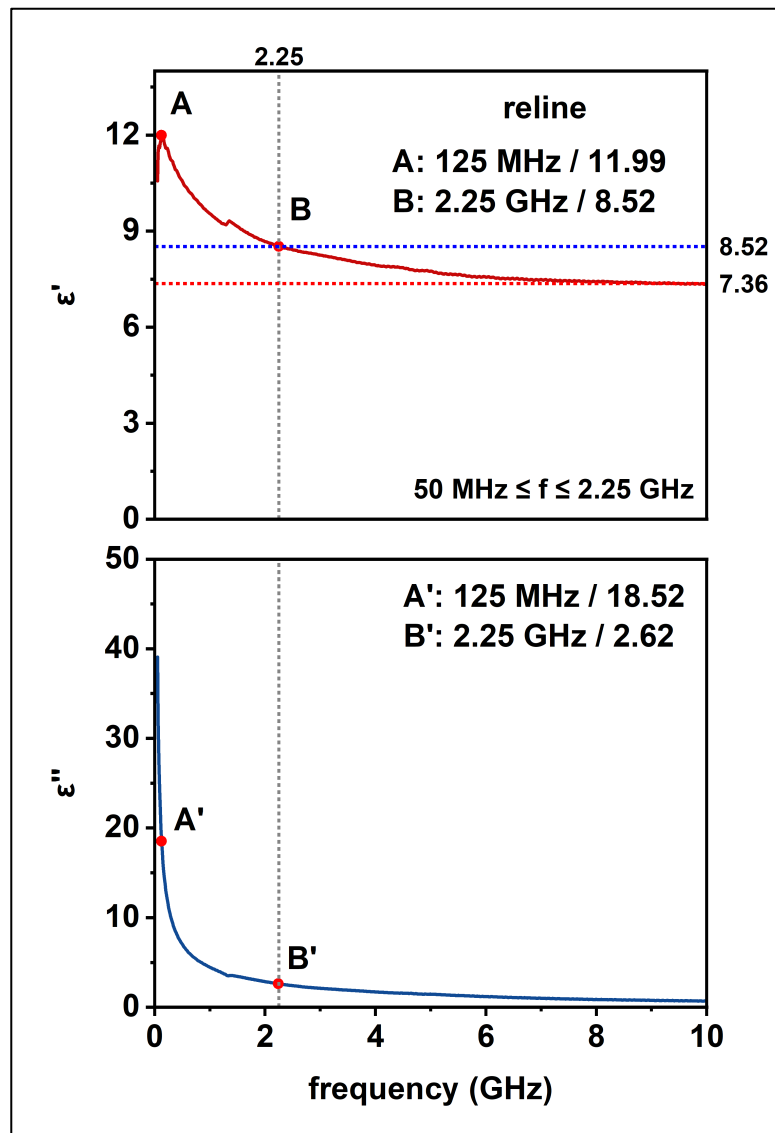


Figure 20. High frequency spectrum of reline ($n=3$).

For ethaline (**Figure 21**), the dielectric constant starts at around 3.57 at very low frequencies and rapidly increases to a peak of 17.69 at 388 MHz. This difference between the starting value and the peak is notably larger compared to reline. As frequency increases, the dielectric constant decreases, crossing the $\epsilon_{75\%}$ line at 3.49 GHz with a value of 10.53. The minimum value of the dielectric constant, marked by the $\epsilon_{\text{plateau}}$, is around 8.14, indicating ethaline's strong ability to maintain polarization over a wide frequency range.

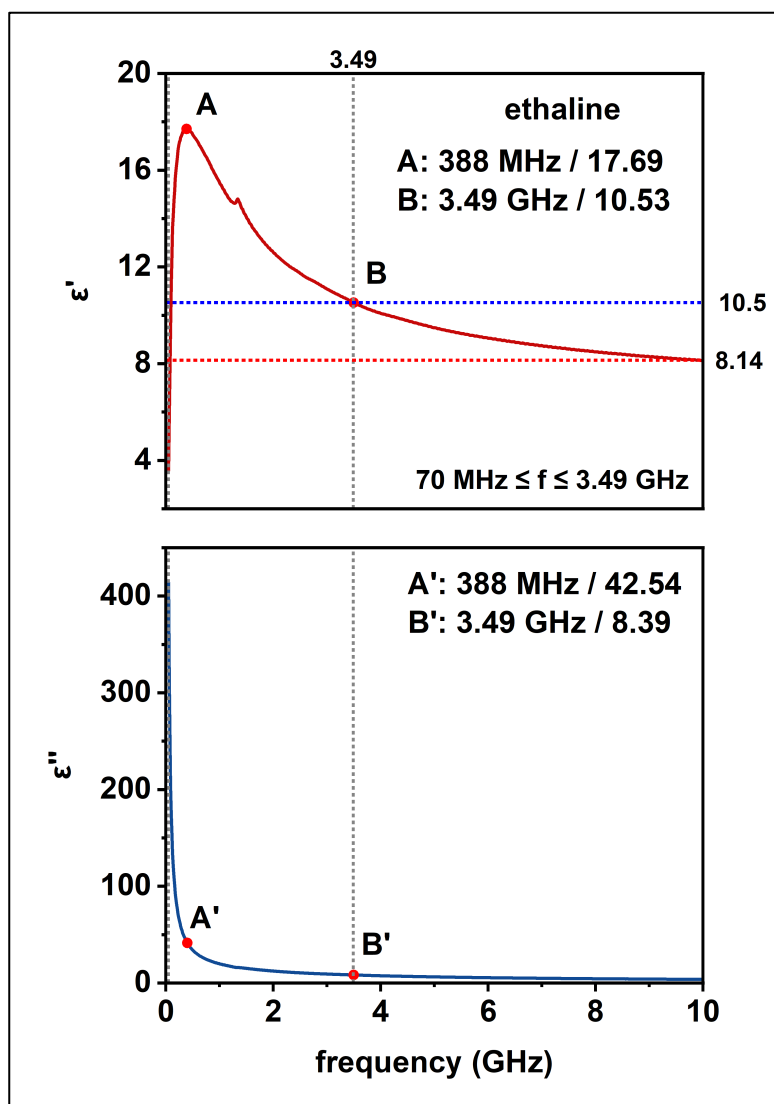


Figure 21. High frequency spectrum of ethaline ($n=3$).

The frequency range where ϵ is above the $\epsilon_{75\%}$ line is 70 MHz to 3.49 GHz. The dielectric loss at 388 MHz is 42.54, showing substantial dielectric losses that diminish significantly at higher frequencies, decreasing to 8.39 at 3.49 GHz.

As for glyceline (**Figure 22**), the dielectric constant starts around 9.62 at low frequencies and peaks rapidly at 138 MHz with a value of 15.18.

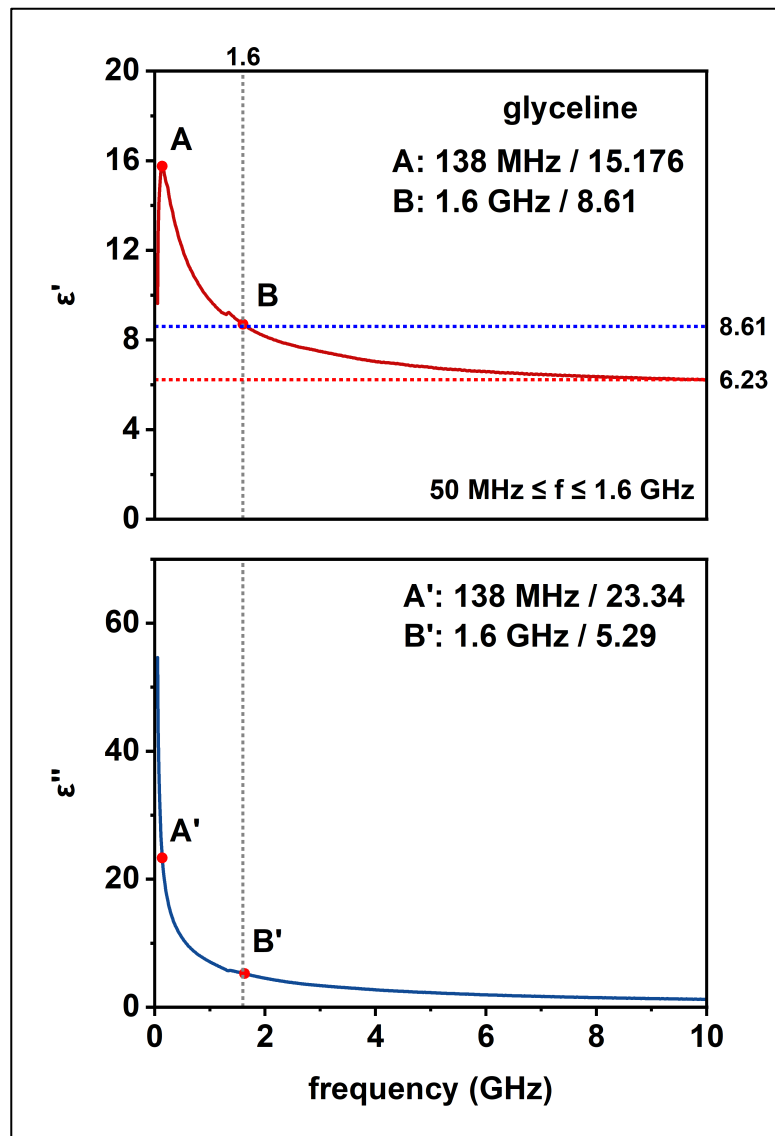


Figure 22. High frequency spectrum of glyceline (n=3).

As frequency increases, the dielectric constant decreases, crossing the $\epsilon_{75\%}$ line at 1.6 GHz with a value of 8.61, indicating a broad range over which glyceline maintains its polarization capability. The $\epsilon_{\text{plateau}}$ marks the minimum ϵ' value of 6.23. The frequency range where ϵ is above the $\epsilon_{75\%}$ line is 50 MHz to 1.6 GHz. The dielectric loss at 138 MHz is 23.34, which decreases to 5.29 at 1.6 GHz, suggesting significant dielectric losses at lower frequencies that reduce at higher frequencies.

In the case of glycoline (**Figure 23**), the dielectric constant starts at around 7.55 at low frequencies and peaks at 150 MHz with a value of 14.11.

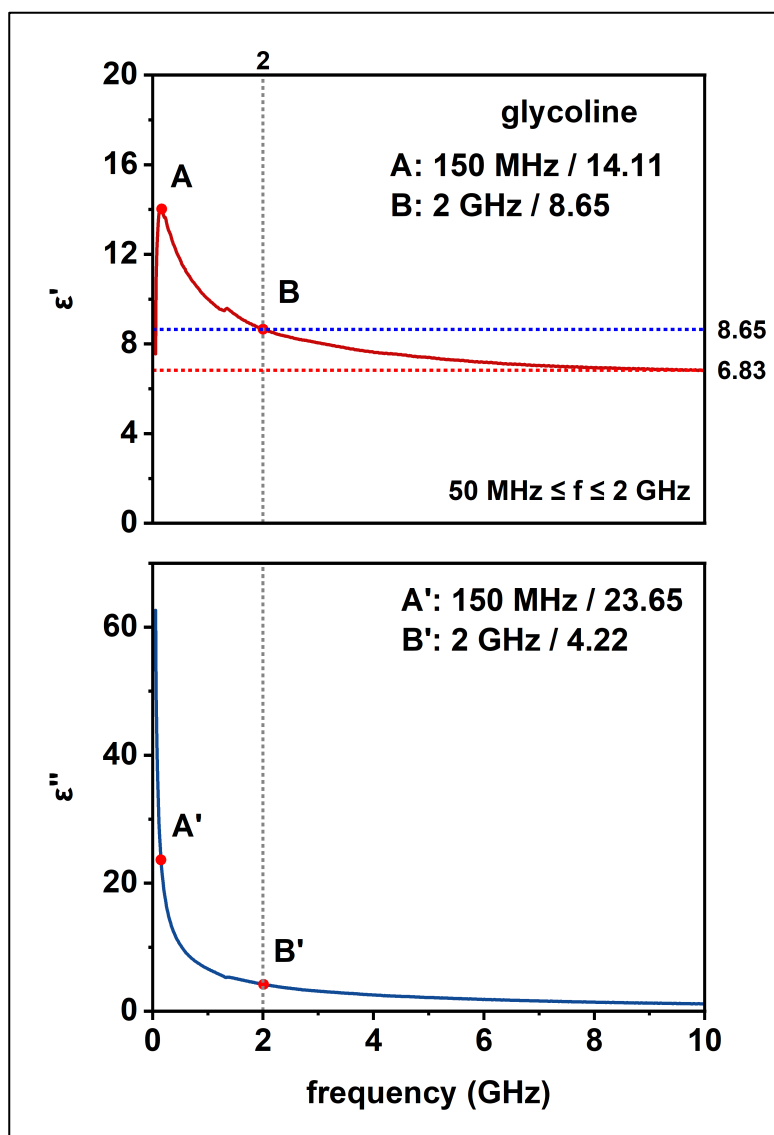


Figure 23. High frequency spectrum of glycoline ($n=3$).

This shows a notable increase similar to glyceline. As frequency increases, the dielectric constant decreases, crossing the $\epsilon_{75\%}$ line at 2 GHz with a value of 8.65, indicating a broad frequency range for maintaining polarization. The $\epsilon_{\text{plateau}}$ marks the minimum ϵ' value of 6.83. The frequency range where ϵ is above the $\epsilon_{75\%}$ line is 50 MHz to 2 GHz. The dielectric loss at 150 MHz is 23.65, which decreases to 4.22 at 2 GHz, highlighting significant losses that decrease with frequency.

For T-glycoline (**Figure 24**), the dielectric constant starts at around 4.48 at low frequencies and peaks at 238 MHz with a value of 5.76.

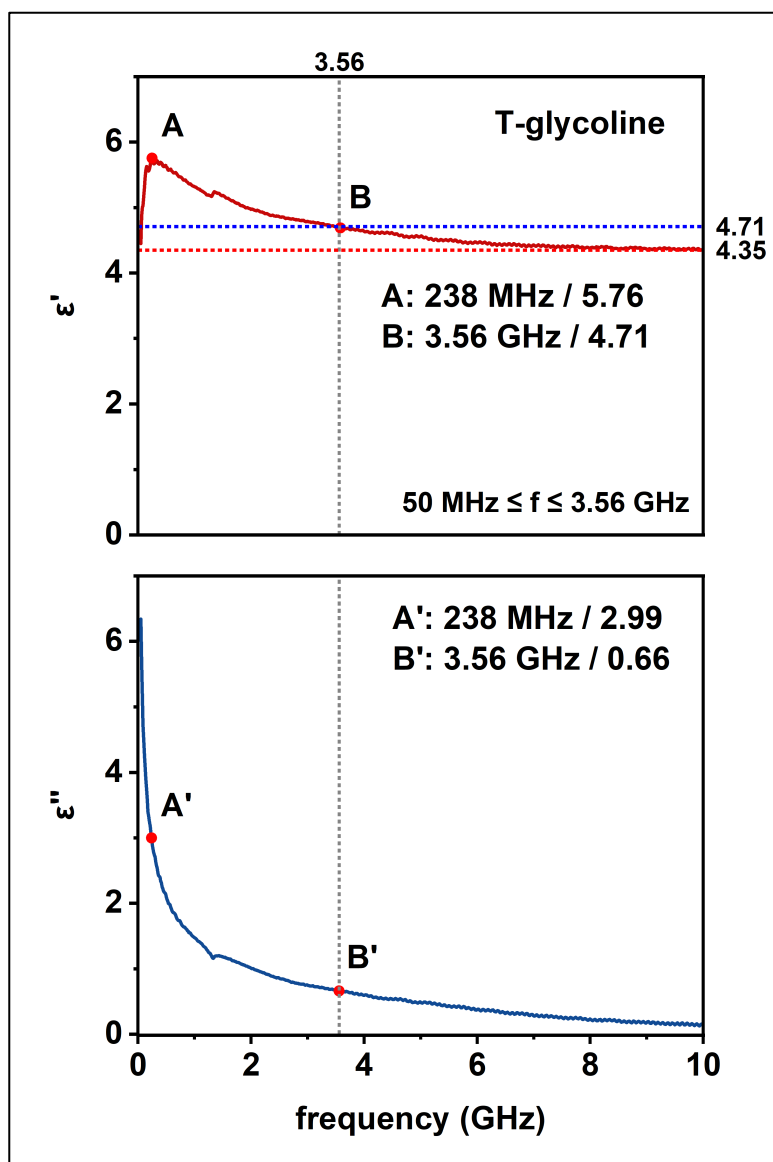


Figure 24. High frequency spectrum of T-glycoline (n=3).

As frequency increases, the dielectric constant decreases, crossing the $\epsilon_{75\%}$ line at 3.56 GHz with a value of 4.71, indicating T-glycoline's ability to maintain polarization over a wide frequency range. The $\epsilon_{\text{plateau}}$ marks the minimum ϵ' value of 4.35. The frequency range where ϵ is above the $\epsilon_{75\%}$ line is 50 MHz to 3.56 GHz. The dielectric loss at 238 MHz is 2.99, decreasing to 0.66 at 3.56 GHz, indicating moderate losses that further reduce at higher frequencies.

In the case of maloline (**Figure 25**), the dielectric constant starts at around 8.67 at low frequencies and peaks at 138 MHz with a value of 10.56.

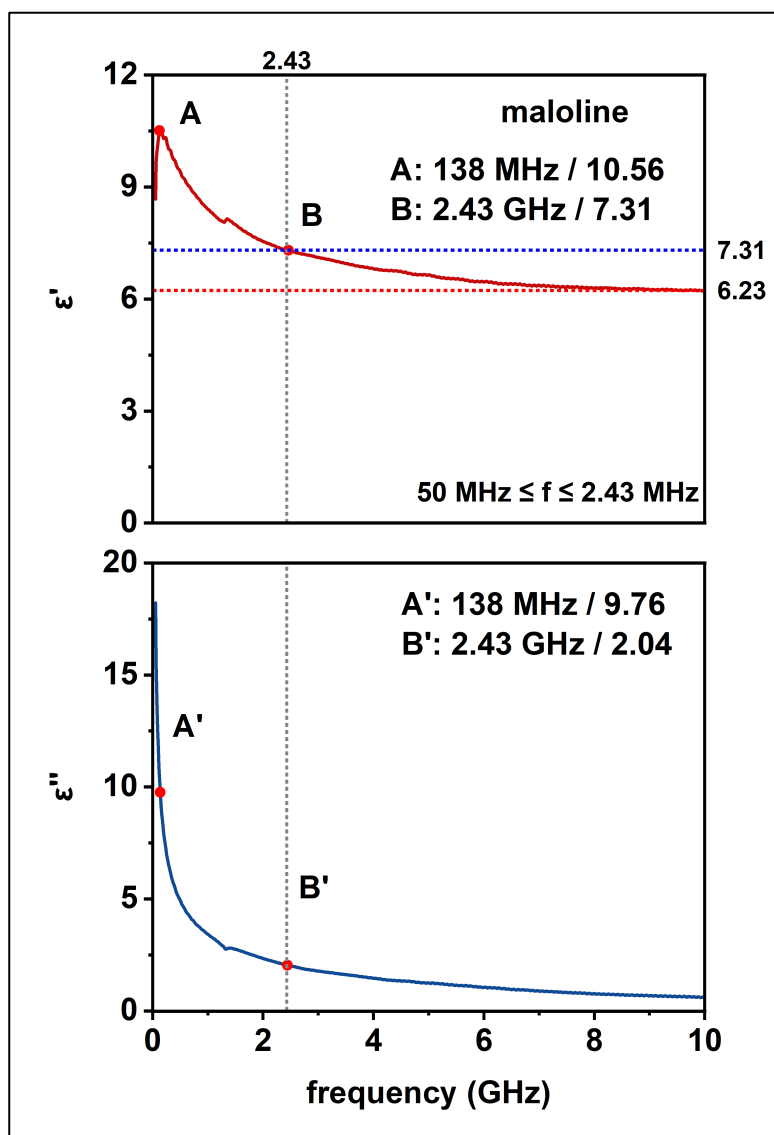


Figure 25. High frequency spectrum of maloline ($n=3$).

As frequency increases, the dielectric constant decreases, crossing the $\epsilon_{75\%}$ line at 2.43 GHz with a value of 7.31, indicating a wide range of maintained polarization capability. The $\epsilon_{\text{plateau}}$ marks the minimum ϵ' value of 6.23. The frequency range where ϵ is above the $\epsilon_{75\%}$ line is 50 MHz to 2.43 GHz. The dielectric loss at 138 MHz is 9.76, decreasing to 2.04 at 2.43 GHz, indicating moderate dielectric losses that significantly decrease at higher frequencies.

The spectrum of maline (**Figure 26**) reveals a dielectric constant starting at 5.67 at low frequencies, peaking at 250 MHz with a value of 6.39.

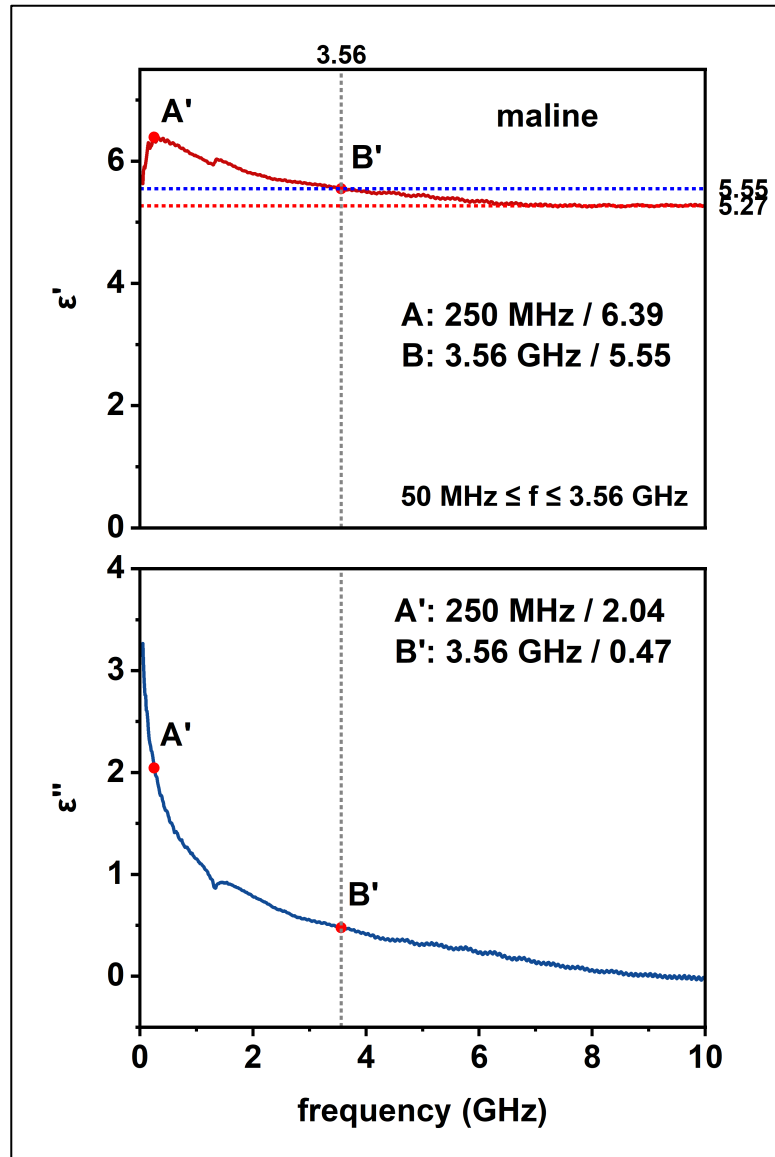


Figure 26. High frequency spectrum of maline (n=3).

The increase from the starting value to the peak is relatively small. As frequency increases, the dielectric constant decreases, crossing the $\epsilon_{75\%}$ line at 3.56 GHz with a value of 5.55. The $\epsilon_{\text{plateau}}$ marks the minimum ϵ' value of 5.27. The frequency range where ϵ is above the $\epsilon_{75\%}$ line is 50 MHz to 3.56 GHz. The dielectric loss at 250 MHz is 2.04, decreasing to 0.47 at 3.56 GHz, indicating relatively low losses that further reduce at higher frequencies.

For oxaline (**Figure 27**), the dielectric constant starts at around 7.24 at low frequencies and peaks at 150 MHz with a value of 8.33, showing a small increase compared to others.

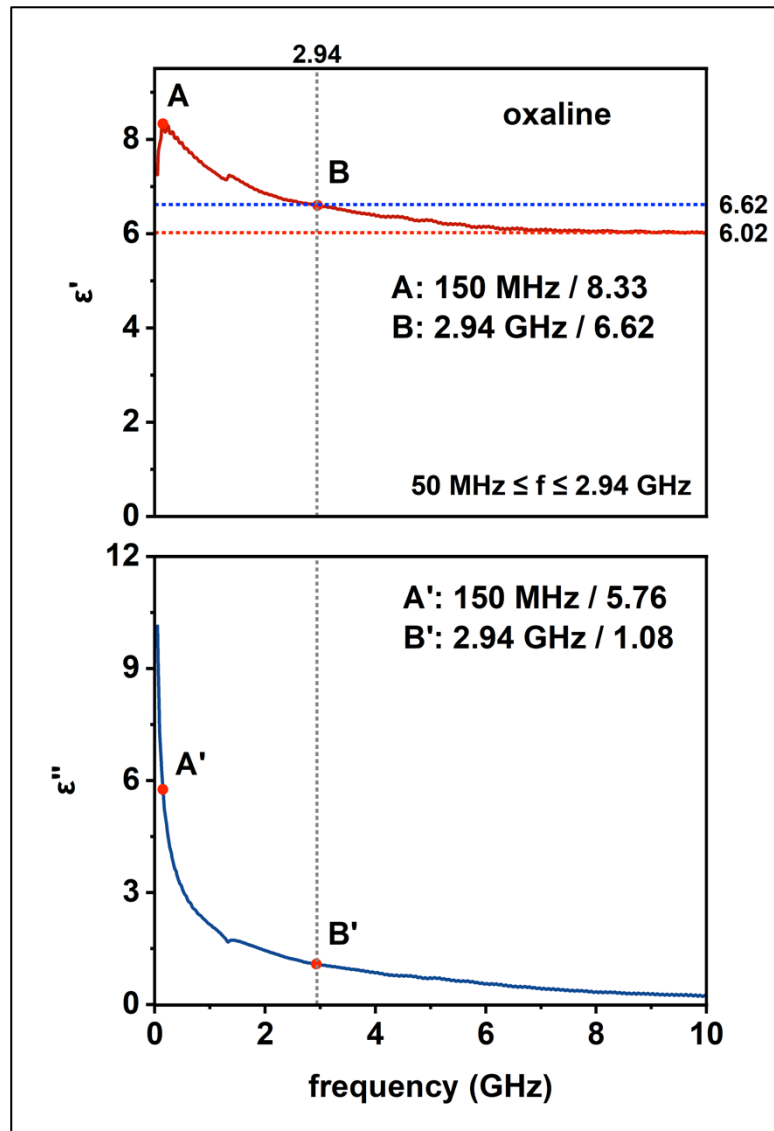


Figure 27. High frequency spectrum of oxaline ($n=3$).

As frequency increases, the dielectric constant decreases, crossing the $\epsilon_{75\%}$ line at 2.94 GHz with a value of 6.62, indicating a broad range for maintained polarization. The $\epsilon_{\text{plateau}}$ marks the minimum ϵ' value of 6.02. The frequency range where ϵ is above the $\epsilon_{75\%}$ line is 50 MHz to 2.94 GHz. The dielectric loss at 150 MHz is 5.76, decreasing to 1.08 at 2.94 GHz, indicating moderate initial losses that reduce significantly at higher frequencies.

The dielectric properties of various DESs reveal distinctive behaviors across a range of frequencies (**Table 12**). Comparing conventional DESs (reline, ethaline, glyceline) and OA-DESs (glycoline, T-glycoline, maloline, maline, oxaline) highlights significant differences in their dielectric constant and dielectric loss, as well as their ability to maintain polarization and exhibit dielectric losses.

Table 12. Summary of the DESs microwave characteristics

Sample		Points Frequencies and Values				Frequency Range
		ϵ'		ϵ''		
		A	B	A'	B'	
OA-DESs	glycoline	150 MHz 14.11	2 GHz 8.65	150 MHz 23.65	2 GHz 4.22	50 MHz ≤ f ≤ 2 GHz
	T-glycoline	238 MHz 5.76	3.56 GHz 4.71	238 MHz 2.99	3.56 GHz 0.66	50 MHz ≤ f ≤ 3.56 GHz
	maloline	138 MHz 10.56	2.43 GHz 7.31	138 MHz 9.76	2.43 GHz 2.04	50 MHz ≤ f ≤ 2.43 GHz
	maline	250 MHz 6.39	3.56 GHz 5.55	250 MHz 2.04	3.56 GHz 0.47	50 MHz ≤ f ≤ 3.56 GHz
	oxaline	150 MHz 8.33	2.94 GHz 6.62	150 MHz 5.76	2.94 GHz 1.08	50 MHz ≤ f ≤ 2.94 GHz
Conventional DESs	reline	125 MHz 11.99	2.25 GHz 8.52	125 MHz 18.52	2.25 GHz 2.62	50 MHz ≤ f ≤ 2.25 GHz
	ethaline	388 MHz 17.69	3.49 GHz 10.53	388 MHz 42.54	3.49 GHz 8.39	70 MHz ≤ f ≤ 3.49 GHz
	glyceline	138 MHz 15.18	1.6 GHz 8.61	138 MHz 23.34	1.6 GHz 5.29	50 MHz ≤ f ≤ 1.6 GHz

Reline, ethaline, and glyceline display high initial values for both the dielectric constant and the dielectric loss, indicating strong electric energy storage and significant dielectric losses. The dielectric loss at their respective peak frequencies are substantial, indicating important initial dielectric losses that decrease significantly at higher frequencies. Ethaline's dielectric loss is the highest, making it potentially more effective for applications requiring high energy dissipation at specific frequencies. In contrast, glycoline, T-glycoline, maloline, maline, and oxaline present lower initial values for both the dielectric constant and the dielectric loss compared to conventional DESs, indicating they may have lesser dielectric losses and potentially lower heating capabilities. The dielectric losses at their respective peak frequencies are lower compared to conventional DESs,

indicating moderate initial losses that generally decrease with increasing frequency.

Conventional DESs exhibit higher initial dielectric constants and larger differences between starting and peak values compared to OA-DESs, suggesting stronger polarization capabilities and significant dielectric losses suitable for applications requiring efficient energy storage and dissipation. Ethaline, with the highest dielectric loss at its peak frequency, may be particularly effective for dielectric heating applications, making it suitable for disrupting bacterial cells and biofilms. Glyceline, with its higher initial dielectric constant and substantial dielectric loss, also shows promise for such applications. OA-DESs, with their lower initial dielectric constants and dielectric loss, may be less effective for direct heating, but could serve as effective solvents or carriers for antibacterial agents. Their moderate dielectric losses suggest they can enhance the delivery and efficacy of active ingredients in topical treatments or surface decontamination processes without causing excessive heating. For instance, maloline and oxaline, with their relatively stable dielectric constants and moderate losses, could be useful in formulations requiring consistent performance over a range of frequencies.

The significant dielectric losses of conventional DESs at lower frequencies imply efficient conversion of electromagnetic energy to heat, beneficial for bacterial infection treatments and surface decontamination. Ethaline and reline, with their broad frequency ranges and substantial dielectric losses, stand out as particularly suitable for applications requiring effective dielectric heating. These properties can be leveraged to disrupt bacterial cells and biofilms, making them ideal for treating bacterial infections and decontaminating surfaces. OA-DESs, on the other hand, with their more moderate dielectric properties, may be more suitable for applications where lower heating, but effective solvent properties are desired. They can serve as carriers for antibacterial agents, enhancing the delivery and efficacy of active ingredients in topical treatments or surface decontamination

processes. Maloline and oxaline, with their relatively stable dielectric constants and moderate losses, could be particularly useful in such applications.

4.2 Antibacterial Evaluation of DESs

4.2.1 Disk Diffusion Test (DD)

All the DESs were screened for their antibacterial properties towards *P. aeruginosa*, MRSA, and *E. coli*[†] using a modified disk-diffusion test (DD) (**Table 13, Figure A12-Figure A14**). The disk-diffusion test allows the assessment of a microorganism's susceptibility to antibiotics. In this method, antibiotic-impregnated disks are placed on an agar plate inoculated with the test microorganism. As the antibiotic diffuses into the agar, it inhibits microbial growth, forming a clear zone around the disk called the zone of inhibition. The size of this zone is measured and compared to standardized charts to determine if the microorganism is susceptible, intermediate, or resistant to the antibiotic. However, it can also be used for more general evaluation just by the observation of the presence or absence of the inhibition halo^[271].

OA-DESs were effective towards all of the investigated strains, with inhibition halos above 16 mm, even surpassing the referral antibiotics. Inhibition halos ranged from 16-17 mm for maline up to 24-27 for oxaline against *E. coli* (13-14 mm for ampicillin); 28-30 mm for oxaline against *P. aeruginosa* (29-30 mm for ciprofloxacin); 23-26 mm for glycoline and 27-30 mm for oxaline against MRSA (23-24 mm for tetracycline). For *E. coli*, the effectiveness increased in the order of R-glyceline < maline < maloline < glycoline < T-glycoline < oxaline; for *P. aeruginosa*: R-glyceline < T-glycoline < maloline < maline < glycoline < oxaline; for MRSA: R-glyceline < maline < maloline < T-glycoline < glycoline < oxaline. In contrary, reline, glyceline and ethaline were not effective at inhibiting the bacteria, while R-

[†] Every time *E. coli* is mentioned, it refers to the *E. coli* K-12 strain unless stated otherwise.

glyceline exhibited minor inhibition. Oxaline, the OA-DES composed of OxA and ChCl, was the most effective material, with inhibition halos in the range of 24-30 mm (**Figure 28**).

Table 13. Inhibition halos diameters in mm for the investigated DESs (ampicillin (10 µg) for *E. coli*, ciprofloxacin (30 µg) for *P. aeruginosa*, and tetracycline (5 µg) for MRSA (n=3).

		Inhibition halo size (mm)		
		<i>E. coli</i>	<i>P. aeruginosa</i>	MRSA
OA-DESs	glycoline	19-20	20-24	23-26
	T-glycoline	23-25	17-19	22-23
	maloline	18-20	18-20	19-23
	maline	16-17	16-22	19-20
	oxaline	24-27	28-30	27-30
Conventional DESs	reline	–	–	–
	ethaline	–	–	–
	glyceline	–	–	–
	R-glyceline	7-8	7-8	8-9
antibiotic		13-14	29-30	23-24

“–” indicates that no inhibition zone was observed.

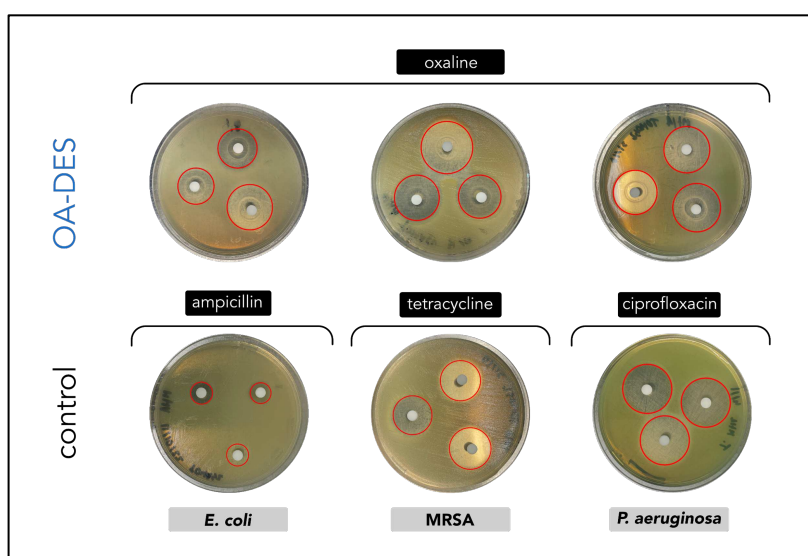


Figure 28. Disk-diffusion test results of oxaline tested against *E. coli*, MRSA, and *P. aeruginosa*, with the control experiments performed using ampicillin, tetracycline, and ciprofloxacin (n=3).

Interestingly, when compared, T-glycoline and glycoline where the only difference is a swap between ChCl and TBACl, it is clearly seen that the behavior was not the same, inferring that both PSs influenced the total effectiveness of the OA-DESs and not only the organic acid.

Interestingly, the inhibition halos for MRSA were greater when compared to *P. aeruginosa* and *E. coli*. Gram-negative bacteria are usually harder to kill as their thin peptidoglycan layer in their cell wall is surrounded by an outer membrane composed of lipopolysaccharides and proteins. This outer membrane acts as an additional physical barrier to the bacteria, inhibiting the penetration of the membrane by drugs. Additionally, Gram-negative bacteria present efflux pump proteins that actively pump out antibiotics from the bacterial cell, further contributing to their resistance. However, since this is a modified version of the DD^[190], where DESs were tested in pure, non-diluted form, one cannot assess their antibacterial properties based solely on this test. Also, DD was used just as a preliminary test for a quick qualitative assessment rather than quantitative.

The main limiting factor that contributes to this test' uncertainty is that DES are viscous liquids, and because of that, the diffusion occurs differently than for the dried substances (such as referral antibiotics)^[168]. This means that these results serve screening purposes only and that antibacterial properties of our formulations must be investigated more in-depth using other techniques as well.

4.2.2 Minimum Inhibitory and Bactericidal Concentration (MIC/MBC)

It has to be noted that DD is not the best test to evaluate the antimicrobial properties of DESs. This is due to the lack of standardization of the test to DESs and liquids, in general^[168]. To further assess the antibacterial properties, all the DESs were investigated for their MIC and MBC values. In order to have a broader view on the potential effectiveness of the DESs, a total of twelve bacteria strains were tested, consisting of six Gram-positive (**Figure A15-Figure A20**) and six

Gram-negative (**Figure A21-Figure A26**), including three strains exhibiting resistance towards antibiotics (CRAB, MRSA and MDRSA).

4.2.2.1 Organic Acid-based Deep Eutectic Solvents

The MIC values determined for classical OA-DESs are summarized in **Figure 29**. The data indicated that all MIC values were below 1% v/v, with the majority falling within the range of 0.15 to 0.4% v/v. However, noteworthy variations were observed in the activity of the investigated OA-DESs among different strains. Specifically, we found that glycoline exhibited the highest performance against *K. pneumoniae*, while maloline showed superior efficacy against *E. coli* and *A. baumannii*.

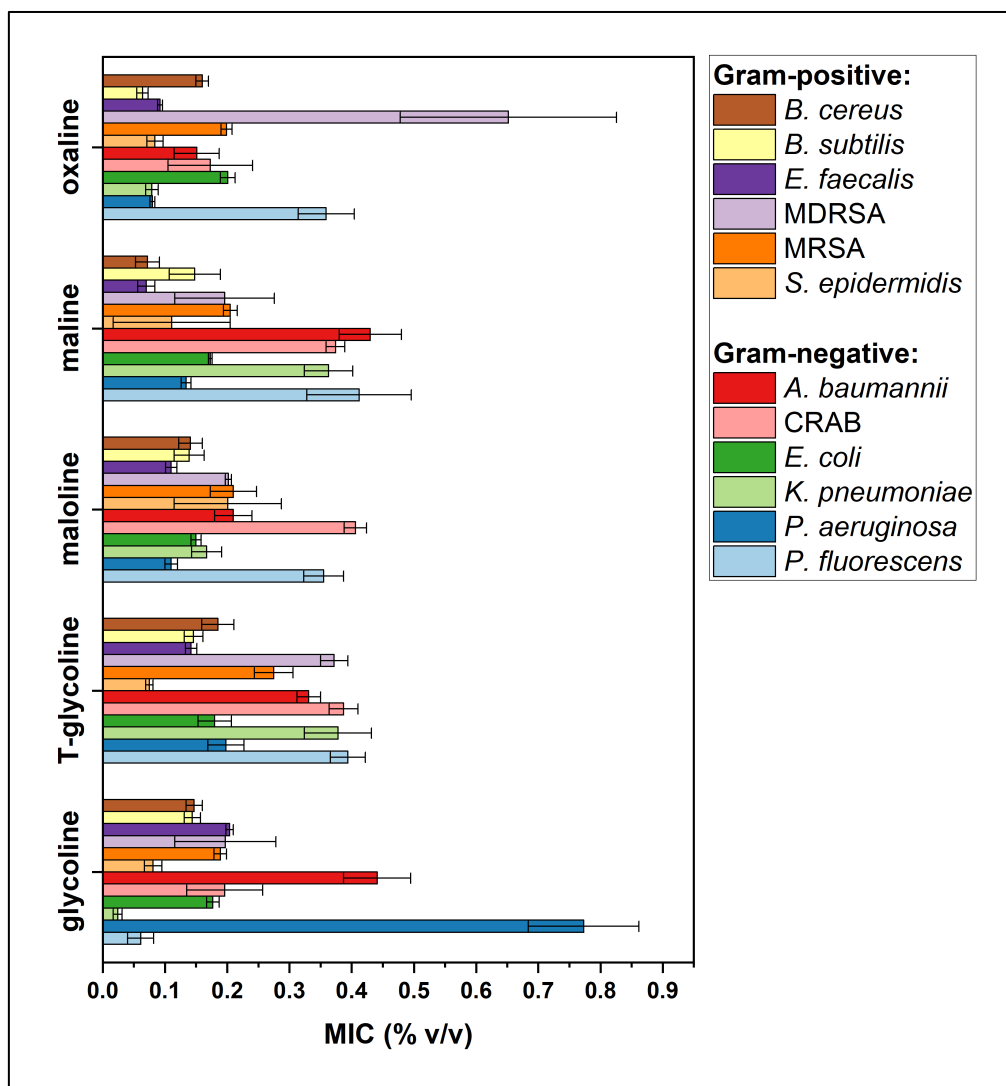


Figure 29. MIC values of OA-DESs towards six Gram-positive strains (n=3).

Additionally, maline demonstrated notable effectiveness against *B. cereus* and *E. faecalis* and oxaline displayed favorable results against *K. pneumoniae* and *P. aeruginosa*. Among the OA-DESs, T-glycoline achieved the greatest effectiveness against *E. coli* and *P. aeruginosa*, with the results in this particular group demonstrating more uniformity. On the other hand, *P. fluorescens* appeared to be the most resistant bacteria, as it consistently maintained medium MIC values for four out of the five tested OA-DESs. These findings provided valuable insights into the varying responses of Gram-negative bacteria to the tested OA-DESs and emphasized the importance of considering bacterial strain-specific differences in antimicrobial activity. After conducting the Gram-negative bacteria assessment, we investigated the effectiveness of the OA-DESs against Gram-positive bacteria. Similar to the previous test, all MIC values for these substances against Gram-positive bacteria were below 1% v/v. However, a majority of the values fall below 0.2% v/v, resulting in an overall average MIC value lower than that observed for Gram-negative strains, which goes along with the general resistance differences between Gram-negative and Gram-positive bacteria. The activity of the investigated substances showed comparable results across the majority of the strains. However, MRSA and MDRSA exhibited the highest MIC values. Nevertheless, the effectiveness of all the investigated substances was similar across the strains. Considering the statistical error, we can assume that the most effective substance was maloline.

4.2.2.2 Conventional Deep Eutectic Solvents

To compare the total effectiveness of our formulations, MIC assessment was also carried out for conventional DESs, namely: glyceline, R-glyceline, ethaline, and reline. Activity profile differed greatly between Gram-negative and Gram-positive strains. For the Gram-positive strains, we were not able to achieve MIC values in most cases (**Figure 30**). The only examples were *B. subtilis* (reline) and MDRSA (glyceline), which exhibited MIC values of 12.97 ± 2.74 and 22.93 ± 1.98 % v/v, respectively. Also, R-glyceline displayed great variation of the efficiency, ranging

from 2.72 ± 0.26 % v/v for MDRSA to lack of activity for *E. faecalis*. On the other hand, the activity of the same DESs towards Gram-negative bacteria differed very much. Investigated DESs were able to inhibit the growth of the majority of the bacteria with only *K. pneumoniae* being resistant to glyceline and ethaline, and *P. fluorescens* being resistant to ethaline and R-glyceline.

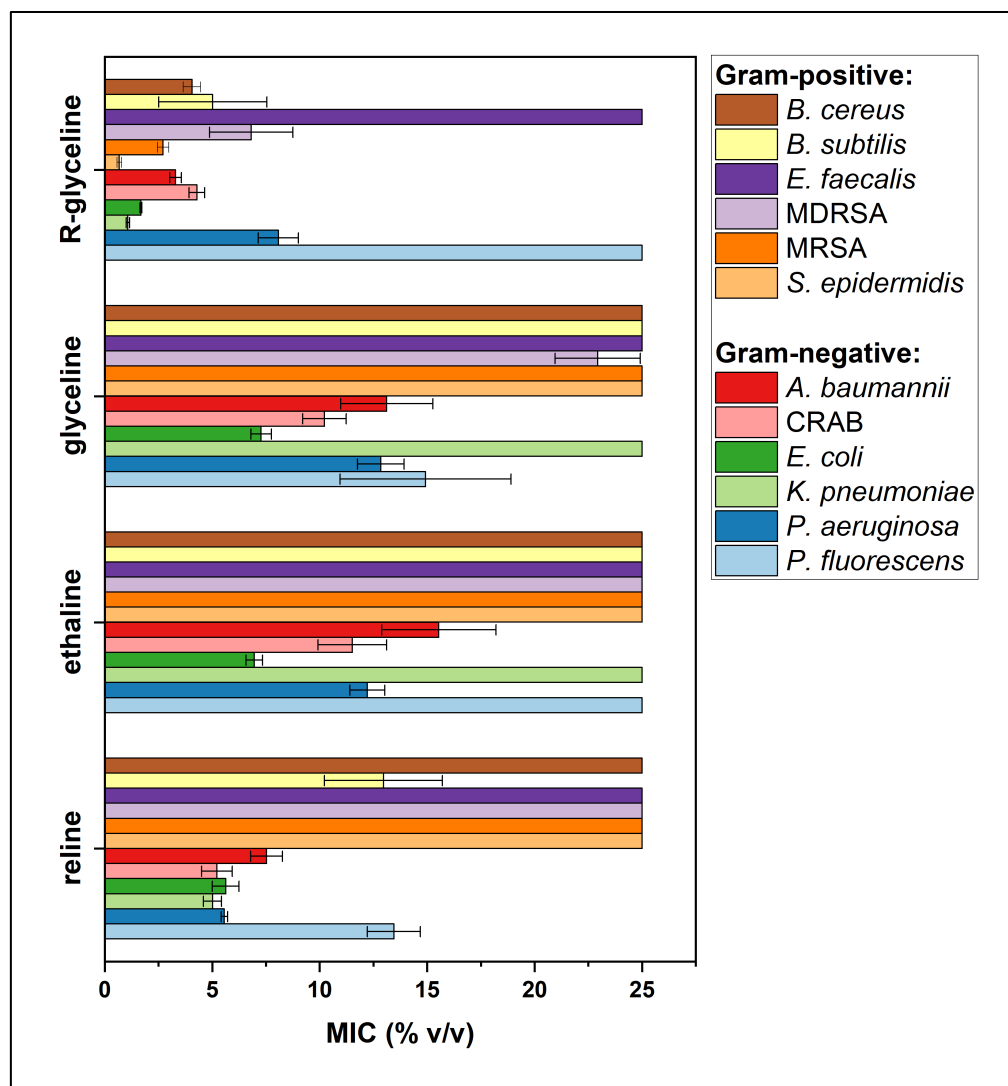


Figure 30. MIC values of conventional DESs (lack of error bars indicates that MIC value was not observed; n=3).

All the other strains were inhibited in the range of concentration between 5-15% v/v. Of all the investigated DESs of this group, R-glyceline was the most effective with a MIC as low as $1.08 (\pm 0.05)$ % v/v for *K. pneumoniae* and having the overall lowest average of the MIC values.

4.2.2.3 Comparative Studies with Parent Substances

Since most of the carboxylic acids exhibit antimicrobial effect, it was necessary to first compare their activity with that of DESs on chosen bacterial strains. To that end, MIC assessments were performed for all the PSs (**Table 14-Table 16; Figure A27**) and DESs (**Table A1** and **Table A2**) on *E. coli*, *P. aeruginosa*, and MRSA. The data was originally expressed as mmol/mL and later recalculated to mmol/L (mM). For all the liquids their MIC values have been converted from % v/v after assessing their densities (DESs (**Table 10**); glycerol and ethylene glycerol according to the manufacturer data sheets). In instances where fitting with the Gompertz model proved unattainable, turbidity was assessed through visual observation.

Table 14. MIC values against *E. coli* of parent substances compared to their concentration at MIC value of the DES formulation (n=3).

DES	PS ₁	PS ₂	MIC DES (mM)	MIC PS ₁ (mM)	C PS ₁ (DES) (mM)	MIC PS ₂ (mM)	C PS ₂ (DES) (mM)
glycoline	ChCl	GlycA	7.34	273*	7.34	89 ± 4	14.68
T-glycoline	TBACl	GlycA	4.35	38 ± 1	4.35	89 ± 4	8.71
maloline	ChCl	MaloA	6.28	273*	6.28	68 ± 1	6.28
maline	ChCl	MalA	6.57	273*	6.57	60 ± 1	6.57
oxaline	ChCl	OxA	9.89	273*	9.89	38 ± 1	9.89
reline	ChCl	U	253.46	273*	253.47	132 ± 12	506.94
ethaline	ChCl	EG	295.75	273*	295.75	-	591.51
glyceline	ChCl	GLY	265.04	273*	265.04	-	530.09
R-glyceline	Arg	GLY	33.87	-	33.87	-	135.49

"-" indicates that no inhibition was observed in the investigated range; "*" indicates visual MIC assessment.

Table 15. MIC values against *P. aeruginosa* of parent substances compared to their concentration at MIC value of the DES formulation (n=3).

DES	PS ₁	PS ₂	MIC DES (mM)	MIC PS ₁ (mM)	C PS ₁ (DES) (mM)	MIC PS ₂ (mM)	C PS ₂ (DES) (mM)
glycoline	ChCl	GlycA	32.06	-	32.06	74 ± 3	64.13
T-glycoline	TBACl	GlycA	4.79	944 ± 12	4.79	74 ± 3	9.58
maloline	ChCl	MaloA	18.96	-	18.96	62 ± 2	18.96
maline	ChCl	MalA	5.09	-	5.09	55 ± 2	5.09
oxaline	ChCl	OxA	3.94	-	3.94	38 ± 2	3.94
reline	ChCl	U	250.81	-	250.81	451*	501.62
ethaline	ChCl	EG	518.81	-	518.81	849*	1037.62
glyceline	ChCl	GLY	468.14	-	468.14	728*	936.27
R-glyceline	Arg	GLY	162.31	2007*	162.31	728*	649.23

"-" indicates that no inhibition was observed in the investigated range; "*" indicates visual MIC assessment.

Table 16. MIC values against MRSA of parent substances compared to their concentration at MIC value of the DES formulation (n=3).

DES	PS ₁	PS ₂	MIC DES (mM)	MIC PS ₁ (mM)	C PS ₁ (DES) (mM)	MIC PS ₂ (mM)	C PS ₂ (DES) (mM)
glycoline	ChCl	GlycA	7.84	-	7.84	78 ± 3	15.68
T-glycoline	TBACl	GlycA	6.65	37 ± 3	6.65	78 ± 3	13.30
maloline	ChCl	MaloA	8.79	-	8.79	65*	8.79
maline	ChCl	MalA	7.79	-	7.79	57 ± 2	7.79
oxaline	ChCl	OxA	9.79	-	9.79	36 ± 1	9.79
glyceline	ChCl	GLY	-	-	-	-	-
R-glyceline	Arg	GLY	54.56	-	54.564	-	218.26
ethaline	ChCl	EG	-	-	-	-	-

reline	ChCl	U	-	-	-	-	-
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"-" indicates that no inhibition was observed in the investigated range; "*" indicates visual MIC assessment.

Our results showed that formulation of PSs into DESs had improved antibacterial activity with at least four times lower MIC values with some minor exemptions. Interestingly, substances, such as choline chloride, urea, glycerol, ethylene glycol and L-arginine, did not exhibit MIC values even at highest concentrations that correspond to their maximum concentration at DES formulation. These results confirm, the alteration of biological properties of PSs when formulated in the form of DESs.

4.2.2.4 Minimum Bactericidal Concentration of Deep Eutectic Solvents

After each of the above-mentioned tests, an assessment of MBC was conducted. The MBC values of each of the bacterial strain were estimated based on the observable absence of bacterial colonies on the suitable agar plates (**Table 17**). All the OA-DESs exhibited MBC, with a value being usually 2-4 times higher than MIC. On the contrary, conventional DESs were not able to completely eradicate investigated bacteria. For a selected group of Gram-negative strains, *E. coli*, *P. aeruginosa*, CRAB, and *A. baumannii*, DESs were capable to eradicate the bacteria in most cases, while for others varied activity was recorded. Almost all of the Gram-positive strains were able to survive even the highest concentrations, with the exception of R-glyceline, which was effective in the eradication of four out of six strains. Interestingly, within the same genera, some differences in the susceptibility were evidenced. The *Bacillus* genus displayed minimal variations in its characteristics. For the majority of the tested DESs, they demonstrated comparable MIC and MBC values, differing by no more than 20%. However, *B. cereus* and *B. subtilis* responded differently to maline and oxaline. *B. cereus* exhibited higher susceptibility to maline (MIC = 0.07% v/v), which was twice as low as the MIC observed for *B. subtilis*. Conversely, *B. subtilis* showed greater

sensitivity to oxaline (MIC = 0.06% v/v), while nearly three times the dosage was required to inhibit *B. cereus*. Additionally, relene only exhibited inhibitory effects on the growth of *B. subtilis*, with no activity observed towards the other species. Both species also required nearly identical concentrations for complete eradication. Staphylococcus, another genus examined in our study, also displayed distinct behaviors in the presence of DESs. Two species (three strains) demonstrated different behaviors. Generally, *S. epidermidis* exhibited inhibition at doses at least twice as low as those required for MRSA, often resulting in MIC values 2-3 times lower. However, both species maintained similar MBC values, suggesting that comparable doses of OA-DESs were required for their complete eradication. In comparison to MRSA, MDRSA resistance was not observed. MDRSA displayed relatively low MIC values, and higher dosages were only required for T-glycoline, oxaline, and R-glyceline. Achieving complete bacterial eradication needed at least twice the concentration for MDRSA compared to MRSA, with no eradication observed for glycoline and R-glyceline. Among the Gram-negative group, the *Pseudomonas* genus displayed significant variation. Particularly, *P. aeruginosa* demonstrated lower MIC values when exposed to all, but two of the DESs, indicating susceptibility to almost all conventional DESs as well. On the other hand, *P. fluorescens* exhibited inhibition at concentrations more than ten times lower for glycoline, and approximately only 20% lower for maloline, as compared to *P. aeruginosa*. Furthermore, *P. fluorescens* showed greater resistance to conventional DESs, specifically exhibiting resistance to R-glyceline and ethaline. While the MBC values for these species revealed slight differences, the most notable disparity was observed for oxaline, which featured a preferable eradication effect on *P. aeruginosa*. Two strains of the *A. baumannii* species demonstrated different responses depending on the DES used. Maloline and maline exhibited favorable inhibition of *A. baumannii*, with MIC values approximately 20 times lower. Additionally, glycoline inhibited CRAB at a concentration 2 times lower. However, CRAB strain required at least 2-4 times

higher concentrations of DESs for complete eradication, with MBC values reaching as high as 1.56% v/v.

Table 17. MBC values of the investigated DES

Strain	OA-DES					Conventional DES			
	glycoline	T-glycoline	matoline	matine	oxaline	glyceline	R-glyceline	ethaline	reline
MRSA	0.78	0.39	0.78	0.78	0.39	-	3.12	-	-
MDRSA	0.39	0.78	0.78	0.78	1.56	-	-	-	-
<i>S. epidermidis</i>	0.19	0.19	0.78	0.78	0.39	-	1.56	-	-
<i>B. cereus</i>	0.19	0.39	0.19	0.19	0.39	-	12.50	-	-
<i>B. subtilis</i>	0.19	0.39	0.39	0.19	0.39	-	6.25	-	-
<i>E. faecalis</i>	0.39	0.39	0.19	0.19	0.19	-	-	-	-
<i>E. coli</i>	0.19	0.39	0.39	0.78	3.13	6.25	3.13		
<i>K. pneumoniae</i>	0.05	0.78	0.39	0.78	0.19	-	3.12	-	6.25
<i>P. aeruginosa</i>	1.56	0.39	1.56	0.39	0.19	-	12.50	12.50	6.25
<i>P. fluorescens</i>	0.78	0.78	0.78	0.78	0.78	25.00	-	-	25.00
CRAB	0.39	1.56	0.78	1.56	0.39	-	6.25	-	6.25
<i>A. baumannii</i>	0.78	0.78	0.39	0.39	0.39	-	6.25	-	12.50

Our findings are in accordance with previously reported data. As an example, bacterial inhibition towards *E. coli*, *S. aureus* and *P. aeruginosa* was observed for both reline and oxaline^[197]. However, in the work of Hayyan et al.^[272], no bacterial inhibition was found for reline, ethaline, and glyceline towards *E. coli*, *S. aureus*,

P. aeruginosa and *B. subtilis*. In another work, by Zhao et al.^[273], similar formulations (reline, ethaline, glyceline, maloline, and maline) were assessed towards *E. coli* and *S. aureus*. No activity was recorded for the first three DESs, while inhibition halos were observed for organic acid-based DESs, confirming their activity. The discrepancy of these and our findings in case of classical DESs can be attributed to at least two factors. First point is that different strains may exhibit differed behaviors to DESs, which was also confirmed in our work (as seen for MDRSA, MRSA, CRAB and *A. baumannii*). Another even more important point is that DD was used as a method of choice. As already mentioned in our work, as well as in work of Marchel et al.^[168], due to physical properties of DESs, DD is not a suitable method to describe the antimicrobial potential of DESs. This is also confirmed by our findings in previous section where classical DESs exhibited barely observable inhibition halos, and only MIC/MBC assessment using microbroth dilution method correlated with initial screening of PSs enabled us to describe antibacterial potential of all of the nine DESs.

4.2.3 Time-Kill Kinetics Assay (TK)

In order to better understand the effectiveness of the investigated DESs, a time-kill kinetics assay was performed. The purpose of this assay is to better understand the nature of interaction of the substance with the bacteria, namely its bactericidal/bacteriostatic activity, time-dependent activity, and concentration-time correlated activity^[274,275]. Furthermore, it enables to recognize the DESs that own enhanced efficiency in terms of their speed of bacterial eradication. TK were performed on three strains, similarly to DD, namely *E. coli*, *P. aeruginosa*, and MRSA – representatives of Gram-positive and Gram-negative bacteria. The tests were conducted for the two most effective representatives in both groups of the tested substances – R-glyceline and oxaline. From the findings presented in **Table 18** and **Figure A28**, a clear trend emerges in the activity levels of the investigated DESs. Across most scenarios, these DESs exhibit remarkably swift actions, often falling below the 2-hour mark. Notably, in the case of *P. aeruginosa* and *E. coli* –

representatives of Gram-negative bacteria – their eradication occurred almost instantaneously, within an hour. Conversely, the Gram-positive MRSA required a considerably longer duration to surpass the $t_{99.9\%}$ threshold, necessitating higher DES concentrations. Intriguingly, despite originating from distinct families (OA-DESs and conventional DESs), both types showcased a similar efficacy, hinting at a shared mechanism of action, although this hypothesis is questioned by subsequent TEM findings.

Table 18. TK results – time for eradication of 99.9% bacteria ($t_{99.9\%}$) against the concentration of DESs (n=3).

DES	MIC factor	$t_{99.9\%}$ (h)		
		<i>E. coli</i>	<i>P. aeruginosa</i>	MRSA
oxaline	1	< 8	< 2	-
	2	< 4	< 1	< 8
	4	< 1	< 1*	< 4
	8	< 1*	< 1*	< 4
R-glyceline	1	< 2	< 2	-
	2	< 2	< 2	< 8
	4	< 1	< 1	< 4
	8	< 1*	< 1*	< 1

“*” indicates further increase of the concentration resulted in instant eradication of bacteria within the time interval

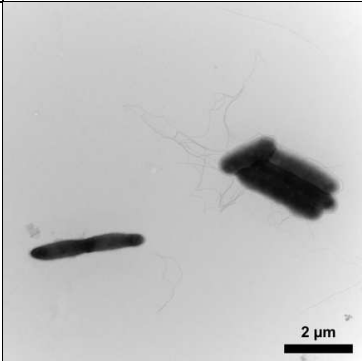
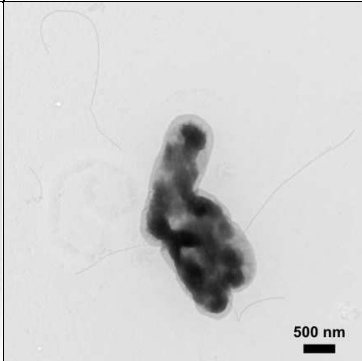
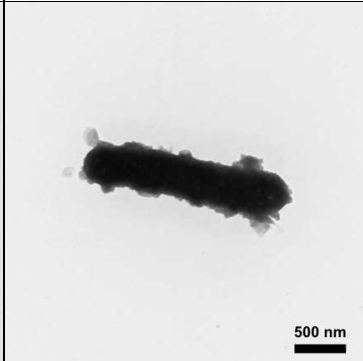
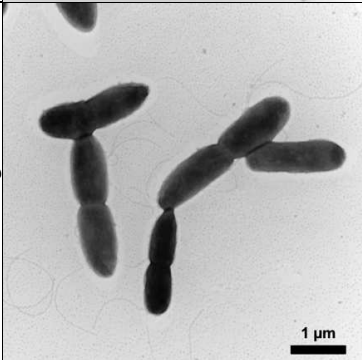
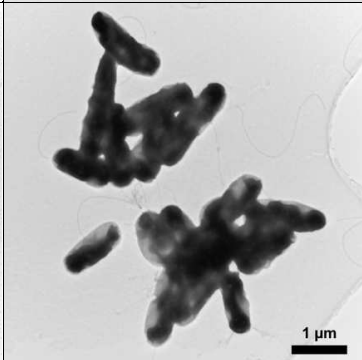
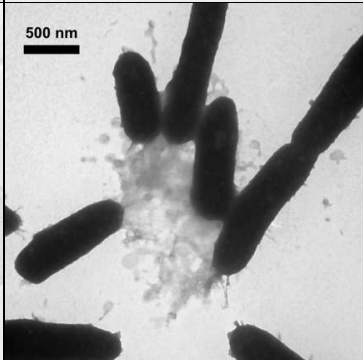
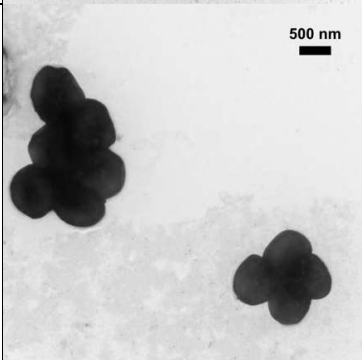
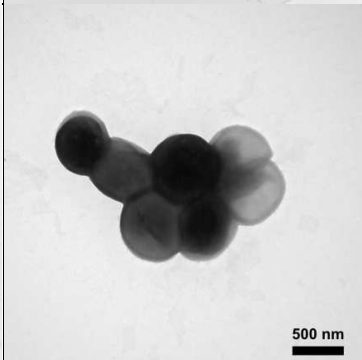
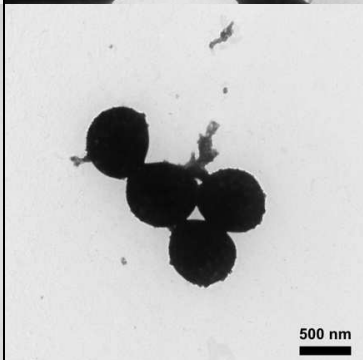
Moreover, the data highlights the proximity between MBC and MIC values, underscoring the need to comprehend the less precise methodology behind MBC determination, despite its close association with MIC values. These observations offer valuable insights into the rapid action of DESs against various bacterial strains, shedding light on their potency and potential shared mechanisms. However, the discrepancies unearthed between the two DES families and the

nuances in assessing MBC values underscore the complexity of these compounds' actions, necessitating further exploration for a comprehensive understanding.

4.2.4 Transmission Electron Microscopy (TEM)

Following physicochemical characterization and microbiological evaluation, microscopic analysis using transmission electron microscopy was performed (Table 19).

Table 19. TEM Micrographs of the investigated strains treated with OA-DES (oxaline) and conventional DESs (R-glyceline).

	Non-treated	R-glyceline (Conventional DES)	oxaline (OA-DES)
<i>E. coli</i>			
<i>P. aeruginosa</i>			
MRSA			

Same as for DD and TK, we investigated three main strains of interest: *E. coli*, *P. aeruginosa*, and MRSA. These strains were treated with most effective (in terms of TK and MIC) DESs, namely: R-glyceline and oxaline. Bacterial cultures of the OD₆₀₀ were treated overnight with 8×MIC value of the investigated DESs to ensure total eradication.

As seen in the micrographs, conventional DESs bacteria maintained their shape and uniformity. Their shape is well-preserved, the bacterial wall seems intact, their interior is uniform and for Gram-negative strains, the flagella/fimbriae are visible and attached to the cell. Bacterial strains treated with conventional DESs were characterized by the presence of the flagella/fimbriae and being partially emptied out. This is clearly seen for *E. coli* and is indicated by the bright areas inside the cell. The dark spots inside are possibly due to remaining cytoplasm. Similar morphology was observed for *P. aeruginosa*. For MRSA, the number of emptied bacteria was a little bit lower, but when spotted, cells were totally emptied out, without dark spots present inside. The observed morphology might suggest the formation of nanopores through which cytoplasm spills out, effectively leading to the death of the cells. On the contrary, bacteria treated with OA-DESs exhibited major differences. Gram-negative strains lost their fimbriae/flagella and for all the observed strains, disruption of the bacterial wall was observed. This may be a result of the interaction of the DESs with either proteins present on the surface of the wall or the wall itself.

4.3 Investigation of Eradication Efficiency of DESs Towards MRSA and *E. coli* Biofilms

The presence of biofilm presents a significant threat to human health. While occurring naturally in both the environment and the human body, biofilms are among the leading causes of bacterial-related deaths. Due to their composition and structure, they resist the effectiveness of many antibiotics, often requiring either mechanical removal or the use of antibiotics in higher concentrations.

However, neither solution is permanent, and each has its drawbacks. In the first approach, biofilm rebuilds quickly due to residual bacterial cells left on contaminated surfaces, leading to chronic infections. In the second scenario, increased antibiotic concentrations can foster AMR and heightened toxicity to human cells. Both approaches diminish healthy cell populations, prolonging treatment duration by hindering the repair of damaged areas.

Given the challenges in treating biofilms, it is essential to explore the potential of DESs in eradicating biofilm-encased bacteria. This could be achieved through the inherent antibacterial properties of DESs, coupled with their high permeability. Specifically, all the OA-DESs, which have demonstrated superior antibacterial properties, were tested against biofilms formed by two commonly found bacteria, namely *E. coli* and MRSA, using colony forming assay coupled with Live/Dead bacterial staining. In addition to the eradication of biofilms, the study also focused on the potential emergence of persister cells following DES treatment – a subpopulation of dormant bacteria that can survive antibiotic treatments and potentially lead to recurrent infections via rebuilding of biofilms.

4.3.1 Colony Forming Assay (CF)

The primary method used for the evaluation of biofilm eradication was the colony-forming assay. Unlike the well-known technique of crystal violet staining, it is a quantitative method that directly measures the number of viable bacteria within the biofilm. While crystal violet staining assesses the total biomass (including both live and dead cells, as well as extracellular matrix), the CF assay specifically reflects the ability of bacteria to proliferate, providing a more accurate measure of the biofilm's viability after treatment^[276,277]. The colony forming assay involved cultivating MRSA or *E. coli* cells in a 24-well microplate, followed by treatment, homogenization, and plating onto agar-coated dishes. MRSA biofilms were grown for 24 hours, while *E. coli* biofilms required 72 hours to mature. Subsequently, all

biofilms were treated with OA-DESs for a time of 4 hours, at concentrations matching the MIC assessment, with 10 concentrations ranging from 25% v/v.

4.3.1.1 MRSA Biofilm

As seen in the **Figure 31** and **Figure 32**, all the MRSA biofilms reached a bacterial load between 6 to 7.5 log units suggesting a good growth conditions for this strain. Slight differences in the controls may arise from the minor differences during biofilm initial inoculation, however, they are not of great importance as the control was grown for each of the experiment separately. It is evident that all tested biofilms reached a substantial bacterial load, indicating favorable growth conditions for the MRSA strain across the board.

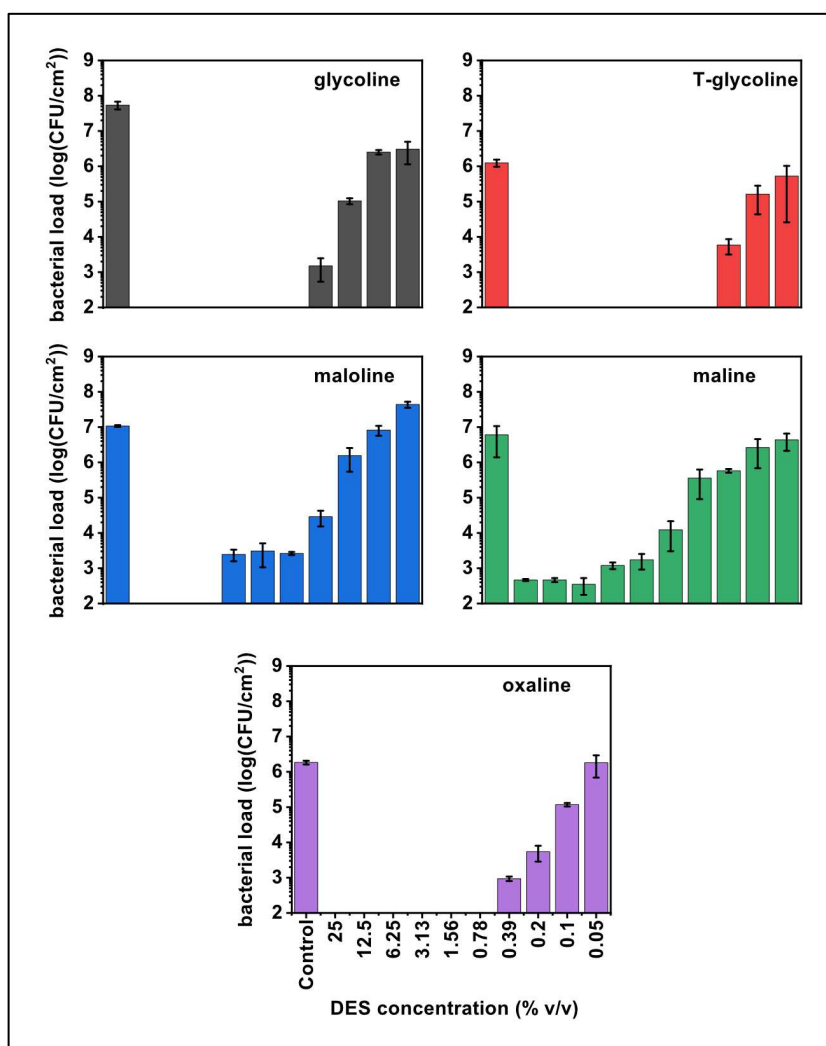


Figure 31. CF assay of MRSA biofilm after treatment with OA-DESs.

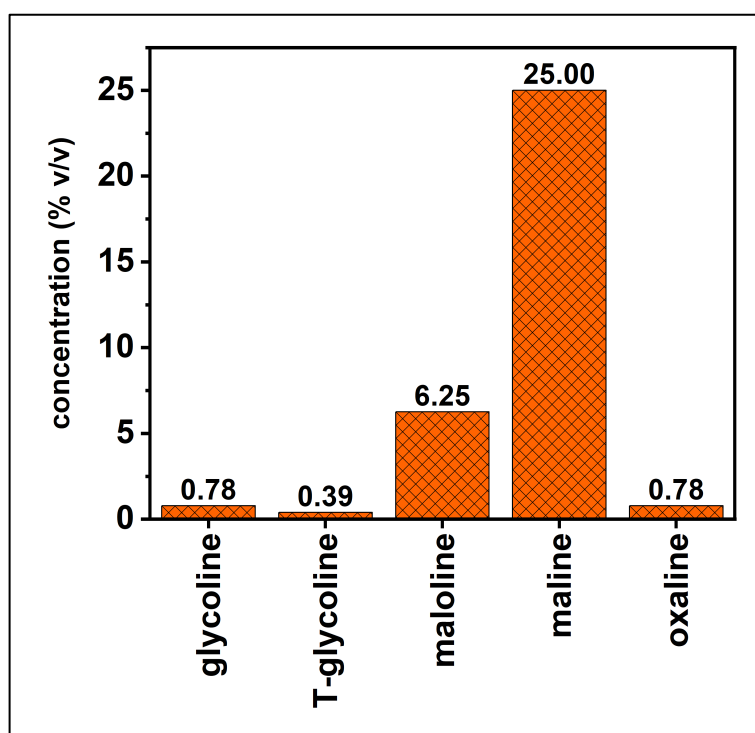


Figure 32. Comparison of eradication concentration values of the OA-DESs against MRSA biofilm.

Of notable interest is the remarkable anti-biofilm activity demonstrated by T-glycoline, which proved highly effective at a concentration of 0.4% v/v, only 1.5 times higher than its MIC value. Both glycoline and oxaline also exhibited potent antibacterial properties, eradicating all MRSA cells at concentrations as low as 0.78% v/v, albeit at nearly four times their respective MIC values.

Maloline emerged as the third most effective OA-DES, albeit with a higher eradication value of 6.25% v/v, representing a 30-fold increase from its MIC value. Interestingly, maloline displayed an unconventional pattern of bacterial load reduction, with a plateau observed between 0.78% to 3.13% v/v concentrations.

On the contrary, maline failed to achieve complete MRSA eradication within the biofilm structure. While a steady decrease in bacterial load was observed up to a concentration of 6.25% v/v, a notable presence of MRSA cells persisted even at the highest concentration, with approximately 2.5 log units still detected.

These findings underscore the potential of OA-DES solutions, particularly T-glycoline, glycoline, and oxaline, as promising candidates for combating MRSA

biofilms. However, further investigation is warranted to elucidate the underlying mechanisms behind maloline's unique efficacy profile and to address the limitations encountered with maline.

4.3.1.2 *Escherichia coli* Biofilm

When it comes to the treatment of *E. coli* biofilm, we can clearly see that control values of bacterial load were much less scattered and ranged between 7 to 8 log units. When compared to MRSA controls the overall difference in bacterial load is easily explained by the prolonged maturation time, as well as average cell division time which is faster for *E. coli* than for MRSA.

The findings from **Figure 33** and **Figure 34** continue to highlight the effectiveness of T-glycoline as an antimicrobial agent, surpassing other OA-DES solutions in eradicating *E. coli* biofilms. Notably, T-glycoline exhibited an eradication concentration value of 0.39% v/v, which is merely double its MIC value. What is intriguing is the sharp decline in bacterial load observed within a narrow concentration range, indicating a threshold effect where bacterial eradication abruptly occurs beyond a certain concentration. This suggests a potentially critical concentration threshold for T-glycoline's antimicrobial activity against *E. coli* biofilms.

Following T-glycoline, oxaline emerged as the second most effective OA-DES, requiring an eradication concentration of 0.78% v/v, approximately four times its MIC value, to achieve complete eradication of *E. coli* cells within the biofilm matrix.

Maloline demonstrated considerable efficacy as the fourth best OA-DES, eradicating all *E. coli* cells at a concentration of 1.56% v/v, representing a more than 15-fold increase from its MIC value.

In contrast, maline and glycoline displayed lower efficacy, requiring concentrations as high as 12.5% v/v to eliminate biofilm-encased bacteria, corresponding to an over 80-fold increase from their MIC values.

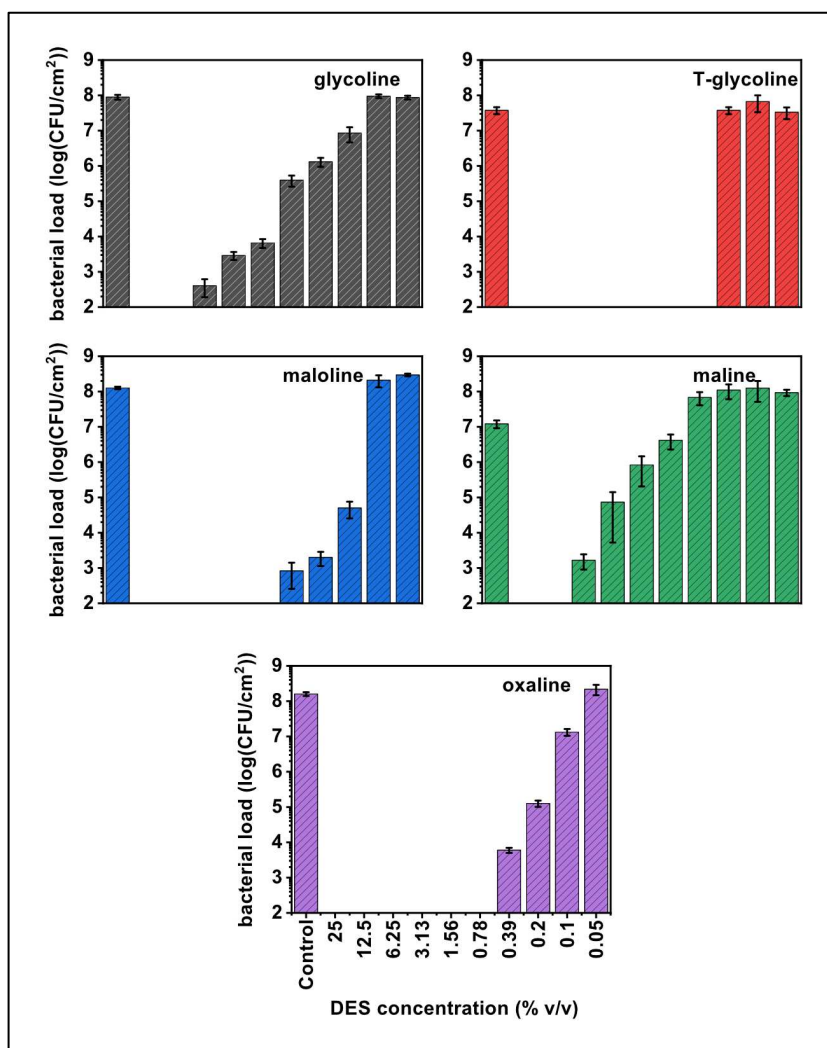


Figure 33. CF assay of *E. coli* biofilm after treatment with OA-DESs.

Comparing the efficacy of OA-DES solutions against MRSA and *E. coli* biofilms reveals intriguing nuances in antimicrobial activity that warrant further exploration. While T-glycoline consistently demonstrates potent antibiofilm properties across both bacterial strains, the substantial difference in eradication concentrations between MRSA and *E. coli* underscores the complexity of bacterial responses to antimicrobial agents. This variation could stem from inherent differences in the genetic makeup, cell wall structure, or biofilm composition between the two pathogens, influencing their susceptibility to specific compounds like T-glycoline.

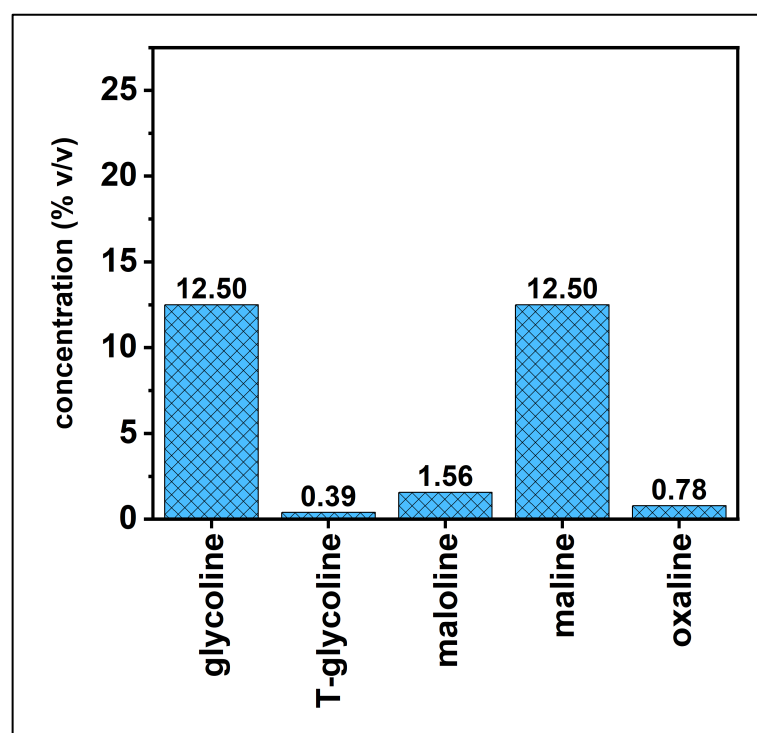


Figure 34. Comparison of eradication concentration values of the OA-DESs against *E. coli* biofilm.

The observed concentration thresholds for T-glycine against each bacterium add another layer of complexity to the discussion. The sharp decline in MRSA bacterial load within a narrow concentration range suggests a potential threshold effect, where incremental increases in T-glycine concentration led to abrupt and total eradication. Conversely, *E. coli* biofilms exhibit a different response profile, requiring a higher eradication concentration of T-glycine, without displaying the same threshold behavior. This disparity highlights the need for a nuanced understanding of species-specific responses to antimicrobial agents, considering not only the MIC values but also concentration-dependent effects and potential threshold phenomena.

Furthermore, the varying effectiveness of other OA-DES solutions against MRSA and *E. coli* biofilms underscores the importance of tailoring antimicrobial strategies to specific bacterial pathogens and biofilm contexts. Investigating the underlying mechanisms driving these differences could yield valuable insights into the complex interplay between bacterial biofilms and antimicrobial agents. Such

insights could inform the development of more targeted and effective treatment approaches against biofilm-associated infections, potentially expanding the arsenal of therapeutic options available for combating antibiotic-resistant pathogens like MRSA and clinically relevant bacteria such as *E. coli*.

4.3.2 Live/Dead Staining (LD)

All the above mentioned biofilm both before and after the treatment were subjected to Live/Dead staining. These procedure has many variations, but in this case a pair of propidium iodide (PI) and SYTO™ 9 fluorescent labels were used. The first stain is a fluorescent dye that is impermeable to intact cell membranes. It penetrates only cells with compromised membranes, such as dead or damaged cells. Once inside these cells, PI binds to nucleic acids (DNA/RNA) and emits red fluorescence when excited by an appropriate light source. SYTO™ 9 on the other hand is a green, fluorescent dye that easily penetrates both live and dead cells regardless of membrane integrity. It binds to nucleic acids in the cell and emits green fluorescence when excited by light.

This technique provides invaluable insights into the distribution of live and dead bacteria within these complex microbial communities. In planktonic cultures, LD staining aids in assessing bacterial viability and susceptibility to antimicrobial agents. In biofilms, it unveils the spatial organization of live and dead cells within the biofilm matrix, offering critical information on biofilm architecture, antibiotic resistance, and microbial interactions. Ultimately, LD staining serves as a powerful tool for understanding bacterial physiology, antibiotic efficacy, and the dynamics of microbial communities in various environmental settings.

Biofilms formed by MRSA (**Figure 35**), when treated with designated eradication concentrations of DESs, underwent staining and subsequent imaging to assess their structural integrity and microbial viability. The initial structure of MRSA biofilms (control) at the microscale was described as heterogeneous, loosely packed, multilayered, and resembling cotton.

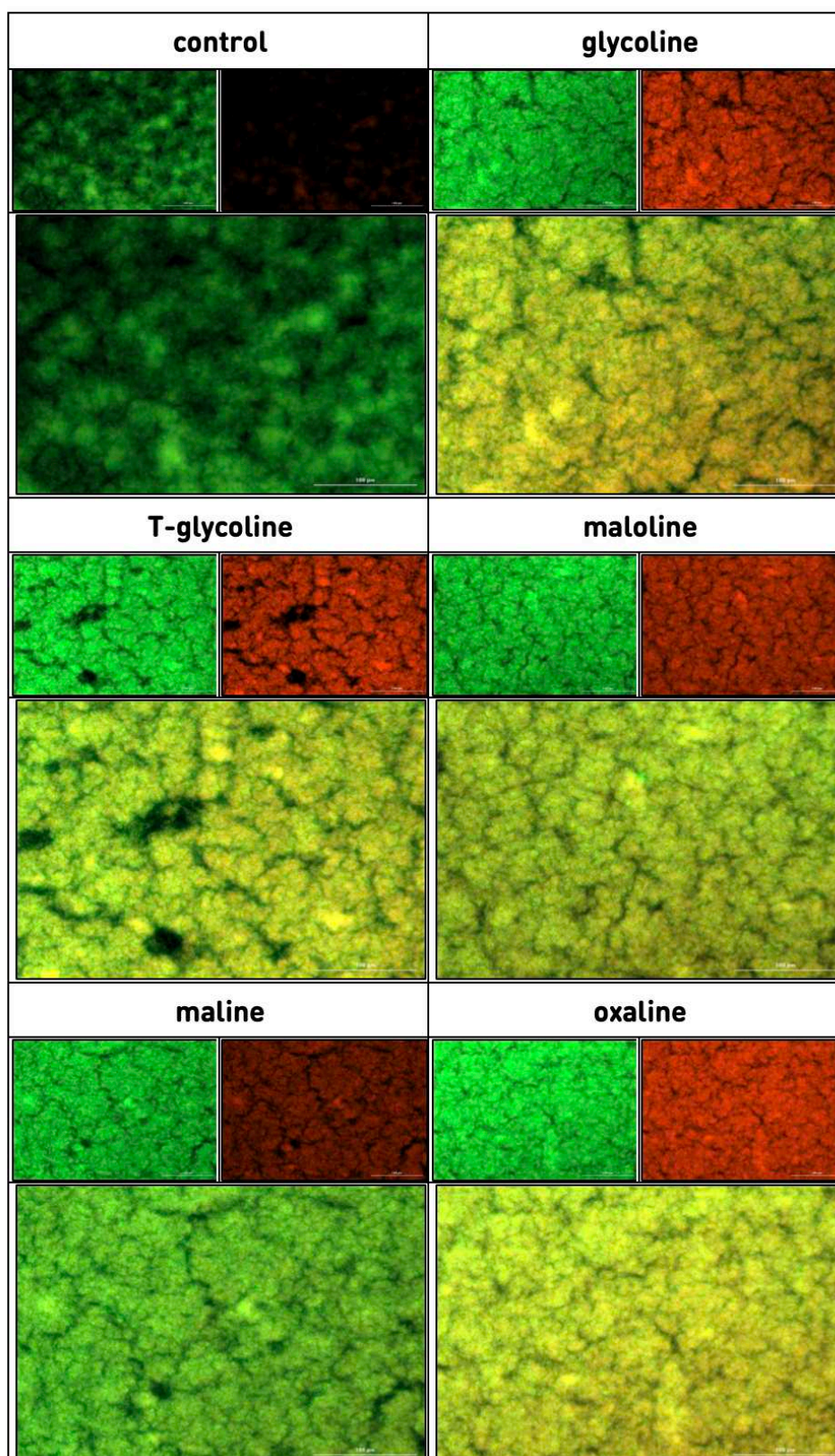


Figure 35. LD staining of MRSA biofilms treated with OA-DESSs.

The presence of dead cells was relatively low, attributed to the mild release of PI. Upon treatment with OA-DESs, the biofilms underwent a significant transformation, becoming denser with visible crevices, indicating potential interaction between the OA-DESs and EPS, possibly through protein denaturation caused by organic acids. Notably, T-glycoline induced the formation of pores in the biofilms, potentially facilitating better penetration of antimicrobial agents into the structure. Additionally, maline resulted in a notably lower number of dead cells, evident in the composite image by a more pronounced green hue.

The complementary SYTO™ 9 stain, while also shedding light on bacterial load, requires careful interpretation due to its potential interaction with both dead bacteria and nucleic acids within the extracellular polymeric substance matrix. Nonetheless, its application underscores the likely high efficacy of the DES treatment in eradicating the biofilm. These findings suggest that DESs hold promise as effective agents in disrupting biofilms, highlighting the need for further investigation into their mechanisms and potential clinical applications.

As seen in the **Figure 36**, *E. coli* not-treated biofilms display rather non-homogenous, strand like structure, with visible multilayering. Due to long growth cycle (three times longer than for MRSA biofilms) the number of dead cells is definitely much greater as seen on red light-emission channel.

When subjected to T-glycoline, maloline, and glycoline biofilms' structure changed, now exhibiting a strikingly uniform and well-dispersed architectural arrangement. This uniformity was notably discernible under microscopic examination, with both staining agents penetrating the biofilm matrix effectively, revealing a substantial population of dead cells, as indicated by intense red staining. A noteworthy observation emerged from the composite images of maloline-treated biofilms, which displayed slightly heightened green areas, indicative of an increased presence of live bacteria within the matrix.

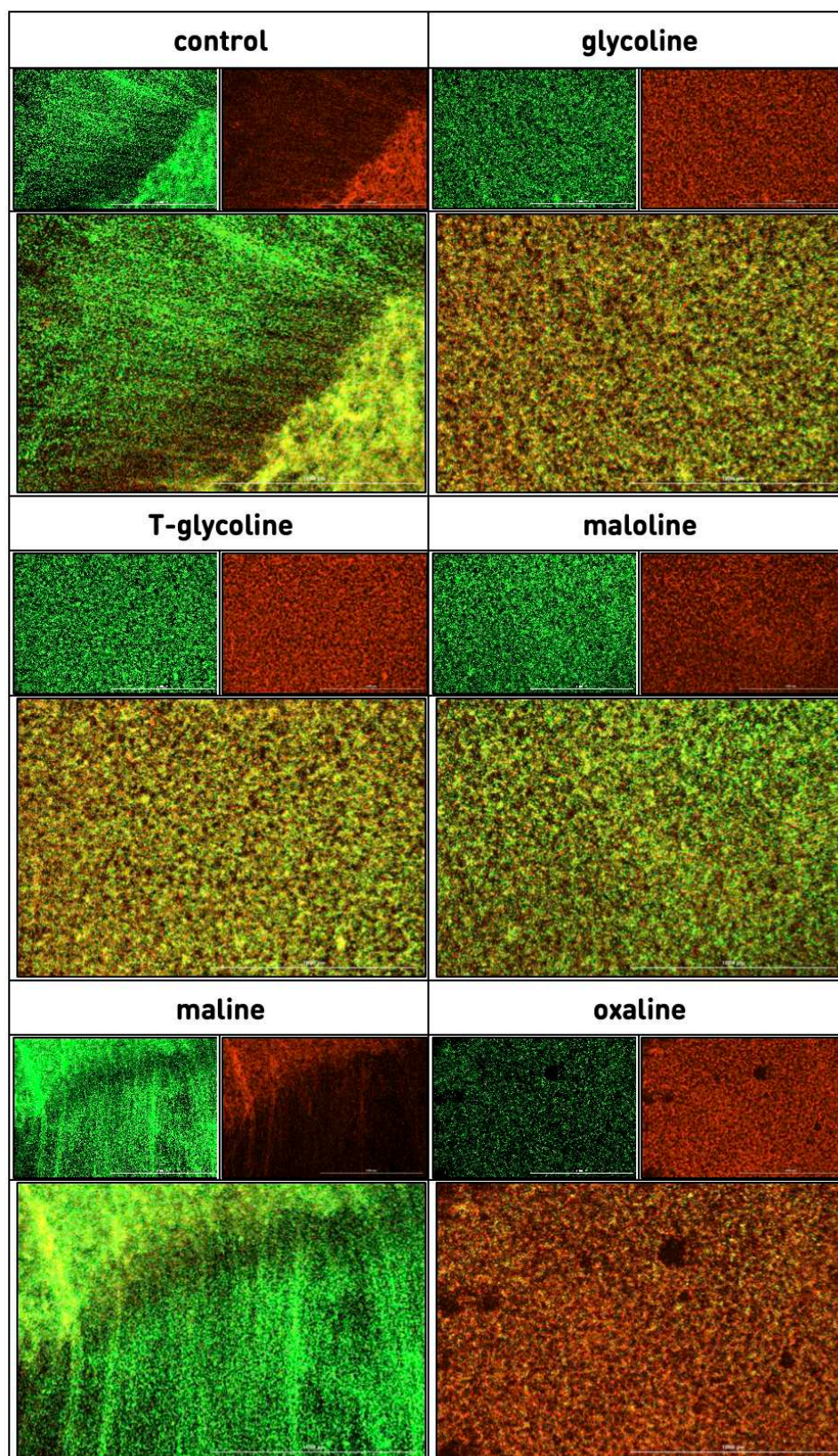


Figure 36. LD staining of *E. coli* biofilms treated with OA-DESs.

In contrast, the biofilms treated with maline, despite the presence of the antimicrobial agent, exhibited a less uniform structure, hinting at potential overgrowth. This disparity was further accentuated by a comparatively weaker red stain signal in maline-treated samples, juxtaposed with a more pronounced green stain signal, corroborating the heightened viability of bacteria within this cohort. Of particular intrigue was the comparative efficacy of oxaline, which, despite sharing an equivalent eradication concentration with glycoline, exhibited a surprising degree of effectiveness, probably due to formation of holes in the biofilms' structure. This was evidenced by a notably high dead/live ratio, prominently discernible in the composite image. Such findings underscore the nuanced interplay between DES composition and their efficacy in combating MRSA biofilms, suggesting avenues for further exploration and optimization in antimicrobial strategies.

4.3.3 Monitoring of Persister Cells Growth in MRSA and *E. coli* Biofilms

As mentioned before biofilms pose an imminent danger in healthcare. Their eradication poses a great challenge due to its structure but also due to persister-induced resilience. As proved before, the concentrations required for biofilm eradication are much higher than those needed for planktonic bacteria, posing a threat of the emergence of persister cells – a subpopulation of bacteria, that can withstand higher dosages of antimicrobial agents, and in turn rebuild the biofilm, once the antimicrobial agent is no longer present. To determine if DESs can prevent the emergence of persister cells, OA-DESs that were effective in biofilm eradication were tested against MRSA and *E. coli*.

4.3.3.1 MRSA Persisters

For oxaline-treated MRSA (**Figure 37**) initially, a steady bacterial load of approximately 10^6 CFU/mL up to the 5-minute mark was observed. A steady decrease of around 1 log unit occurred until the -minute mark. Then, a sudden drop in bacterial population was observed, and after the 2-hour mark, no growth

was observed until 48 hours into the experiment, indicating no regrowth or emergence of persister cells

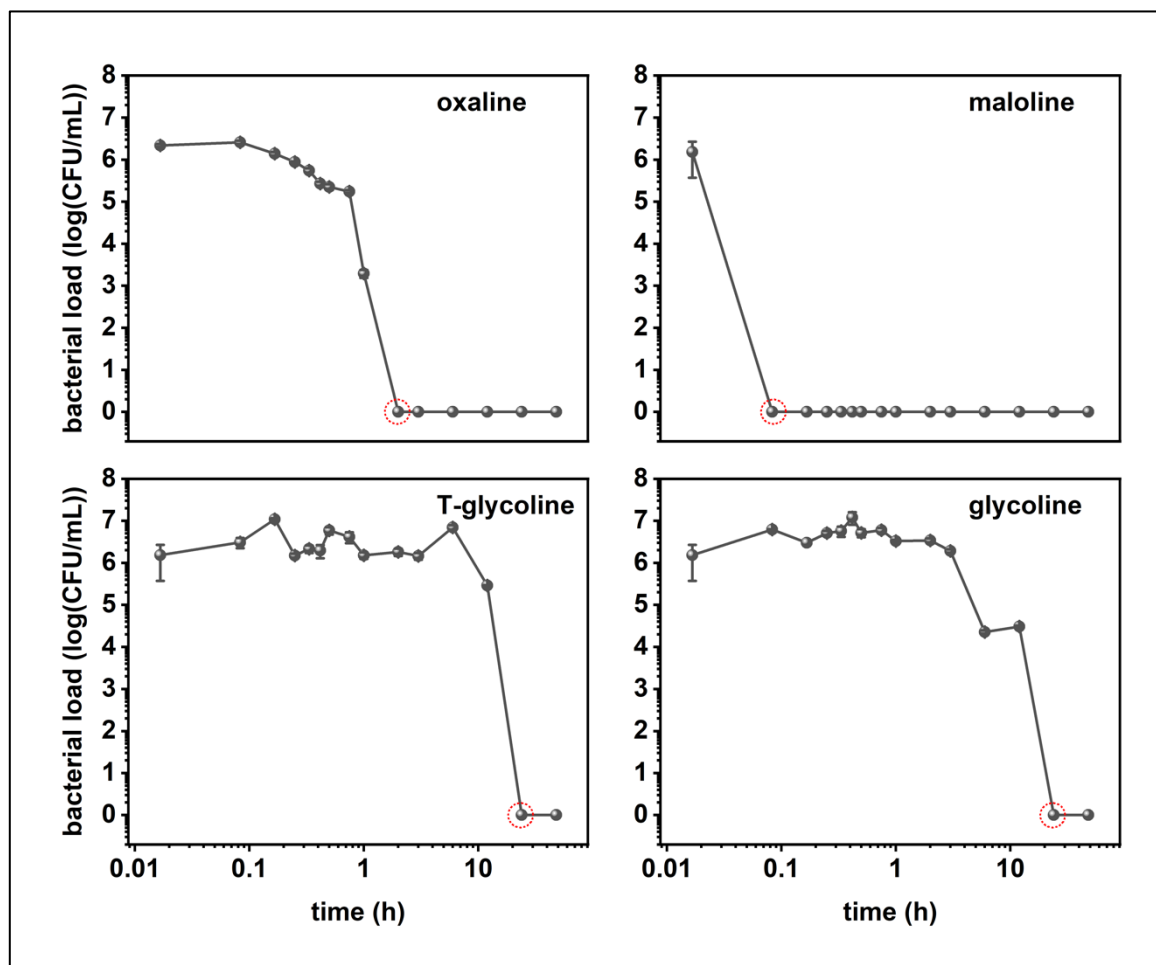


Figure 37. Persister cell emergence test for MRSA using OA-DESs.

As for maloline-treated MRSA a rapid decrease in bacterial load was observed shortly after treatment, with eradication occurring as early as 1 minute. This rapid efficacy suggests maloline's extremely potent antimicrobial properties. The bacterial load remained undetectable throughout the subsequent time points, indicating no survival of persister cells.

Unlike oxaline and maloline, the bacterial load of T-glycoline-treated MRSA remained relatively stable for an extended period, up to the 6-hour mark. However, a dramatic decline in bacterial load was observed thereafter, with complete eradication occurring around 12 hours. The bacterial load remained at 0 CFU/mL

for the rest of the observation period, indicating the eventual effectiveness of T-glycoline in eliminating persister cells.

Similar to T-glycoline, the bacterial load of glycoline-treated MRSA initially remained stable and even fluctuated slightly before a significant reduction was observed. Complete eradication was achieved after the 12-hour mark, with no bacterial regrowth detected throughout the remaining time points.

The obtained results underscore the efficacy of DESs in counteracting the emergence of persister cells. Both oxaline and maloline demonstrated rapid and sustained eradication, highlighting their potential as effective antimicrobial agents. The rapid action of maloline, in particular, suggests that it could be especially useful in acute clinical settings where swift eradication of bacterial load is crucial. However, it has to be noted, that such rapid action should be attributed to extremely high concentration of this DES. T-glycoline and glycoline, while slower to act, ultimately achieved complete eradication without the emergence of persister cells. The delay in action observed with these DESs could be attributed to their different mechanisms of action or slower penetration into biofilms. Nonetheless, their ultimate effectiveness in preventing persister cell formation is a promising finding.

4.3.3.2 *Escherichia coli* Persisters

E. coli forms a morphologically different biofilms. However, it is known for its much faster division time (around 20 minutes) which directly translates to its survivability. To determine if OA-DESs can prevent the emergence of persister cells, five OA-DESs that were effective in biofilm eradication were tested.

The bacterial load over time for oxaline-treated *E. coli* is shown in the **Figure 38**. Initially, there was a steady bacterial load of approximately 10^8 CFU/mL. A gradual decline in bacterial load began after the 1-minute mark, with a noticeable steady decrease of 6 log units until the 6-hour mark. After this point, a sharp decline was observed, and by the 12-hour mark, no bacterial growth was detected. The

bacterial load remained at 0 CFU/mL for the duration of the observation period, indicating that oxaline effectively prevented the emergence of persister cells.

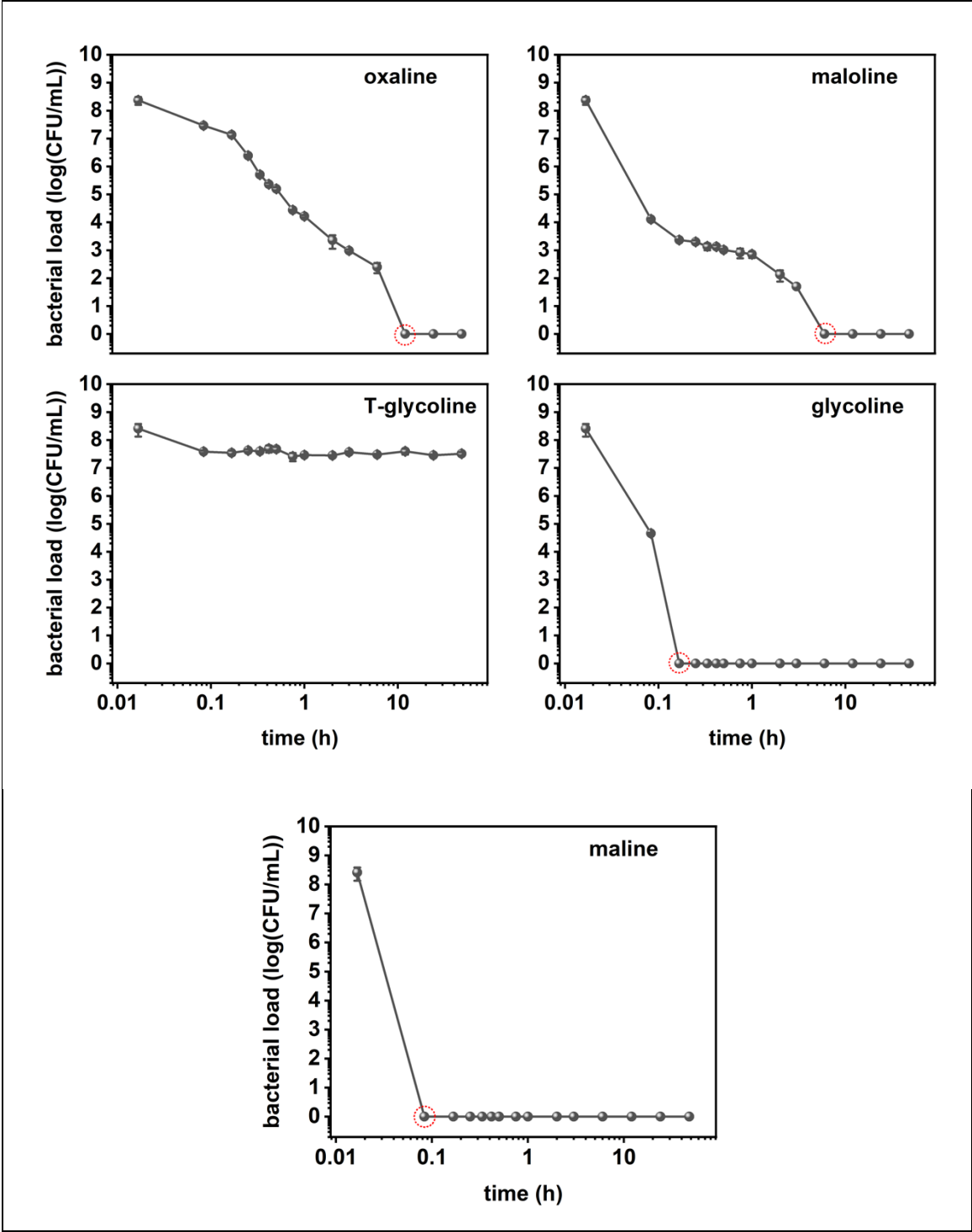


Figure 38. Persister cell emergence test for *E. coli* using OA-DESSs.

For maloline-treated *E. coli*, a rapid decrease in bacterial load was observed shortly after treatment, with a significant reduction from 10^8 CFU/mL to around

10^3 CFU/mL within the hour. The bacterial load continued to decline steadily, and complete eradication was achieved by the 12-hour mark. No bacterial regrowth was detected afterwards, suggesting that maloline is highly effective in eradicating *E. coli* and preventing the formation of persister cells.

The bacterial load for T-glycoline-treated *E. coli*, does not show a significant decrease over time. The bacterial load remains relatively stable at around 10^8 CFU/mL throughout the observation period. This indicates that T-glycoline did not effectively reduce the bacterial population suggesting the emergence of persister cells. Unlike the other OA-DESs, T-glycoline failed to prevent the emergence of persister cells or achieve a significant reduction in bacterial load.

The bacterial load for glycoline-treated *E. coli* initially starts at around 10^8 CFU/mL. There is a rapid and significant decline in bacterial load, dropping to around 10^5 CFU/mL by the 5-minute mark. Complete eradication is achieved shortly after at 10-minute mark, with no bacterial regrowth detected throughout the remaining time points, up to 48 hours. This indicates that glycoline is highly effective in eradicating *E. coli* and preventing the emergence of persister cells.

For maline-treated *E. coli*, a rapid decrease in bacterial load was observed, similar to maloline, with a significant reduction from 10^8 CFU/mL to undetectable levels within the first hour. The bacterial load remained at 0 CFU/mL for the duration of the observation period, indicating that maline effectively prevents the emergence of persister cells.

The results obtained in this experiment underscore the efficacy of OA-DESs in counteracting the emergence of persister cells in *E. coli* biofilms. Oxaline and maloline demonstrated rapid and sustained eradication, highlighting their potential as highly effective antimicrobial agents. The rapid action of maloline, in particular, suggests that it could be especially useful in acute clinical settings where swift eradication of bacterial load is crucial. T-glycoline and glycoline, while slower to act, ultimately achieved complete eradication without the emergence of

persister cells. The delay in action observed with these DESs could be attributed to their different mechanisms of action or slower penetration into biofilms. Nonetheless, their ultimate effectiveness in preventing persister cell formation is a promising finding.

The absence of persister cells in most of the treatments indicates that the concentrations of DESs used were sufficient to eliminate all bacterial subpopulations, including those with enhanced survival capabilities. This is a critical finding, as the ability to prevent the emergence of persister cells could significantly reduce the risk of recurrent infections and improve treatment outcomes for patients with *E. coli* and MRSA infections. The promising results from these DESs highlight their potential as valuable additions to the arsenal of antimicrobial agents used to combat biofilm-associated infections. Further research is warranted to explore the mechanisms underlying the efficacy of these DESs and to optimize their use in clinical settings.

4.4 Use of DESs as Antimicrobial Drug Enhancers

4.4.1 Conventional Antibiotics Enhancement – Ampicillin, Carbenicillin

During the first stage of our studies, we chose to explore the enhancement of antibacterial effects of DES on conventional, small-molecule antibiotics. For this part, we tested two representative strains – Gram-negative *E. coli* K-12[‡] and Gram-positive MRSA. Both bacteria are a part of ESKAPEE strains, thus posing a potential threat to a human health, and exhibiting fast emerging resistance towards antibacterial substances. This choice is also important due to morphological differences of both bacteria, as possible DESs effect might be hindered by the thick layer of peptidoglycan of Gram-positive bacteria. That being

[‡] From now on *E. coli* K-12 will be mentioned also using its strain identifier.

said, we verified if the presence of DESs in the system would influence the efficiency of antibiotics in direct contact with planktonic bacteria.

In order to acquire valuable data, antibiotics with high MIC values were considered, as the potential effect of DES is expected to be more pronounced. To this end, we have tested five antibiotics, namely – ampicillin (AMP), carbenicillin (CAR), ciprofloxacin (CIP), kanamycin (KAN) and tetracycline (TET) against both strains (*E. coli* K-12 and MRSA). MIC value assessments (**Table 20** and **Figure A29**) revealed high activity of TET and CIP towards both strains with MIC values falling below 8 µg/mL. As expected, AMP and KAN were not effective as antibiotics, due to resistance of MRSA towards beta-lactams (AMP) and aminoglycosides (KAN). This is also further confirmed by in MIC value of CAR of around 256 µg/mL, which is also a beta-lactam. As for *E. coli* K-12, both beta-lactams were also characterized by high MIC values of 128 and 32 µg/mL for AMP and CAR, respectively, indicating moderate-high resistance to this group of antibiotics. KANs' MIC value was slightly lower, around 16 µg/mL and both CIP and TET exhibited low MIC values of 2 and 8 µg/mL, respectively.

Table 20. MIC values of the tested antibiotics against *E. coli* K-12 and MRSA using visual assessment (more detailed data is available in the appendix (**Figure A29**))^[271].

Strain	Antibiotic				
	AMP	CAR	CIP	KAN	TET
<i>E. coli</i> K-12	128 ^R	32	2 ^R	16 ^S	8 ^I
MRSA	–	256	4 ^R	–	4 ^S

“R” indicates resistance; “S” indicates susceptibility; “I” indicates intermediate susceptibility; “–” indicates that MIC have not been observed in the given range.

Based on the data above, we have focused on two bacteria-antibiotic pairs – that is, *E. coli* K-12/AMP and MRSA/CAR. Both antibiotics are beta-lactams and are characterized by similar mode-of-action, which excludes possible influence of other mechanisms. They are also characterized by high MIC values, which should enable us to better observe potential effect of DESs. Also, based on the MIC values

alone, one could assume that these strains exhibit resistance towards these antibiotics, and thus any possible enhancement of their activity by DESs would provide information into the nature of the resistance.

After this initial screening, both antibiotics were tested in the presence of the DESs. Based on our previous results, DESs concentration was set below their maximum MIC values, that is: 6.4% v/v for glyceline, 12.5% v/v for ethaline, and 4.5% v/v for relene. In this manner, we could prevent the observation of bacteriotoxic effect caused by the DESs themselves. However, to ensure this, in parallel to sterility control, bacteria with DESs were included as additional controls, during all the manipulations.

The MIC assessments of antibiotics mixed with DESs (**Figure 39**, **Figure A30**, and **Figure A31**) revealed that addition of DES to the system improved greatly the effectiveness of both antibiotics. In the case of *E. coli* K-12/AMP, where effective MIC was 79.43 ± 8.13 $\mu\text{g/mL}$, addition of DES decreased its value to 7.81 ± 0.09 $\mu\text{g/mL}$ for glyceline, 7.06 ± 0.77 $\mu\text{g/mL}$ for ethaline, and 7.96 ± 1.07 $\mu\text{g/mL}$ for relene.

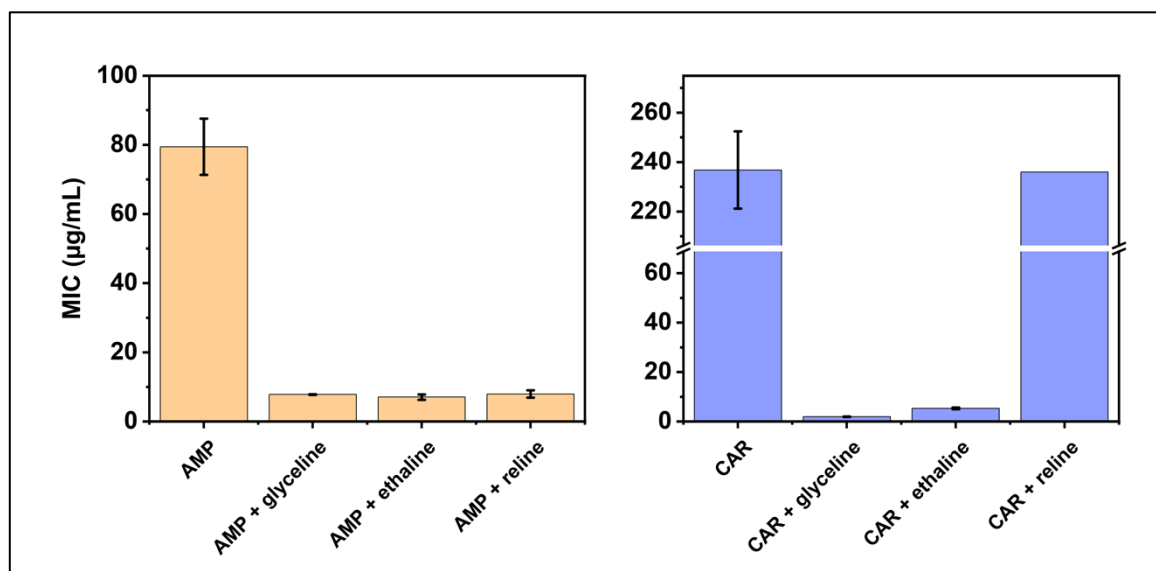


Figure 39. MIC values of the antibiotic/DES systems (ampicillin against *E. coli* K-12 and carbenicillin against MRSA; lack of error bar indicates that MIC value was not observed in the given range; n=3).

These results translate into ca. 10-fold decrease of MIC of AMP when combined with the DESs. In the case of CAR, which MIC was determined to be 236.82 ± 15.64 $\mu\text{g/mL}$, the addition of DES resulted in even greater improvement in the case of glyceline – 1.92 ± 0.08 $\mu\text{g/mL}$ (120-fold decrease), followed by ethaline – 5.32 ± 0.33 $\mu\text{g/mL}$ (44-fold decrease). Interestingly, the addition of reline did not improve the efficacy of the system, and MIC value was not observed at all within the tested range of CAR concentration.

The nature of the bacterial resistance towards the investigated antibiotics may vary and include the synthesis of beta-lactamases (commonly observed for *E. coli*), the reduction of permeability via outer membrane-porins modification, the alteration of penicillin-binding proteins as well as the presence of efflux pumps, which eagerly remove the antibiotics from/within the bacterial cell. The substantial reduction in MIC values indicates enhanced efficacy, either by improved antibiotic solubility/stability which protects the antibiotic from beta-lactamases and efflux-pumps or alterations in the bacterial membrane, allowing better penetration of the antibiotics into the bacterial cells. The 10 to 120-fold decrease in MIC values infers a substantial improvement in the effectiveness of the antibiotics against their respective bacterial strains. In the case of MRSA, the substantial efficacy improvement with glyceline and ethaline compared to reline hints at the differential effects of DESs on the interaction between antibiotics and bacterial targets and/or increased stabilization of the antibiotic by the DES in the solution.

4.4.2 Antimicrobial Peptides Enhancement – Colistin, RAP-29

Since DESs proved to enhance the activity of the conventional antibiotics, we have further tested their stabilization effect on bigger molecules such as antimicrobial peptides – colistin and RAP-29.

Colistin is a peptide derived from *Paenibacillus polymyxa*, that is often used as a last resort against multidrug-resistant bacteria, particularly those from ESKAPEE

group. It belongs to the class of antibiotics called polymyxins. Its mode-of-action relies on disruption of the bacterial cell membrane, causing the cell contents to leak out and leading to the death of the bacteria. Colistin, employed since the 1940s, fell out of favor due to concerns over its potential nephrotoxicity and neurotoxicity^[278]. However, it has become more important in recent decades due to rising bacterial resistance to other antibiotics. Due to its potency and potential side effects, colistin is typically reserved for serious infections that are resistant to other antibiotics. Therefore, it appears relevant to increase its efficacy, by potentially increasing its bioavailability, which may lead to decreased MIC and MBC values.

With that in mind, we explored whether DESs could enhance the stability of colistin. We conducted microbiological assessments using the microbroth dilution method specifically against *E. coli*, a bacterium known for its susceptibility to this drug. Our aim was to explore the potential for further improving the already effective performance of colistin.

MIC assessment of colistin against *E. coli* K-12 (**Figure 40** and **Figure A32**) revealed a relatively low MIC value for pure colistin, equal to $1.38 \pm 0.13 \mu\text{g/mL}$, indicating high susceptibility of *E. coli* K-12 towards this drug. Addition of DES resulted in decrease of MIC value to $0.37 \pm 0.01 \mu\text{g/mL}$ for glyceline, $0.35 \pm 0.01 \mu\text{g/mL}$ for ethaline and $0.20 \pm 0.04 \mu\text{g/mL}$ for reline, which translates into 3.75 to 6.80-fold decrease of MIC value of colistin in the presence of the investigated DESs. Despite much lower decrease of MIC value of colistin, when compared to conventional, small-molecule antibiotics, it is worth emphasizing that such an enhancement holds significant importance, as it lowers MIC value below $1 \mu\text{g/mL}$ which may directly result in decrease of cytotoxicity of colistin towards mammalian cells^[278] and potentially lowering the risk of the emergence of bacterial resistance.

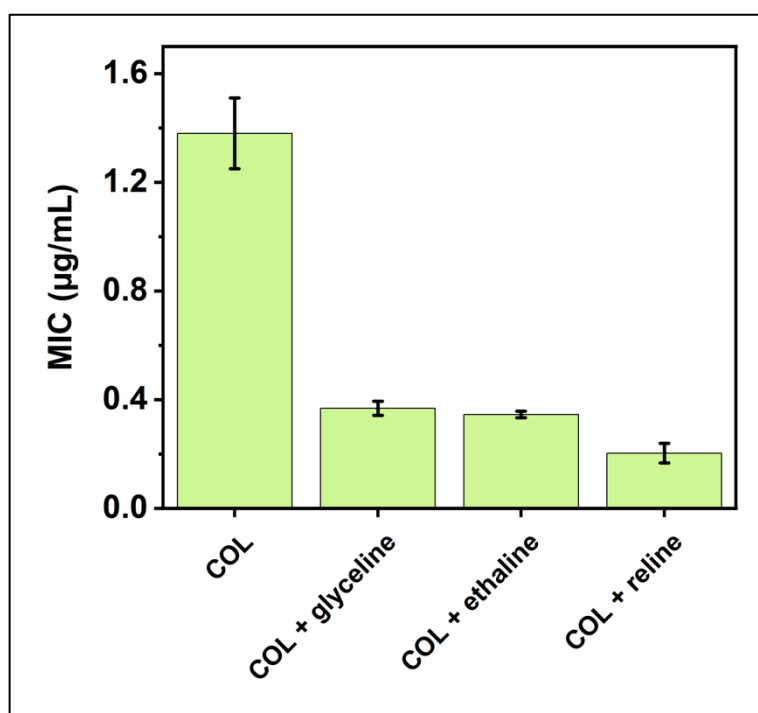


Figure 40. MIC values of the colistin/DES (n=3).

To understand the nature of the mechanism or mode-of-action of colistin on *E. coli* in the presence of DES, we have assessed MIC of colistin in the presence and absence of DES towards two colistin-resistant strains – *E. coli* 184 and 289. For these strains, colistin resistance is encoded in plasmid DNA on mcr (mobilized colistin resistance) gene. The mcr genes constitute a crucial family of genetic elements implicated in conferring resistance to colistin. Initially, uncovered in 2015^[279] with the identification of mcr-1 in *E. coli* strains from diverse sources, such as livestock, food products and clinical isolates, subsequent research has unveiled several variants spanning mcr-1 to mcr-10^[280]. These genes encode phosphoethanolamine transferase enzymes that catalyze the addition of phosphoethanolamine groups to lipid A, a component of the bacterial outer membrane. This modification alters the charge and structure of lipid A, consequently reducing the binding affinity of colistin to the membrane. As a result, the bactericidal action of colistin is compromised, rendering the bacteria resistant to its effects. The widespread dissemination of mcr genes, facilitated by horizontal gene transfer mechanisms, poses a significant challenge to public health by

limiting treatment options and exacerbating the global crisis of antibiotic resistance. For instance, while strain 184 harbors the *mcr-1* gene and 289 contains the *mcr-4* gene, the latter scenario introduces additional complexity as potential mutations, such as those affecting *mgrB*, *phoP*, *phoQ*, *pmrB*^[147,281], may further alter the mechanism of colistin resistance.

Due to highest effectiveness in decreasing the MIC of colistin against *E. coli* K-12 in a previous experiment, relne was selected for further evaluation. In this study, relne in its sub-MIC value was tested alongside changing concentrations of colistin against *E. coli* 189 and 284. These strains were also subjected to colistin alone within the same concentration range for comparison. The results, illustrated in **Figure 41**, revealed that colistin displayed significantly high MIC values against both investigated strains, approximately 10 µg/mL, indicating their resistance to this antibiotic. Conversely, when relne was present, the MIC of colistin against *E. coli* 189 notably decreased to 0.06 ± 0.01 µg/mL, indicating a reduction of approximately 150-fold. In contrast, *E. coli* 284 exhibited subdued growth despite exposure to sub-MIC levels of relne. Nevertheless, visual assessment suggested a MIC value of approximately 2.5 µg/mL, corresponding to a four-fold decrease. This variance likely stems from differing resistance mechanisms in this strain. While this experiment does not definitively elucidate the mechanism of relne action, it does not rule out the involvement of DES in interacting with the lipid A of the *E. coli* membrane.

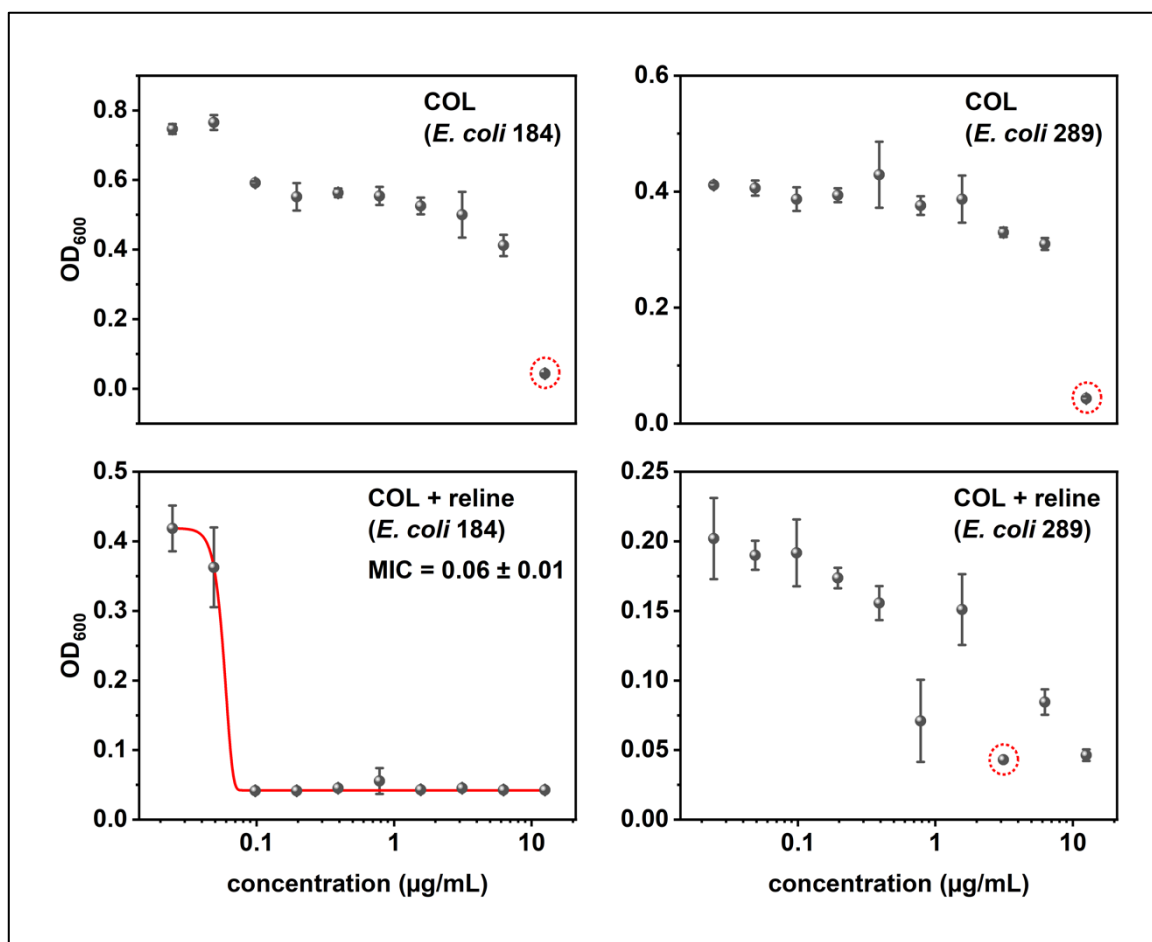


Figure 41. MIC assessment of colistin without (top) and with (bottom) relene in its sub-MIC value against *E. coli* 189 and 284 (red line indicates Gompertz model fitting; circled points indicate to visual MIC assessment when Gompertz model was unable to fit the curve; $n = 3$).

Another approach of counteracting bacterial infections may involve the use of antimicrobial peptides (AMPs). AMPs are widely distributed in nature, but our interest is in AMPs hidden in the primary sequences of larger proteins, specifically endolysins (lysins) – lytic enzymes of phage origin. Phage-encoded endolysins play a pivotal role during the end of bacteriophage infection. Endolysins act by targeting the peptidoglycan layer of the bacterial cell wall and now are considered as a novel class of therapeutics^[282]. Previous study on antibacterial activity of PhiKo endolysins from *Thermus thermophilus* bacteriophage phiKo (AYJ74695.1) showed its high antibacterial effect against thermophilic bacteria and moderate antibacterial efficiency against mesophiles^[112]. Going further, using bioinformatics

tools, it was shown that in the C-terminal region of PhiKo endolysin, there is a hidden antibacterial peptide RAP-29 (RAPGWKSLAWLVREL RKHDSGLRLRLVRH) with high activity against mesophiles represented by staphylococci and Gram-negative bacteria^[112]. By targeting bacterial membranes, this kind of regions in endolysins may facilitate their passage across the outer membrane of Gram-negative bacteria. Consequently, we investigated this newly discovered, synthesized AMP RAP-29 in the present study.

RAP-29 solutions were prepared by serial dilution. Reline was selected as a DES of choice, owing to its lowest MIC values with colistin. RAP-29 antibacterial activity was assessed with lack (control) and presence of relin at a concentration of 2.5, 3.5, and 4.5% v/v, towards *P. aeruginosa*, a strain that exhibited, a high tolerance towards RAP-29 when compared to *E. coli* KPD 217 and *Staphylococcus hominis* (**Figure A33**). The range of relin was selected based on our previous studies^[283], where we assessed antimicrobial activity of various DESs, and relin MIC value was estimated to be $\approx 5.5\%$ v/v This approach helps eliminate any influence of the DES on the bacteria themselves. As seen on **Figure 42**, *P. aeruginosa* exhibited very high MIC values of around $236.14 \pm 5.02 \mu\text{g/mL}$.

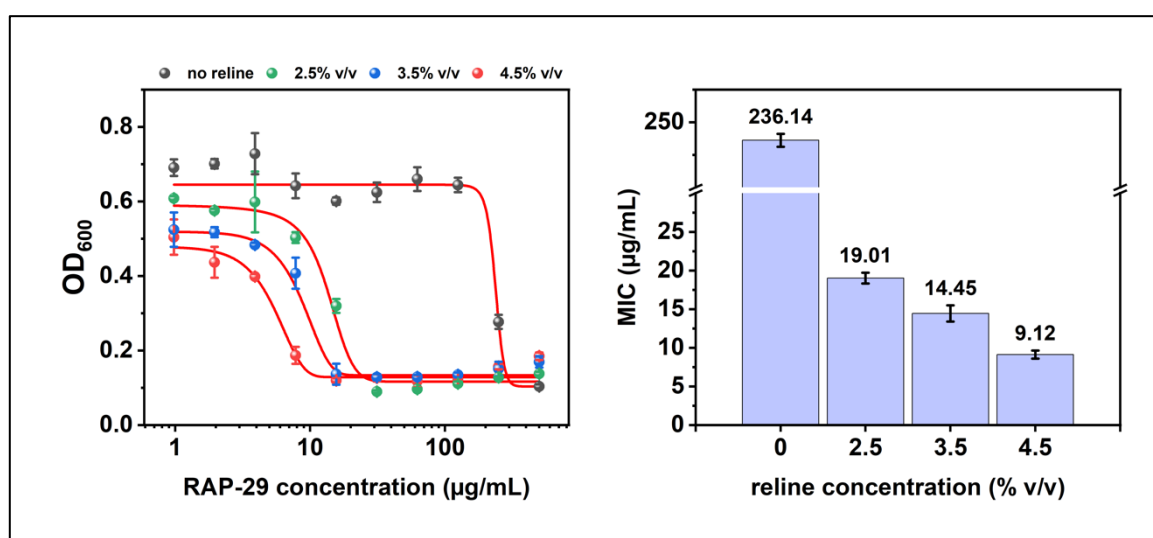


Figure 42. MIC values for pure RAP-29 and RAP-29 with increasing concentration of relin against *P. aeruginosa* (red lines indicate Gompertz model fitting; $n=3$).

However, the addition of relene (2.5% v/v) resulted in a substantial decrease of the MIC value down to $19.01 \pm 0.70 \mu\text{g/mL}$. Further addition of relene improved the results, by lowering MIC values of RAP-29 down to $14.45 \pm 1.05 \mu\text{g/mL}$ and $9.12 \pm 0.53 \mu\text{g/mL}$ for 3.5 and 4.5% v/v of relene, respectively.

These results are promising and demonstrate the capabilities of relene as a potential stabilizing agent for proteins. During our manipulations, we have noticed that when RAP-29 was mixed with liquid bacterial medium (Mueller-Hinton broth), the solution became cloudy, inferring the destabilization and aggregation of the peptide. This process can greatly impact the peptide's effectiveness against bacteria and result in structure-derived activity of the peptide, decreased solubility (bioavailability) and/or altered kinetics. Therefore, RAP-29 solutions of $500 \mu\text{g/mL}$ were premixed in microplate with relene resulting in final concentration of DES of 2.5, 3.5, and 4.5% v/v. After 5 minute-long incubation at 35°C , the microplate was read at 600 nm to estimate the turbidity. As seen on **Figure 43**, pure solution of RAP-29 exhibited high absorbance of around 0.846 ± 0.049 indicating increased turbidity.

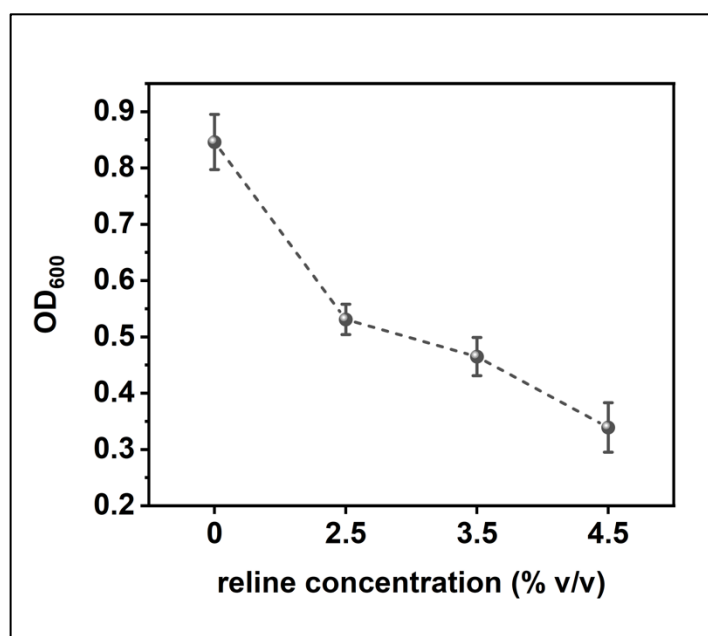


Figure 43. RAP-29 solution turbidity in the presence of relene in the concentration range from 2.5 to 4.5% v/v (n=3).

However, the addition of reline significantly reduced the OD, down to 0.531 ± 0.027 for 2.5% v/v of reline content. Following increase of the concentration of reline resulted in further decrease of OD down to 0.339 ± 0.044 , which overall decreased the turbidity of the solution, suggesting the stabilization of the peptide.

4.4.3 Eradication of MRSA Biofilm Using Nisin Combined with DES

Nisin, a bacteriocin produced by certain strains of *Lactococcus lactis*, is a potent antimicrobial peptide known for its broad-spectrum activity against a range of Gram-positive bacteria, including various foodborne pathogens. This naturally occurring peptide consists of a polycyclic structure with unusual amino acid modifications, contributing to its stability and efficacy. Nisin exerts its antimicrobial effect by disrupting bacterial cell membrane integrity, leading to pore formation and ultimately cell death. Due to its unique mechanism of action and selective targeting of bacteria, nisin has found widespread application as a natural preservative in the food industry^[284]. Its use extends beyond food preservation to include potential applications in pharmaceuticals and healthcare, showcasing its versatility as an antimicrobial agent^[285]. The safety profile of nisin, coupled with its effectiveness in inhibiting bacterial growth, positions it as a promising candidate for addressing microbial challenges in various fields, making it a subject of interest in scientific research and practical applications. Nisin's efficacy, while notable against planktonic bacteria, poses limitations in addressing biofilm-associated infections. Biofilms, complex communities of bacteria encased in a self-produced matrix, exhibit heightened resistance to antimicrobial agents, including nisin. The protective matrix shields embedded bacteria, reducing nisin's penetration and impact within the biofilm structure. Consequently, the effectiveness of nisin in eradicating biofilms is limited, highlighting the challenges in combating these resilient bacterial communities.

In our approach, we investigated the ability of nisin to eradicate the bacteria enveloped in MRSA biofilm by using DESs. For this purpose, we have considered

glyceline, which proved to partially solubilize the biofilm. In our preliminary investigations, MRSA biofilms, cultivated for 24 h, underwent treatment with varying concentrations of glyceline and were verified using colony forming assay (CF), crystal violet staining (CV) and brightfield microscopy (BFM). As depicted in **Figure 44**, despite the use of notably high concentrations of DES, the majority of the biofilm mass remained intact. An exception was noted within a narrow concentration range, approximately from ≈ 0.78 to $\approx 0.19\%$ v/v, where observable reduction in biofilm mass occurred. This reduction was visually apparent and corroborated by BFM, with the losses more prominently evident in the microscopic images. Such low concentration of DES will likely contribute to decrease of the cytotoxic effect of nisin-DES systems.

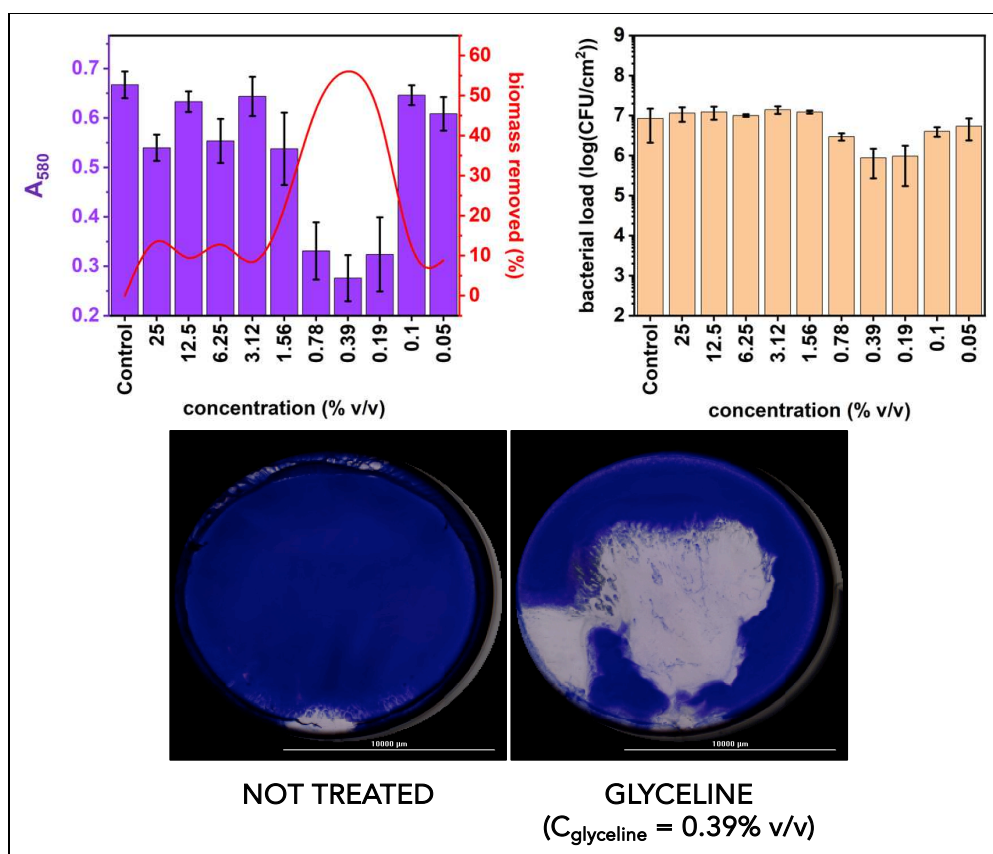


Figure 44. Colony forming assay (top left) and crystal violet staining (top right) for MRSA biofilm treated with glyceline (n=3). Brightfield micrographs (bottom) for MRSA biofilm not treated and treated with $\approx 0.39\%$ v/v glyceline (blue color indicates the presence of the biofilm).

As glycine holds potential for both stabilizing biomolecules and solubilizing biofilms, we devised two testing strategies (**Figure 45**). In the initial approach, varying concentrations of nisin (covering its MIC value, **Figure A34**) were pre-mixed with 0.39% v/v glycine and used to treat MRSA biofilm. In the second method, the biofilm underwent treatment with glycine before exposure to nisin. This sequencing was considered crucial due to the typical availability of nisin as a 2.5-3.5% mixture with proteins like caseins. The lower concentration of 0.39% v/v glycine might not exert its stabilizing effect on these proteins, limiting its interaction with nisin. Conversely, introducing nisin to the biofilm after sensitization with glycine could potentially facilitate its penetration into compromised biofilm structures.

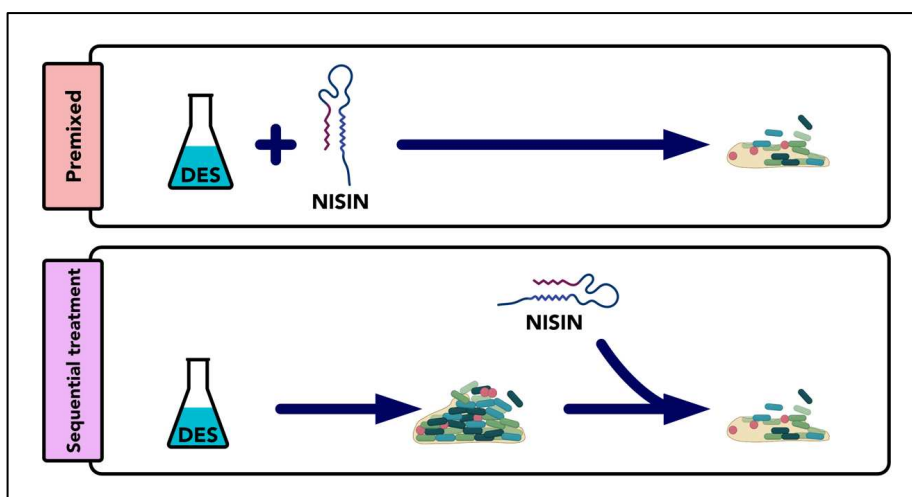


Figure 45. Two strategies of introducing nisin to the biofilm: as a premixed solution of nisin in the glycine (top) or as a subsequent treatment (bottom).

As seen in the **Figure 46**, nisin alone was indeed unable to reach the bacteria and eradicate them effectively. Bacterial load remained on the similar levels starting from 3.90 up to 15.63 $\mu\text{g/mL}$, and then slowly drops by around 3 log units, until reaching the highest concentration of 250 $\mu\text{g/mL}$. A very similar result was observed for nisin premixed with glycine, with only minor difference at higher concentrations.

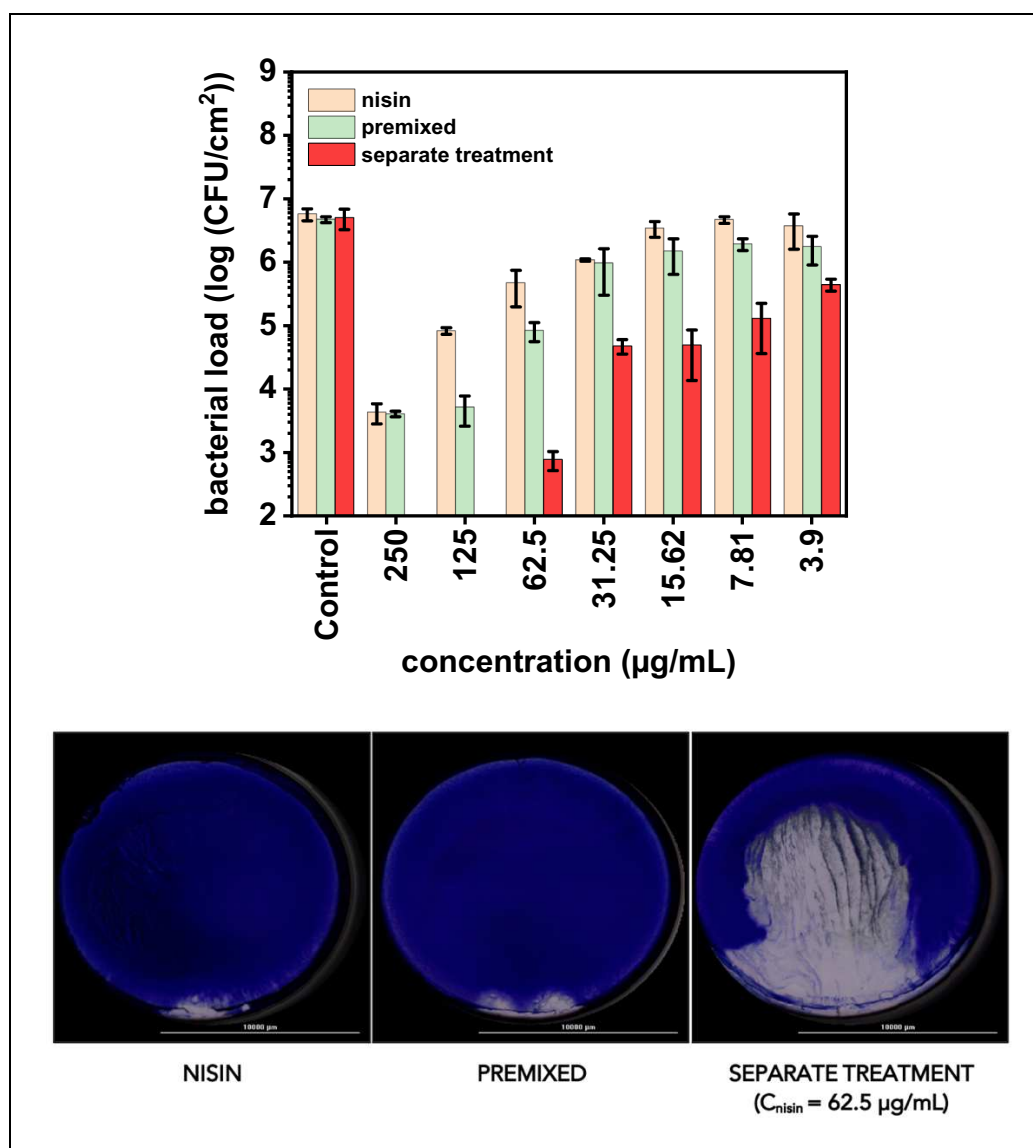


Figure 46. Colony forming assay (top, n=3) and brightfield micrographs (bottom) for MRSA biofilm treatment with: nisin alone, nisin in premixed solution with glycine, as well as nisin after the biofilm has been treated with glycine.

However, when nisin was used after the biofilm was treated with glycine first, we could clearly see improved effectiveness of the peptide starting from its lowest concentration with single log unit reduction compared to nisin alone, then slowly decreasing up until the concentration of 62.5 μg/mL where a 4 log reduction was noted. Concentrations higher than this value were able to effectively eradicate all of the bacteria, proving this strategy effective. This is well illustrated by the CV of the biofilm, where the biofilm remains intact for both treatments with nisin alone

and when premixed with DES. However, a subsequent treatment resulted in reduction of biofilm. Interestingly, most of the biofilm reduction occurs in the middle of the well, possibly because the biofilm is thinner there due to pipetting, providing an entry point.

4.5 Perspectives of DESs Application Against Fungal Infections

Our previous findings demonstrated the effectiveness of DES against bacteria in various approaches, whether as a pure antimicrobial agent or as a co-drug. Nonetheless, it's imperative to recognize that bacteria represent just one kingdom of microbes posing a threat to human health. Yeast and fungi, as microbial entities, present significant hazards due to their potential to induce a broad spectrum of infections and illnesses. Notably, *Candida albicans*, *Candida auris*, and *Aspergillus fumigatus* are pathogens of particular concern for their adverse effects on human health. *C. auris*, notably, emerges as a multidrug-resistant fungal strain, capable of causing severe bloodstream infections, particularly in healthcare environments.^[286] *C. albicans*, a common inhabitant of the human microbiota^[287] can transition to a pathogenic state, causing a range of infections from mild mucosal to severe systemic diseases, especially in immunocompromised individuals^[288,289]. Similarly, *A. fumigatus* is a primary cause of invasive aspergillosis, predominantly afflicting individuals with weakened immune systems or underlying respiratory conditions.^[290] Understanding the risks posed by these fungal strains is vital for devising effective strategies to mitigate their impact on human health, particularly in healthcare settings where infection risks are elevated. With this awareness, we have chosen to explore the potential of DES in combating fungal infections by evaluating their antifungal properties. To this end, we conducted an antifungal effect assay (AE) and verified yeast morphology using optical microscopy (OM).

All the DESs, both conventional and OA-DESs have been prepared so that their final concentration corresponds to 1, 5 and 10% v/v. PSs have also been prepared,

so that their final concentration corresponds to that at the 1, 5, and 10% v/v concentrations of DESs.

4.5.1.1 *Aspergillus fumigatus*

As seen in the **Figure 47** all the tested DESs displayed antifungal activity, in the highest tested concentration, that is 10% v/v. Six out of the tested substances inhibited the growth effectively, with OD₆₀₀ values being below 20% compared to the control (glycoline, T-glycoline, maline, oxaline, reline and ethaline). Two of the DESs were characterized by the relative OD₆₀₀ values between 20-30% (maloline, R-glyceline) and one around 60% (glyceline). When compared separately, though, we can see that the most effective DESs from this group were glycoline, maline and T-glycoline, as no growth was observed in both 5 and 10% v/v. It is worth nothing, though that maline was the only DES that seemed to increase the proliferation of the yeast in its lowest concentration, that is 1% v/v. The effectiveness of other DESs is also noteworthy, especially that of oxaline in the group of OA-DESs, as well as reline and ethaline in the group of conventional DESs, which also quite effectively inhibited the growth of the *A. fumigatus*. In the case of OA-DESs maloline and in the case of conventional DES R-glyceline, exhibited mediocre effectiveness, not being able to effectively inhibit the growth of yeast. At the very end, glyceline, a representative of conventional DESs was unable to significantly inhibit the growth of the *A. fumigatus* in all the tested concentrations.

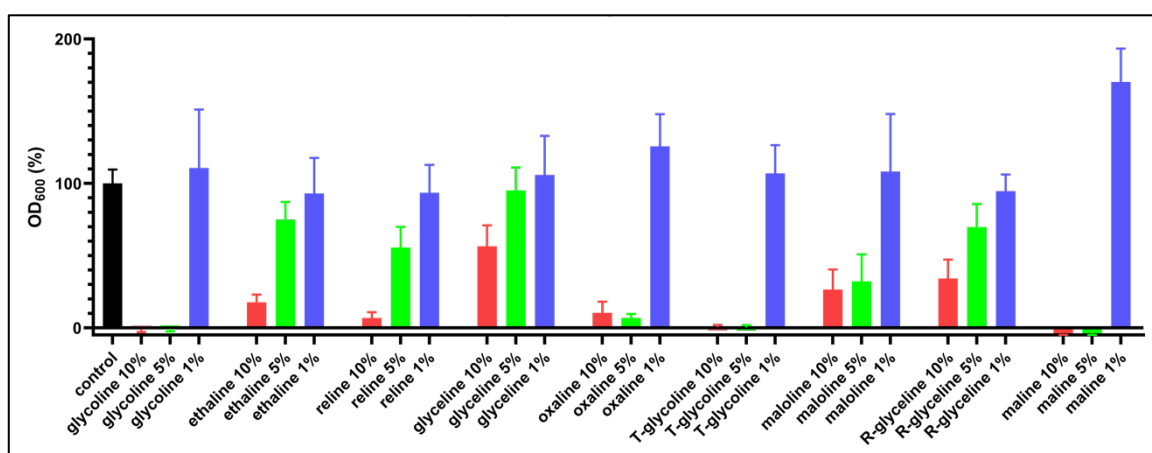


Figure 47. AE assay of the investigated DESs against *A. fumigatus* strain.

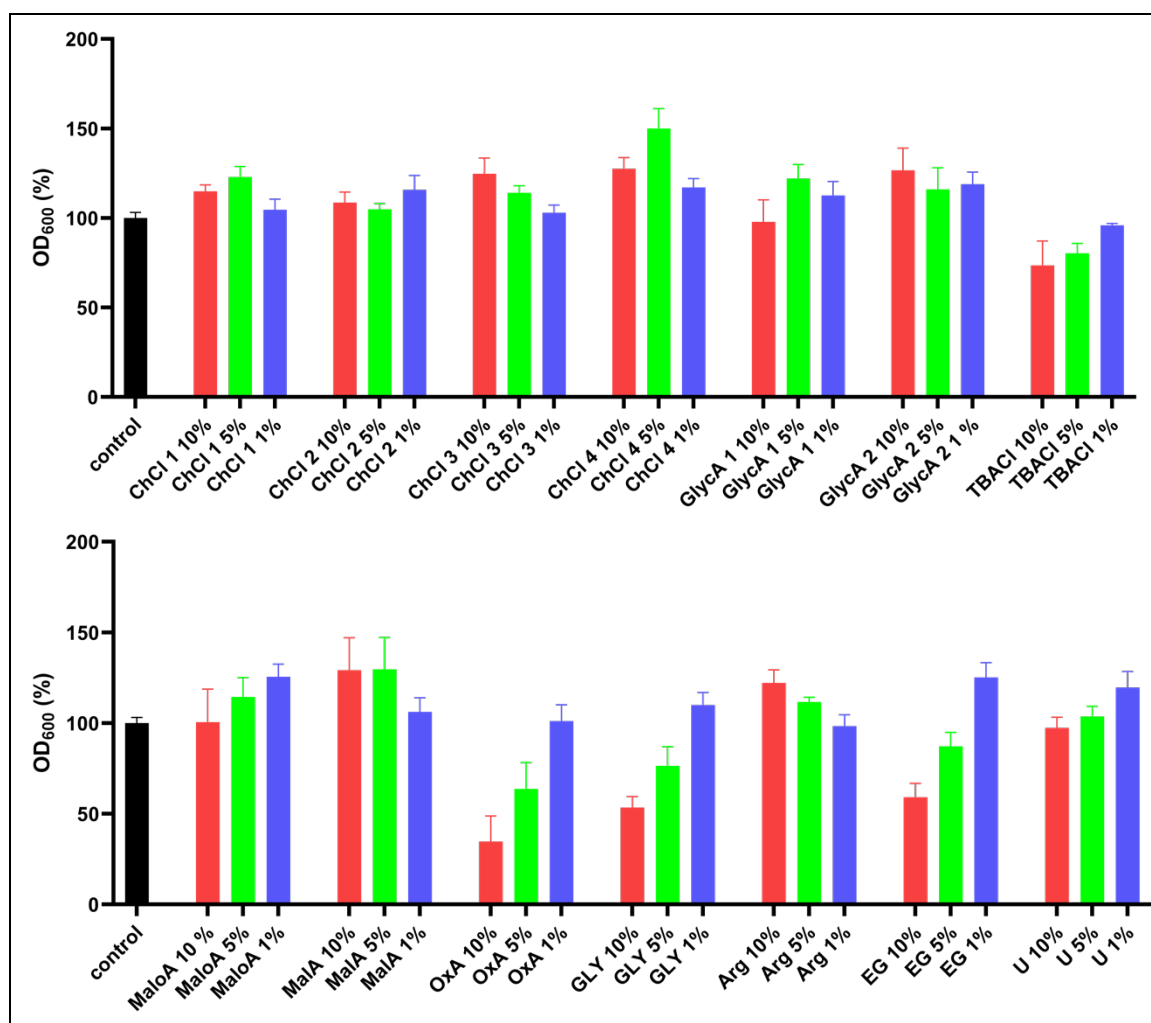


Figure 48. AE assay of the PSs of the investigated DESs against *A. fumigatus* strain ["ChCl #" indicates the concentration of ChCl corresponding to that of # = 1 – glycoline, maline and ethaline 2 – maloline and reline, 3 – glyceline, 4 – oxaline; "GlycA #" indicates the concertation of GlycA corresponding to that of # = 1 – glycoline, 2 -T-glycoline).

Upon comparing PSs (**Figure 48**) with DESs, it becomes apparent that, unlike DESs, none of the PSs significantly impeded the growth of *A. fumigatus*. Interestingly, the only substance that exhibited notable inhibition of yeast growth was oxalic acid, particularly at a relative concentration of 10%. These results prompt the formulation of a hypothesis similar to the one derived from comparing MIC values of DESs and PSs against bacteria – suggesting the possibility of enhancing or "activating" certain PSs through solubilization in the form of DESs. Furthermore, an intriguing pattern emerged regarding glycerol, which displayed a

similar inhibitory trend as glyceline, indicating that the formation of DESs might not exert adverse effects on fungal cells.

Optical microscopy imaging full confirms the measured antifungal effect of the investigated DESs (**Figure A35**). In fact, under the threshold of 20% relative OD₆₀₀ no growth was observed with only occasional presence of bigger spores/cell aggregates. No morphological changes were observed either which suggests that DESs' mode of action may involve interference with essential metabolic pathways or processes rather than directly damaging the physical structure of the fungal cells. This could indicate inhibition of key enzymes or metabolic pathways necessary for fungal growth and proliferation. Essentially, the substances might be disrupting the internal processes required for fungal survival and replication without necessarily causing visible changes to the external morphology of the cells.

4.5.1.2 *Candida albicans*

The results of AE assay against *C. albicans* are much different than for *A. fumigatus*. As seen in the **Figure 49**, this strain was effectively inhibited by maline and oxaline, which were able to inhibit the growth of *C. albicans* in the concentration of 5 and 10% v/v. They were followed by T-glycoline, which also slightly inhibited the growth at 5% v/v. However, other DESs, such as R-glyceline, ethaline, glycoline, and reline were also effective at their highest concentrations. Glyceline once again was as ineffective and did not significantly inhibit the growth at its highest concentration. Noteworthy is maloline, which did not kill fungal cells at all, and even caused major proliferation of the fungi. A similar behavior was observed for 1% v/v glycoline and 5% v/v glyceline. Suggesting that some components of the DES might interact with fungal cells in a different way. Maline, and maloline in 1 % v/v and R-glyceline in 5% v/v concentrations seemed to also display high relative OD₆₀₀ values, however, taking into the account the statistic error these results are not unequivocal.

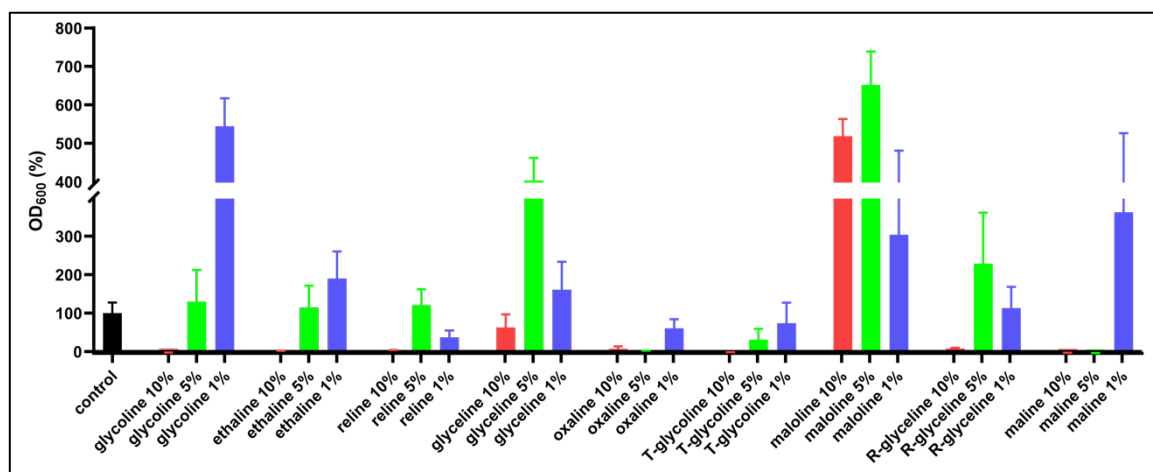


Figure 49. AE assay of the investigated DESs against *C. albicans* strain.

On the other hand, the results of the AE assay of the PS (**Figure 50**) showed that only ethylene glycol was cytotoxic enough to inhibit the growth of *C. albicans*. The other two substances that had any visible effect on the fungi was TBACl and oxalic acid, both in their highest values.

The OM images (**Figure A36**) unveiled several intriguing observations. Firstly, it is worth noting that the investigated strain displays a filamentous, pellet form. This kind of morphology is considered as a virulence factor and thus plays a critical role in the pathogenicity of the organism, enhancing its ability to invade host tissues and evade the immune response. OM images provided confirmation of the efficacy of DES, as evidenced by the minimal or absent growth of fungal colonies in the presence of effective DESs. However, notable morphological alterations were observed in the colonies (or clusters) upon exposure to certain DESs. The first DES to induce such changes was glycoline, which at a concentration of 5% v/v led to the dispersion of colonies from pellets to clumps/aggregates and more dispersed forms. Further increases in its concentration resulted in complete inhibition of *C. albicans* growth. A similar pattern was observed for reline, ethaline, and glyceline. Interestingly, maloline exhibited significant proliferation activity at concentrations of 5% and 10% v/v, yet also caused dispersion of colonies without inhibiting cell growth, suggesting a distinct mechanism at play.

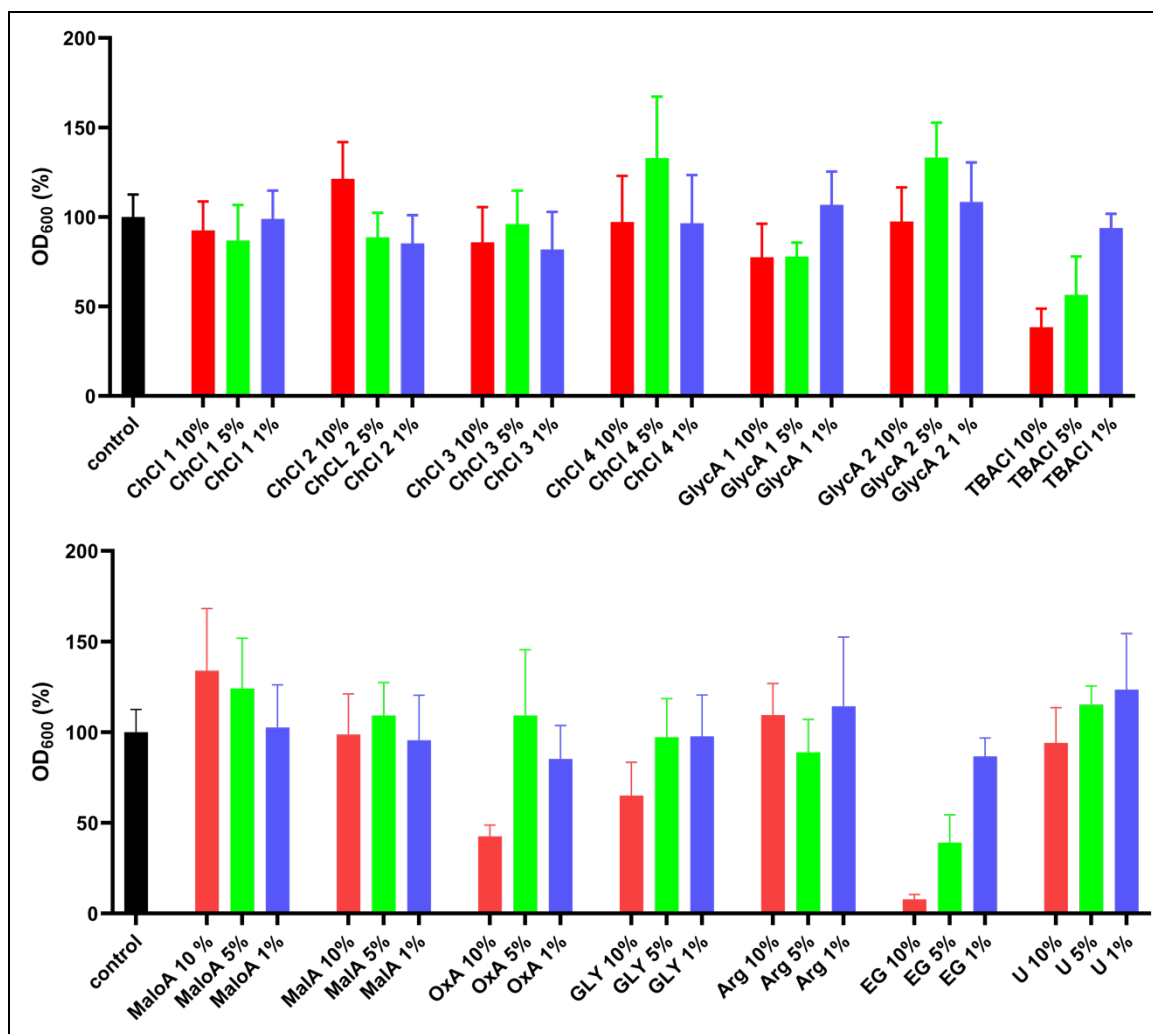


Figure 50. AE assay of the PSs of the investigated DESs against *C. albicans* strain ("ChCl #" indicates the concentration of ChCl corresponding to that of # = 1 – glycoline, maline and ethaline 2 – maloline and relene, 3 – glyceline, 4 – oxaline; "GlycA #" indicates the concentration of GlycA corresponding to that of # = 1 – glycoline, 2 -T-glycoline).

4.5.1.3 *Candida auris*

In the final investigation involving *C. auris*, the results of the antifungal effect (AE) assay (**Figure 51**) once more underscored the variable effectiveness of DESs.

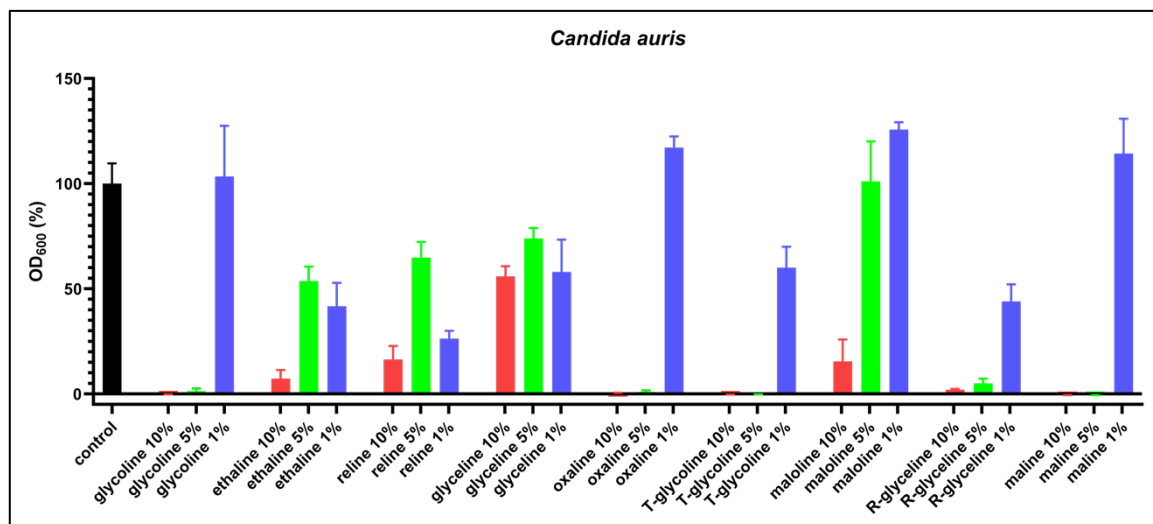


Figure 51. AE assay of the investigated DESs against *C. auris* strain.

Notably, glycoline, T-glycoline, oxaline, and maline appeared to be the most potent, as a concentration of 5% v/v led to complete inhibition of *C. auris* growth. Maloline, along with ethaline and reline, also exhibited moderate inhibition at highest concentration, although they did not achieve full growth suppression. Once again, glyceline demonstrated a benign effect on the yeast, with no observable inhibition.

The analogous test conducted for PSs (**Figure 52**) yielded intriguing results, indicating that only ethylene glycol and glycerol demonstrated antifungal activity. However, neither of these substances was able to completely inhibit fungal growth. Of note, glycerol exhibited a reduction in relative OD₆₀₀ values when used in isolation; however, this activity was not observed when glycerol was formulated in the form of DES.

Optical microscopy images (**Figure A37**) provided further validation of the effectiveness of the DES, as no discernible alteration in the morphology of the colonies was observed.

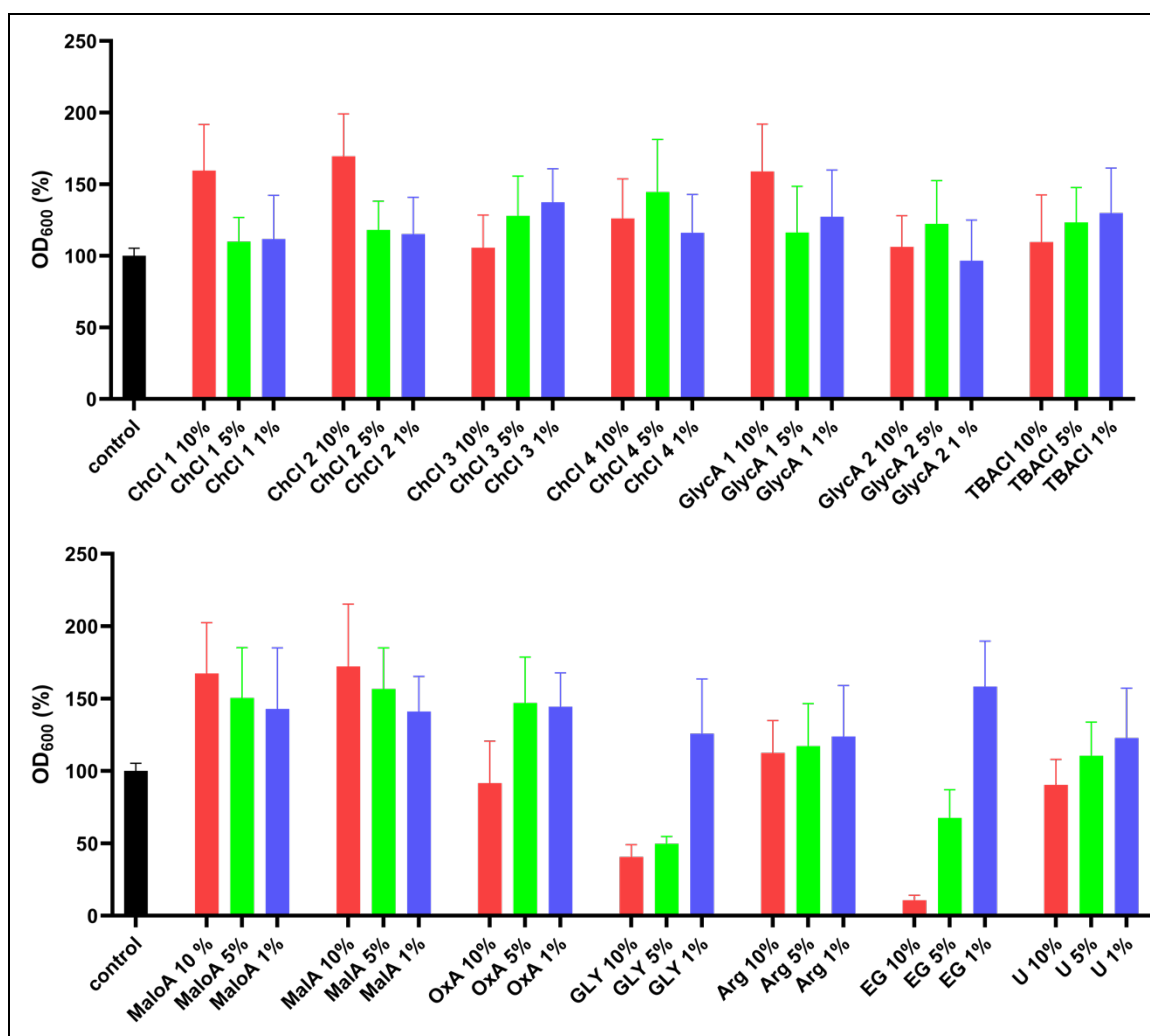


Figure 52. AE assay of the PSs of the investigated DESs against *C. auris* strain ["ChCl #" indicates the concentration of ChCl corresponding to that of # = 1 – glycoline, maline and ethaline 2 – maloline and relene, 3 – glyceline, 4 – oxaline; "GlycA #" indicates the concentration of GlycA corresponding to that of # = 1 – glycoline, 2 -T-glycoline].

The sole exception to this trend was maloline, which, at concentrations of 5% and 10% v/v, induced morphological changes characterized by the aggregation of colonies. This observation suggests that while most DES formulations maintain the typical morphology of fungal colonies, maloline may possess unique properties or concentrations that lead to distinct morphological responses.

The investigation into the antifungal activity of DESs revealed varied effectiveness across different concentrations and formulations. Most of the tested DESs exhibited antifungal properties, with six substances effectively inhibiting growth at

the highest concentration of 10% v/v. Notably, glycoline, T-glycoline, maline, oxaline, reline, and ethaline demonstrated the most potent inhibition, with OD₆₀₀ values usually below 20% compared to controls. However, maline unexpectedly stimulated proliferation of *C. albicans* at 1% v/v. Optical microscopy imaging confirmed the antifungal efficacy of DESs, showing some alterations in colony morphology, suggesting interference with essential metabolic pathways rather than physical damage to fungal cells. The observed de-palletization of fungal colonies is of a key value, as that kind of alteration of the morphology limits the pathogenicity of the yeast.

Comparative analysis with parent substances (PSs) underscored the superior antifungal activity of DESs. PSs, such as ethylene glycol and glycerol, displayed limited antifungal effects, with no complete inhibition of fungal growth observed. Interestingly, glycerol's antifungal activity was attenuated when formulated into DES, suggesting potential synergistic effects within DES compositions. Optical microscopy images further supported the effectiveness of DESs, with notable morphological changes induced by maloline, characterized by colony aggregation. Overall, DESs present promising antifungal properties with potential applications in combating fungal infections. Further research into their mechanisms of action and optimization of formulations, particularly concerning different fungal strains, is warranted to fully realize their therapeutic potential.

5. Summary and Perspectives

This thesis delves into the investigation of various deep eutectic solvents (DESs) for their potential in treating bacterial infections, supported by their structural and high frequency characterization. Nine DESs were synthesized, comprising both conventional DESs (reline, ethaline, glyceline, and R-glyceline) and organic acid-based DESs (OA-DESs) (glycoline, T-glycoline, maloline, maline, and oxaline). While parent substances (PSs) of DESs from the former group lack known antibacterial properties, the latter group includes DESs containing organic acids, which are recognized for such properties.

The first chapter of the studies presented the synthesis of DESs, which followed a standard heating procedure, confirmed by Fourier transform infrared spectroscopy showing hydrogen bond formation and polarized optical microscopy, confirming liquefaction without crystal formation. Microwave spectroscopy was employed to analyze the dielectric properties of the synthesized DESs. The results revealed that conventional DESs display strong electric energy storage and significant dielectric losses compared to OA-DESs. Specifically, the dielectric constant and dielectric loss were measured across a wide frequency range, indicating the ability of these solvents to maintain polarization and dissipate energy efficiently. The results highlighted that the conventional DESs exhibited higher initial dielectric constants and greater differences between starting and peak values than OA-DESs, suggesting their suitability for applications requiring efficient energy storage and dissipation.

The second chapter of this thesis focused on the initial antibacterial assessments. Disk-diffusion (DD) tests against *E. coli*, *P. aeruginosa*, and MRSA supported the hypothesis of antibacterial properties in the investigated DESs. Inhibition zones for OA-DESs ranged from 16 to 30 mm, while conventional DESs generally showed no inhibition zones (except R-glyceline with zones of 7-9 mm). Subsequent experiments assessed minimal inhibitory and bactericidal concentrations

(MIC/MBC) against twelve strains (six Gram-positive and six Gram-negative), revealing additional insights. Although conventional DESs showed varied activity despite lacking antibacterial properties in DD tests, they mostly inhibited Gram-positive strains, with minimal impact on Gram-negative ones (except R-glyceline, which was effective against both groups). MIC values for conventional DESs ranged from 1 to 15% v/v, whereas organic acid-based DESs demonstrated superiority with MIC values often below 1% v/v and sometimes below 0.2% v/v, with consistent effectiveness across Gram groups. Time-kill kinetics (TK) assays and transmission electron microscopy (TEM) analysis of the most effective DES representatives, R-glyceline and oxaline, were conducted against *E. coli*, *P. aeruginosa*, and MRSA. TK assays indicated slower kill kinetics against Gram-positive MRSA but rapid eradication of Gram-negative strains within two hours. TEM images revealed distinct morphological changes for each DES, with R-glyceline seemingly causing partial cytoplasm leakage, and oxaline inducing significant damage to cellular walls and loss of pili/flagella in Gram-negative species, suggesting a more aggressive mode of action.

The third chapter delved into the eradication of biofilms by DESs. Here, OA-DESs proved to be very effective agents, enabling the eradication of both MRSA and *E. coli* biofilms. For MRSA, four out of five DESs eradicated the biofilm within the range of 0.39 to 6.25% v/v. As for *E. coli*, all the OA-DESs successfully eradicated the biofilms, although in a slightly wider range – 0.39 to 12.5% v/v. Live/Dead staining at eradication concentrations proved the OA-DESs' effectiveness, showing major red areas of dead cells as well as morphological changes of the biofilms. To ensure that high concentrations of OA-DESs would not cause recurring biofilm formation, a persister emergence experiment was employed. The results showed that only T-glycoline can induce the emergence of persisters in *E. coli* biofilms, while all the other DESs did not cause the survivability of bacterial subpopulations. The penultimate chapter focused on the application of DESs as co-drugs or enhancers of other antimicrobial agents. Conventional DESs were tested in

combination with ampicillin against *E. coli* as well as carbenicillin against MRSA. Both antibiotics were characterized by high MIC values, suggesting high resistance of the strains towards these antibiotics. However, the addition of DESs in their sub-MIC values resulted in a drastic decrease in MIC value, reaching a 10-fold decrease for ampicillin. When DESs were mixed with carbenicillin, the MIC value of the antibiotic decreased greatly, down to a 44-fold reduction for ethaline and a 120-fold reduction for glyceline, while relene did not affect the effectiveness of the antibiotic. Following this, antimicrobial peptides – naturally occurring colistin and synthetic RAP-29 – were evaluated. The addition of DESs to colistin resulted in a 3.75 to 6.80-fold decrease in MIC value, with relene exhibiting the highest efficacy as a co-drug. This was also evaluated for colistin-resistant *E. coli* strains, where the addition of relene resulted in a decrease in MIC value of colistin, suggesting that relene can overcome the *mcr* gene mutation responsible for colistin resistance. RAP-29, to which *P. aeruginosa* exhibited resistance, was also tested. The addition of relene at 4.5% v/v sensitized the bacteria towards this peptide, and further increases in DES concentration lowered RAP-29's efficacy even more. This phenomenon was investigated by turbidimetry, which indicated DES-induced stabilization of the peptide. The last experiment of this chapter involved the use of nisin, a common bacteriocin used in the food industry. While nisin alone is effective against planktonic bacteria, it does not exhibit good properties in fighting biofilms. The addition of glyceline, a conventional DES which seemed to partially solubilize MRSA biofilm, was tested in two strategies: as a premixed solution of DES and nisin and as a sequential treatment, where glyceline was first added to the biofilm, followed by nisin treatment. While the first strategy was ineffective, the latter proved successful in eradicating the biofilm at concentrations as low as 125 µg/mL.

The final chapter aimed at testing whether DESs can be used to combat fungi, particularly yeasts that pose a threat to human health, such as *C. albicans*, *C. auris*, and *A. fumigatus*. The majority of DESs were able to eradicate the investigated

yeasts at a concentration of 10% v/v, with some being effective at 1% v/v. Comparative studies with PSs showed the effectiveness of DES-based formulations, as all but one PS were ineffective in eradicating yeasts. Interestingly, maloline exhibited a proliferation effect on *C. albicans*. Optical microscopy images showed morphological changes induced by some DESs in *C. albicans* and *C. auris* inocula, changing the colonies from pellet-like forms to dispersed colonies, thus lowering their virulence factor.

In summary, this thesis provides transformative insights into the characterization and antimicrobial activities of deep eutectic solvents, effectively bridging the gap between fundamental research and practical applications. The comprehensive analysis and experimentation conducted in this study reveal the vast potential of DESs as versatile antimicrobial agents. By delving into the synthesis, dielectric properties, and extensive antimicrobial testing of these novel materials, the research has laid a solid foundation for the development of innovative antimicrobial strategies.

The findings from this thesis highlight the unique dielectric properties of DESs, particularly the ability of conventional DESs to store electric energy efficiently and exhibit significant dielectric losses. This characteristic can be harnessed in various technological applications, including the development of advanced electronic and photonic devices. The potential to tailor these materials for specific energy storage and dissipation needs opens up new avenues in materials science and engineering, driving forward the capabilities of next-generation electronic components.

Moreover, the demonstrated antimicrobial properties of OA-DESs position them as promising candidates for addressing critical challenges in medical and healthcare settings. The effective inhibition of bacterial growth, especially against antibiotic-resistant strains, underscores the potential of DESs in developing new treatments for bacterial infections. Their ability to act as co-drugs, enhancing the efficacy of existing antibiotics and antimicrobial peptides, presents a valuable

strategy to combat the escalating issue of antibiotic resistance. This approach could revolutionize combination therapies, making them more effective against resistant bacterial strains and reducing the global burden of bacterial infections.

The potential applications of DESs extend beyond human health into agriculture, where they could play a crucial role in protecting crops from bacterial and fungal pathogens. The use of DESs as antimicrobial agents in agricultural settings could reduce the reliance on traditional chemical pesticides, promoting a more sustainable and environmentally friendly approach to crop protection. This could lead to healthier food production systems and improved food security worldwide. In industrial settings, the ability of DESs to eradicate biofilms presents significant advantages. Biofilms, often resistant to conventional cleaning methods, pose a major challenge in various industries, including food processing, water treatment, and pharmaceuticals. The efficacy of DESs in biofilm eradication could lead to cleaner production environments, reducing contamination risks and enhancing product safety. This, in turn, could improve industrial efficiency and reduce costs associated with biofilm-related issues. The antifungal properties of DESs also offer promising applications in the treatment of fungal infections, which are particularly challenging in immunocompromised individuals. The effectiveness of DESs against yeasts such as *C. albicans* and *C. auris* suggests their potential use in antifungal therapies, providing new options for managing fungal infections that are difficult to treat with existing medications.

Looking forward, future research could further explore the diverse applications of DESs in various fields. In medicine, continued investigation into the synergistic effects of DESs with different antimicrobial agents could lead to the development of highly effective combination therapies. Research could also focus on optimizing the formulation and delivery methods of DES-based treatments to enhance their efficacy and patient compliance. In agriculture, studies could investigate the long-term effects of DESs on crop health and soil ecosystems, ensuring that their use is both effective and sustainable. Exploring the potential of DESs in integrated pest

management systems could provide farmers with new tools to protect their crops while minimizing environmental impact. In industry, research could focus on scaling up the production of DESs and integrating them into existing cleaning and disinfection protocols. Investigating the compatibility of DESs with various industrial materials and processes could facilitate their adoption across different sectors.

Overall, this thesis lays the groundwork for the development of DESs as multifaceted antimicrobial agents, addressing urgent global health challenges and paving the way for innovative applications in medicine, agriculture, and industry. By leveraging the unique properties of DESs, future research and development efforts can contribute to safer, healthier, and more sustainable practices across various domains, ultimately enhancing human health and safety on a global scale.

Literature

- [1] A. Gray, F. Sharara, *Lancet Glob. Health* **2022**, *10*, S2.
- [2] "MICROBE," can be found under <https://vizhub.healthdata.org/microbe>, **n.d.**
- [3] A. Reza, J. M. Sutton, K. M. Rahman, *Antibiotics* **2019**, *8*, 229.
- [4] M. S. Mulani, E. E. Kamble, S. N. Kumkar, M. S. Tawre, K. R. Pardesi, *Front. Microbiol.* **2019**, *10*, 539.
- [5] European Centre for Disease Prevention and Control., World Health Organization., *Antimicrobial Resistance Surveillance in Europe 2023: 2021 Data.*, Publications Office, LU, **2023**.
- [6] "Antimicrobial resistance," can be found under <https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance>, **n.d.**
- [7] B. Mazińska, W. Hryniewicz, *Pol. J. Microbiol.* **2017**, *66*, 309.
- [8] J. Merlino, S. Siarakas, *Microb. Drug Resist.* **2022**, *28*, 536.
- [9] A. J. Browne, M. G. Chipeta, G. Haines-Woodhouse, E. P. A. Kumaran, B. H. K. Hamadani, S. Zaraa, N. J. Henry, A. Deshpande, R. C. Reiner, N. P. J. Day, et al., *Lancet Planet. Health* **2021**, *5*, e893.
- [10] R. Mulchandani, Y. Wang, M. Gilbert, T. P. Van Boeckel, *PLOS Glob. Public Health* **2023**, *3*, e0001305.
- [11] S. Reardon, *Nature* **2023**, *614*, 397.
- [12] B. A. Wee, D. M. Muloi, B. A. D. van Bunnik, *Clin. Microbiol. Infect.* **2020**, *26*, 1612.
- [13] G. Jalloul, I. Keniar, A. Tehrani, C. Boyadjian, *Front. Environ. Sci.* **2021**, *9*.
- [14] R. Rappuoli, P. Young, E. Ron, S. Pecetta, M. Pizza, *One Health Outlook* **2023**, *5*, 5.
- [15] R. Sender, S. Fuchs, R. Milo, *PLOS Biol.* **2016**, *14*, e1002533.
- [16] H. J. Flint, K. P. Scott, P. Louis, S. H. Duncan, *Nat. Rev. Gastroenterol. Hepatol.* **2012**, *9*, 577.
- [17] T. Uebanso, T. Shimohata, K. Mawatari, A. Takahashi, *Mol. Nutr. Food Res.* **2020**, *64*, 2000426.
- [18] J. L. Ellis, J. P. Karl, A. M. Oliverio, X. Fu, J. W. Soares, B. E. Wolfe, C. J. Hernandez, J. B. Mason, S. L. Booth, *Gut Microbes* **2021**, *13*, 1887721.
- [19] K. M. Maslowski, A. T. Vieira, A. Ng, J. Kranich, F. Sierro, Di Yu, H. C. Schilter, M. S. Rolph, F. Mackay, D. Artis, et al., *Nature* **2009**, *461*, 1282.
- [20] T. G. Dinan, J. F. Cryan, *Psychosom. Med.* **2017**, *79*, 920.
- [21] G. D. Ehrlich, N. L. Hiller, F. Z. Hu, *Genome Biol.* **2008**, *9*, 225.
- [22] A. R. Ahmad, S. Ridgeway, A. A. Shibl, Y. Idaghdour, A. R. Jha, *PLOS ONE* **2024**, *19*, e0293895.
- [23] M. L. Hutchison, D. J. I. Thomas, A. H. Small, S. Buncic, M. Howell, *J. Food Prot.* **2007**, *70*, 1633.
- [24] J. S. Weese, M. B. Fulford, in *Companion Anim. Zoonoses*, John Wiley & Sons, Ltd, **2011**, pp. 109–240.
- [25] K. Kwiecien, A. Zegar, J. Jung, P. Brzoza, M. Kwitniewski, U. Godlewska, B. Grygier, P. Kwiecinska, A. Morytko, J. Cichy, *Cytokine Growth Factor Rev.* **2019**, *49*, 70.
- [26] K. F. Johnstone, M. C. Herzberg, *Front. Oral Health* **2022**, *3*, DOI 10.3389/froh.2022.958480.
- [27] A. M. McDermott, *Exp. Eye Res.* **2013**, *117*, 53.

- [28] R. Schnetler, W. D. H. Gillan, G. Koorsen, *Afr. Vis. Eye Health* **2012**, *71*, 123.
- [29] A. J. M. Ligtenberg, Ed. , *Saliva - Secretion and Functions: And 15 Tables*, Karger, Basel Freiburg, **2014**.
- [30] N. Parker, M. Schneegurt, A.--Hue T. Tu, B. M. Forster, P. Lister, *Microbiology*, OpenStax College, Rice University, Houston, Texas, **2016**.
- [31] M. A. Caligiuri, **2008**, *112*.
- [32] E. M. Van Beek, F. Cochrane, A. N. Barclay, T. K. Van Den Berg, *J. Immunol.* **2005**, *175*, 7781.
- [33] D. J. Witt, D. E. Craven, W. R. McCabe, *Am. J. Med.* **1987**, *82*, 900.
- [34] T. Bjarnsholt, *APMIS* **2013**, *121*, 1.
- [35] M. K. Ibrahim, M. Zambruni, C. L. Melby, P. C. Melby, *Clin. Microbiol. Rev.* **2017**, *30*, 919.
- [36] J. Videcnik Zorman, L. Lusa, F. Strle, V. Maraspin, *Infection* **2013**, *41*, 909.
- [37] P. W. Stone, *Expert Rev. Pharmacoecon. Outcomes Res.* **2009**, *9*, 417.
- [38] J. A. Al-Tawfiq, P. A. Tambyah, *J. Infect. Public Health* **2014**, *7*, 339.
- [39] A. Revelas, *Niger. Med. J.* **2012**, *53*, 59.
- [40] J. A. Otter, G. L. French, *J. Hosp. Infect.* **2011**, *79*, 189.
- [41] G. W. KAATZ, S. D. GITLIN, D. R. SCHABERG, K. H. WILSON, C. A. KAUFFMAN, S. M. SEO, R. FEKETTY, *Am. J. Epidemiol.* **1988**, *127*, 1289.
- [42] L. Dijkshoorn, A. Nemec, H. Seifert, *Nat. Rev. Microbiol.* **2007**, *5*, 939.
- [43] C. Verdial, I. Serrano, L. Tavares, S. Gil, M. Oliveira, *Biomedicines* **2023**, *11*, 1221.
- [44] E. Bouza, Á. Asensio, J. A. G. Navarro, P. González, M. Á. A. Benito, J. Aguilar, J. Barberán, J. Cabrera, J. Díez-Manglano, C. Fernández, et al., *Rev. Esp. Quimioter.* **2023**, *36*, 552.
- [45] K. Zay Ya, P. T. N. Win, J. Bielicki, M. Lambiris, G. Fink, *JAMA Netw. Open* **2023**, *6*, e2253806.
- [46] L. Zaffiri, J. Gardner, L. H. Toledo-Pereyra, *J. Invest. Surg.* **2012**, *25*, 67.
- [47] "FDA Approves Sirturo to Treat Multi-Drug Resistant Tuberculosis," can be found under <https://www.drugs.com/newdrugs/fda-approves-sirturo-multi-resistant-tuberculosis-3619.html>, **n.d.**
- [48] H. L. Stennett, C. R. Back, P. R. Race, *Antibiotics* **2022**, *11*, 1237.
- [49] J. E. Ferrie, *Int. J. Epidemiol.* **2014**, *43*, 977.
- [50] J. L. Strominger, D. J. Tipper, *Am. J. Med.* **1965**, *39*, 708.
- [51] O. Sköld, *Drug Resist. Updat.* **2000**, *3*, 155.
- [52] M. Baunach, L. Ding, K. Willing, C. Hertweck, *Angew. Chem. Int. Ed.* **2015**, *54*, 13279.
- [53] Q.-Y. Zhang, Z.-B. Yan, Y.-M. Meng, X.-Y. Hong, G. Shao, J.-J. Ma, X.-R. Cheng, J. Liu, J. Kang, C.-Y. Fu, *Mil. Med. Res.* **2021**, *8*, 48.
- [54] C. T. D. Price, I. R. Lee, J. E. Gustafson, *Int. J. Biochem. Cell Biol.* **2000**, *32*, 1029.
- [55] B. D. Davis, *Microbiol. Rev.* **1987**, *51*, 341.
- [56] L. S. Pierson, E. A. Pierson, *FEMS Microbiol. Lett.* **1996**, *136*, 101.
- [57] V. V. H. Le, J. Rakonjac, *PLoS Pathog.* **2021**, *17*, e1009663.
- [58] J. L. Smith, E. D. Weinberg, *J. Gen. Microbiol.* **1962**, *28*, 559.

- [59] A. D. Russell, R. H. Fountain, *J. Bacteriol.* **1971**, *106*, 65.
- [60] J. Ciak, F. E. Hahn, *J. Bacteriol.* **1958**, *75*, 125.
- [61] C. Ayoub Moubareck, *Membranes* **2020**, *10*, 181.
- [62] A. M. Zaher, M. A. Makboul, A. M. Moharram, B. L. Tekwani, A. I. Calderón, *J. Antibiot. (Tokyo)* **2015**, *68*, 197.
- [63] D. Schnappinger, W. Hillen, *Arch. Microbiol.* **1996**, *165*, 359.
- [64] J. M. T. Hamilton-Miller, *J. Antimicrob. Chemother.* **1979**, *5*, 61.
- [65] H. Wank, J. Rogers, J. Davies, R. Schroeder, *J. Mol. Biol.* **1994**, *236*, 1001.
- [66] Z. Eyal, D. Matzov, M. Krupkin, S. Paukner, R. Riedl, H. Rozenberg, E. Zimmerman, A. Bashan, A. Yonath, *Sci. Rep.* **2016**, *6*, 39004.
- [67] N. Vázquez-Laslop, A. S. Mankin, *Trends Biochem. Sci.* **2018**, *43*, 668.
- [68] J. Bi, H. Wang, J. Xie, *J. Cell. Physiol.* **2011**, *226*, 331.
- [69] D. Vazquez, *J. Gen. Microbiol.* **1966**, *42*, 93.
- [70] M. Prieri, R. Frita, N. Probst, A. Sournia-Saquet, M. Bourotte, B. Déprez, A. R. Baulard, N. Willand, *Eur. J. Med. Chem.* **2018**, *159*, 35.
- [71] N. E. Allen, T. I. Nicas, *FEMS Microbiol. Rev.* **2003**, *26*, 511.
- [72] J. Spížek, T. Řezanka, *Biochem. Pharmacol.* **2017**, *133*, 20.
- [73] G. A. Prosser, L. P. S. de Carvalho, *FEBS J.* **2013**, *280*, 1150.
- [74] W. Wehrli, in *Med. Chem.*, Springer-Verlag, Berlin/Heidelberg, **1977**, pp. 21–49.
- [75] P. Fernandes, *Cold Spring Harb. Perspect. Med.* **2016**, *6*, a025437.
- [76] J. L. Davis, M. G. Papich, in *Equine Infect. Dis. Second Ed.* (Eds: D. C. Sellon, M. T. Long), W.B. Saunders, St. Louis, **2014**, pp. 571–584.e5.
- [77] K. Schubert, B. Sieger, F. Meyer, G. Giacomelli, K. Böhm, A. Rieblinger, L. Lindenthal, N. Sachs, G. Wanner, M. Bramkamp, *mBio* **2017**, *8*, e02213.
- [78] N. G. Bush, I. Diez-Santos, L. R. Abbott, A. Maxwell, *Molecules* **2020**, *25*, 5662.
- [79] Y. Cao, Q. Peng, S. Li, Z. Deng, J. Gao, *RSC Adv.* **2019**, *9*, 42204.
- [80] S. Khoshnood, M. Heidary, A. Asadi, S. Soleimani, M. Motahar, M. Savari, M. Saki, M. Abdi, *Biomed. Pharmacother.* **2019**, *109*, 1809.
- [81] M. Talpaert, F. Campagnari, L. Clerici, *Biochem. Biophys. Res. Commun.* **1975**, *63*, 328.
- [82] M. I. El-Gamal, I. Brahim, N. Hisham, R. Aladdin, H. Mohammed, A. Bahaaeldin, *Eur. J. Med. Chem.* **2017**, *131*, 185.
- [83] C. R. Dean, D. T. Barkan, A. Bermingham, J. Blais, F. Casey, A. Casarez, R. Colvin, J. Fuller, A. K. Jones, C. Li, et al., *Antimicrob. Agents Chemother.* **2018**, *62*, e01200.
- [84] B. Bozdogan, P. C. Appelbaum, *Int. J. Antimicrob. Agents* **2004**, *23*, 113.
- [85] T. M. Wood, N. I. Martin, *MedChemComm* **2019**, *10*, 634.
- [86] J. P. Sarathy, G. Gruber, T. Dick, *Antibiotics* **2019**, *8*, 261.
- [87] S. S. Branda, Å. Vik, L. Friedman, R. Kolter, *Trends Microbiol.* **2005**, *13*, 20.
- [88] K. Lewis, *Antimicrob. Agents Chemother.* **2001**, *45*, 999.

- [89] A. Schulze, F. Mitterer, J. P. Pombo, S. Schild, *Microb. Cell* **2021**, *8*, 28.
- [90] J. A. Otter, K. Vickery, J. T. Walker, E. deLancey Pulcini, P. Stoodley, S. D. Goldenberg, J. A. G. Salkeld, J. Chewins, S. Yezli, J. D. Edgeworth, *J. Hosp. Infect.* **2015**, *89*, 16.
- [91] V. Vishwakarma, *J. Basic Microbiol.* **2020**, *60*, 198.
- [92] M. Simões, R. N. Bennett, E. A. S. Rosa, *Nat. Prod. Rep.* **2009**, *26*, 746.
- [93] R. Roy, M. Tiwari, G. Donelli, V. Tiwari, *Virulence* **2018**, *9*, 522.
- [94] I. Banerjee, R. C. Pangule, R. S. Kane, *Adv. Mater.* **2011**, *23*, 690.
- [95] K. Lewis, *Annu. Rev. Microbiol.* **2010**, *64*, 357.
- [96] B. P. Conlon, S. E. Rowe, K. Lewis, in *Biofilm-Based Healthc.-Assoc. Infect.* (Ed: G. Donelli), Springer International Publishing, Cham, **2015**, pp. 1–9.
- [97] A. Harms, in *Persister Cells Infect. Dis.* (Ed: K. Lewis), Springer International Publishing, Cham, **2019**, pp. 39–57.
- [98] M. Fauvart, V. N. De Groote, J. Michiels, *J. Med. Microbiol.* **2011**, *60*, 699.
- [99] R. A. Fisher, B. Gollan, S. Helaine, *Nat. Rev. Microbiol.* **2017**, *15*, 453.
- [100] F. Khan, D. T. N. Pham, N. Tabassum, S. F. Oloketuyi, Y.-M. Kim, *Crit. Rev. Microbiol.* **2020**, *46*, 665.
- [101] G. F. Hatfull, R. M. Dedrick, R. T. Schooley, *Annu. Rev. Med.* **2022**, *73*, 197.
- [102] M. Magana, M. Pushpanathan, A. L. Santos, L. Leanse, M. Fernandez, A. Ioannidis, M. A. Giulianotti, Y. Apidianakis, S. Bradfute, A. L. Ferguson, et al., *Lancet Infect. Dis.* **2020**, *20*, e216.
- [103] Z. Lei, A. Karim, *J. Vet. Pharmacol. Ther.* **2021**, *44*, 281.
- [104] T. R. McCulloch, T. J. Wells, F. Souza-Fonseca-Guimaraes, *Trends Microbiol.* **2022**, *30*, 158.
- [105] S. Rehman, Z. Ali, M. Khan, N. Bostan, S. Naseem, *Rev. Med. Virol.* **2019**, *29*, e2041.
- [106] "Considerations for the Use of Phage Therapy in Clinical Practice," DOI 10.1128/aac.02071-21 can be found under <https://journals.asm.org/doi/epdf/10.1128/aac.02071-21>, **n.d.**
- [107] K. E. Kortright, B. K. Chan, J. L. Koff, P. E. Turner, *Cell Host Microbe* **2019**, *25*, 219.
- [108] M. Podlacha, L. Gaffke, Ł. Grabowski, J. Mantej, M. Grabski, M. Pierzchalska, K. Pierzynowska, G. Węgrzyn, A. Węgrzyn, *Nat. Commun.* **2024**, *15*, 1.
- [109] S. LaVergne, T. Hamilton, B. Biswas, M. Kumaraswamy, R. T. Schooley, D. Wooten, *Open Forum Infect. Dis.* **2018**, *5*, ofy064.
- [110] C. Duplessis, B. Biswas, B. Hanisch, M. Perkins, M. Henry, J. Quinones, D. Wolfe, L. Estrella, T. Hamilton, *J. Pediatr. Infect. Dis. Soc.* **2018**, *7*, 253.
- [111] R. T. Schooley, B. Biswas, J. J. Gill, A. Hernandez-Morales, J. Lancaster, L. Lessor, J. J. Barr, S. L. Reed, F. Rohwer, S. Benler, et al., *Antimicrob. Agents Chemother.* **2017**, *61*, e00954.
- [112] M. Szadkowska, A. M. Kocot, D. Sowik, D. Wyrzykowski, E. Jankowska, L. P. Kozłowski, J. Makowska, M. Plotka, *Front. Microbiol.* **2024**, *14*, 1303794.
- [113] X. Luo, H. Chen, Y. Song, Z. Qin, L. Xu, N. He, Y. Tan, W. Dessie, *Eur. J. Pharm. Sci.* **2023**, *181*, 106363.
- [114] C.-I. Cheigh, Y.-R. Pyun, *Biotechnol. Lett.* **2005**, *27*, 1641.
- [115] M. Abdi, S. Mirkalantari, N. Amirmozafari, *J. Pept. Sci.* **2019**, *25*, e3210.

- [116] M. A. E.-G. El-Sayed Ahmed, L.-L. Zhong, C. Shen, Y. Yang, Y. Doi, G.-B. Tian, *Emerg. Microbes Infect.* **n.d.**, *9*, 868.
- [117] Q. Li, M. Montalban-Lopez, O. P. Kuipers, *Appl. Environ. Microbiol.* **2018**, *84*, e00052.
- [118] E. M. Kościuczuk, P. Lisowski, J. Jarczak, N. Strzałkowska, A. Jóźwik, J. Horbańczuk, J. Krzyżewski, L. Zwierzchowski, E. Bagnicka, *Mol. Biol. Rep.* **2012**, *39*, 10957.
- [119] M. R. Pincus, in *Cell Physiol. Source Book Fourth Ed.* (Ed: N. Sperelakis), Academic Press, San Diego, **2012**, pp. 19–47.
- [120] D. Xu, W. Lu, *Front. Immunol.* **2020**, *11*, 764.
- [121] H. G. Boman, B. Agerberth, A. Boman, *Infect. Immun.* **1993**, *61*, 2978.
- [122] S. Pandidan, A. Mechler, *Sci. Rep.* **2019**, *9*, 10841.
- [123] I. Senyürek, M. Paulmann, T. Sinnberg, H. Kalbacher, M. Deeg, T. Gutschmann, M. Hermes, T. Kohler, F. Götz, C. Wolz, et al., *Antimicrob. Agents Chemother.* **2009**, *53*, 2499.
- [124] Y. Syaputri, H. Iwahashi, *Rev. Agric. Sci.* **2020**, *8*, 124.
- [125] M. Heidary, A. D. Khosravi, S. Khoshnood, M. J. Nasiri, S. Soleimani, M. Goudarzi, *J. Antimicrob. Chemother.* **2018**, *73*, 1.
- [126] P. H. Mygind, R. L. Fischer, K. M. Schnorr, M. T. Hansen, C. P. Sönksen, S. Ludvigsen, D. Raventós, S. Buskov, B. Christensen, L. De Maria, et al., *Nature* **2005**, *437*, 975.
- [127] J. L. Smith, E. D. Weinberg, *Microbiology* **1962**, *28*, 559.
- [128] A. P. Zavascki, L. Z. Goldani, J. Li, R. L. Nation, *J. Antimicrob. Chemother.* **2007**, *60*, 1206.
- [129] B. K. Prasannan, F. C. Mukthar, V. N. Unni, S. Mohan, K. Vinodkumar, *Indian J. Nephrol.* **2021**, *31*, 449.
- [130] S. Anees Ahmad, S. Sachi Das, A. Khatoon, M. Tahir Ansari, Mohd. Afzal, M. Saquib Hasnain, A. Kumar Nayak, *Mater. Sci. Energy Technol.* **2020**, *3*, 756.
- [131] W.-R. Li, X.-B. Xie, Q.-S. Shi, H.-Y. Zeng, Y.-S. OU-Yang, Y.-B. Chen, *Appl. Microbiol. Biotechnol.* **2010**, *85*, 1115.
- [132] W.-R. Li, X.-B. Xie, Q.-S. Shi, S.-S. Duan, Y.-S. Ouyang, Y.-B. Chen, *BioMetals* **2011**, *24*, 135.
- [133] Z. Ferdous, A. Nemmar, *Int. J. Mol. Sci.* **2020**, *21*, 2375.
- [134] Y. K. Jo, J. H. Seo, B.-H. Choi, B. J. Kim, H. H. Shin, B. H. Hwang, H. J. Cha, *ACS Appl. Mater. Interfaces* **2014**, *6*, 20242.
- [135] S. Taheri, A. Cavallaro, S. N. Christo, L. E. Smith, P. Majewski, M. Barton, J. D. Hayball, K. Vasilev, *Biomaterials* **2014**, *35*, 4601.
- [136] N. Dixit, S. P. Singh, *ACS Omega* **2022**, *7*, 5112.
- [137] D. Wang, J. Li, Y. Wang, F. Liu, G. Wang, X. Ding, S. Luo, G. Chen, *ACS Appl. Nano Mater.* **2022**, *5*, 6841.
- [138] S. P. Singh, Y. Li, A. Be'er, Y. Oren, J. M. Tour, C. J. Arnusch, *ACS Appl. Mater. Interfaces* **2017**, *9*, 18238.
- [139] N. T. Huynh, C. Passirani, P. Saulnier, J. P. Benoit, *Int. J. Pharm.* **2009**, *379*, 201.
- [140] R. Valsalakumari, S. K. Yadava, M. Szwed, A. D. Pandya, G. M. Mælandsmo, M. L. Torgersen, T.-G. Iversen, T. Skotland, K. Sandvig, J. Giri, *Int. J. Pharm.* **2021**, *597*, 120217.

- [141] R. P. Moura, C. Pacheco, A. P. Pêgo, A. Des Rieux, B. Sarmento, *J. Controlled Release* **2020**, *322*, 390.
- [142] C. Tapeinos, M. Battaglini, G. Ciofani, *J. Controlled Release* **2017**, *264*, 306.
- [143] R. Cé, B. Z. Pacheco, T. M. Ciocheta, F. S. Barbosa, A. D. C. S. Alves, D. R. Dallemole, V. Lavayen, S. S. Guterres, M. Steppe, A. R. Pohlmann, *React. Funct. Polym.* **2021**, *162*, 104876.
- [144] C. Valcourt, P. Saulnier, A. Umerska, M. P. Zanelli, A. Montagu, E. Rossines, M. L. Joly-Guillou, *Int. J. Pharm.* **2016**, *498*, 23.
- [145] R. Jijie, A. Barras, J. Bouckaert, N. Dumitrascu, S. Szunerits, R. Boukherroub, *Colloids Surf. B Biointerfaces* **2018**, *170*, 347.
- [146] E. Sviridova, A. Barras, A. Addad, E. Plotnikov, A. Di Martino, D. Deresmes, K. Nikiforova, M. Trusova, S. Szunerits, O. Guselnikova, et al., *Biomater. Adv.* **2022**, *134*, DOI 10.1016/j.msec.2022.112697.
- [147] N. Hazime, Y. Belguesmia, I. Kempf, A. Barras, D. Drider, R. Boukherroub, *Pharmaceuticals* **2022**, *15*, 682.
- [148] P. C. Henriques, A. T. Pereira, D. Bogas, J. R. Fernandes, A. M. Pinto, F. D. Magalhães, I. C. Gonçalves, *Carbon* **2021**, *180*, 10.
- [149] O. Akhavan, E. Ghaderi, *ACS Nano* **2010**, *4*, 5731.
- [150] A. P. Abbott, G. Capper, D. L. Davies, R. K. Rasheed, V. Tambyrajah, *Chem. Commun.* **2003**, 70.
- [151] M. Vian, C. Breil, L. Vernes, E. Chaabani, F. Chemat, *Curr. Opin. Green Sustain. Chem.* **2017**, *5*, 44.
- [152] A. E. Ünlü, A. Arıkaya, S. Takaç, *Green Process. Synth.* **2019**, *8*, 355.
- [153] E. L. Smith*, *Trans. IMF* **2013**, *91*, 241.
- [154] H.-G. Liao, Y.-X. Jiang, Z.-Y. Zhou, S.-P. Chen, S.-G. Sun, *Angew. Chem. Int. Ed.* **2008**, *47*, 9100.
- [155] M. Figueiredo, C. Gomes, R. Costa, A. Martins, C. M. Pereira, F. Silva, *Electrochimica Acta* **2009**, *54*, 2630.
- [156] R. Costa, M. Figueiredo, C. M. Pereira, F. Silva, *Electrochimica Acta* **2010**, *55*, 8916.
- [157] E. L. Smith, A. P. Abbott, K. S. Ryder, *Chem. Rev.* **2014**, *114*, 11060.
- [158] S. C. Cunha, J. O. Fernandes, *TrAC Trends Anal. Chem.* **2018**, *105*, 225.
- [159] B. B. Hansen, S. Spittle, B. Chen, D. Poe, Y. Zhang, J. M. Klein, A. Horton, L. Adhikari, T. Zelovich, B. W. Doherty, et al., *Chem. Rev.* **2021**, *121*, 1232.
- [160] Y. Liu, J. B. Friesen, J. B. McAlpine, D. C. Lankin, S.-N. Chen, G. F. Pauli, *J. Nat. Prod.* **2018**, *81*, 679.
- [161] T. El Achkar, H. Greige-Gerges, S. Fourmentin, *Environ. Chem. Lett.* **2021**, *19*, 3397.
- [162] M. H. Zainal-Abidin, M. Hayyan, G. C. Ngoh, W. F. Wong, C. Y. Looi, *J. Controlled Release* **2019**, *316*, 168.
- [163] C. Huang, X. Chen, C. Wei, H. Wang, H. Gao, *Front. Pharmacol.* **2021**, *12*, DOI 10.3389/fphar.2021.794939.
- [164] PubChem, "Urea," can be found under <https://pubchem.ncbi.nlm.nih.gov/compound/1176>, n.d.

- [165] PubChem, "Choline Chloride," can be found under <https://pubchem.ncbi.nlm.nih.gov/compound/6209>, **n.d.**
- [166] C. F. Araujo, J. A. P. Coutinho, M. M. Nolasco, S. F. Parker, P. J. A. Ribeiro-Claro, S. Rudić, B. I. G. Soares, P. D. Vaz, *Phys. Chem. Chem. Phys.* **2017**, *19*, 17998.
- [167] H. Zhang, X. Lu, L. González-Aguilera, M. L. Ferrer, F. Del Monte, M. C. Gutiérrez, *J. Chem. Phys.* **2021**, *154*, DOI 10.1063/5.0049162.
- [168] M. Marchel, H. Cieśliński, G. Boczkaj, *J. Hazard. Mater.* **2022**, *425*, 127963.
- [169] A. P. Abbott, J. C. Barron, K. S. Ryder, D. Wilson, *Chem Eur J* **2007**.
- [170] P. Stott, *J. Controlled Release* **1998**, *50*, 297.
- [171] A. R. C. Duarte, A. S. D. Ferreira, S. Barreiros, E. Cabrita, R. L. Reis, A. Paiva, *Eur. J. Pharm. Biopharm.* **2017**, *114*, 296.
- [172] I. M. Aroso, J. C. Silva, F. Mano, A. S. D. Ferreira, M. Dionísio, I. Sá-Nogueira, S. Barreiros, R. L. Reis, A. Paiva, A. R. C. Duarte, *Eur. J. Pharm. Biopharm.* **2016**, *98*, 57.
- [173] M. Zakrewsky, A. Banerjee, S. Apte, T. L. Kern, M. R. Jones, R. E. D. Sesto, A. T. Koppisch, D. T. Fox, S. Mitragotri, *Adv. Healthc. Mater.* **2016**, *5*, 1282.
- [174] A. Banerjee, K. Ibsen, Y. Iwao, M. Zakrewsky, S. Mitragotri, *Adv. Healthc. Mater.* **2017**, *6*, 1601411.
- [175] E. E. L. Tanner, K. N. Ibsen, S. Mitragotri, *J. Controlled Release* **2018**, *286*, 137.
- [176] K. O. Wikene, E. Bruzell, H. H. Tønnesen, *Eur. J. Pharm. Sci.* **2015**, *80*, 26.
- [177] Z. Li, P. I. Lee, *Int. J. Pharm.* **2016**, *505*, 283.
- [178] H. Maag, in *Prodrugs* (Eds: V. J. Stella, R. T. Borchardt, M. J. Hageman, R. Oliyai, H. Maag, J. W. Tilley), Springer New York, New York, NY, **2007**, pp. 703–729.
- [179] F. S. Menniti, J. Knoth, E. J. Diliberto, *J. Biol. Chem.* **1986**, *261*, 16901.
- [180] H.-K. S. Leiros, S. Kozielski-Stuhrmann, U. Kapp, L. Terradot, G. A. Leonard, S. M. McSweeney, *J. Biol. Chem.* **2004**, *279*, 55840.
- [181] L. L. Mazaleuskaya, K. N. Theken, L. Gong, C. F. Thorn, G. A. FitzGerald, R. B. Altman, T. E. Klein, *Pharmacogenet. Genomics* **2015**, *25*, 96.
- [182] A. Borges, C. Ferreira, M. J. Saavedra, M. Simões, *Microb. Drug Resist.* **2013**, *19*, 256.
- [183] O. Aldulaimi, *Pharmacogn. Rev.* **2017**, *11*, 123.
- [184] E. Rico-Munoz, E. E. Bargiota, P. M. Davidson, *Food Microbiol.* **1987**, *4*, 239.
- [185] M. Mikłasińska-Majdanik, M. Kępa, R. Wojtyczka, D. Idzik, T. Wąsik, *Int. J. Environ. Res. Public Health* **2018**, *15*, 2321.
- [186] S. T. Rutherford, B. L. Bassler, **n.d.**
- [187] H. Asfour, *J. Microsc. Ultrastruct.* **2018**, *6*, 1.
- [188] B. Amrutha, K. Sundar, P. H. Shetty, *Microb. Pathog.* **2017**, *111*, 156.
- [189] J. M. Silva, C. V. Pereira, F. Mano, E. Silva, V. I. B. Castro, I. Sá-Nogueira, R. L. Reis, A. Paiva, A. A. Matias, A. R. C. Duarte, *ACS Appl. Bio Mater.* **2019**, *2*, 4346.
- [190] J. M. Silva, E. Silva, R. L. Reis, A. R. C. Duarte, *Sustain. Chem. Pharm.* **2019**, *14*, 100192.
- [191] E.-S. Park, W.-S. Moon, M.-J. Song, M.-N. Kim, K.-H. Chung, J.-S. Yoon, *Int. Biodeterior. Biodegrad.* **2001**, *47*, 209.

- [192] Y.-J. Zhu, H.-T. Zhou, Y.-H. Hu, J.-Y. Tang, M.-X. Su, Y.-J. Guo, Q.-X. Chen, B. Liu, *Food Chem.* **2011**, *124*, 298.
- [193] Q. Wei, X. Wang, J.-H. Cheng, G. Zeng, D.-W. Sun, *Food Chem.* **2018**, *268*, 220.
- [194] J. J. Carrascal, R. Pinal, T. Carvajal, L. D. Pérez, Y. Baena, *Int. J. Pharm.* **2021**, *607*, 120991.
- [195] S. D. Cook, *Plant Cell Physiol.* **2019**, *60*, 243.
- [196] Y. Kim, J.-Y. Cho, J.-H. Kuk, J.-H. Moon, J.-I. Cho, Y.-C. Kim, K.-H. Park, *Curr. Microbiol.* **2004**, *48*, 312.
- [197] K. Radošević, I. Čanak, M. Panić, K. Markov, M. C. Bubalo, J. Frece, V. G. Srček, I. R. Redovniković, *Environ. Sci. Pollut. Res.* **2018**, *25*, 14188.
- [198] F. Al-Akayleh, R. M. Khalid, D. Hawash, E. Al-Kaissi, I. S. I. Al-Adham, N. Al-Muhtaseb, N. Jaber, M. Al-Remawi, P. J. Collier, *Lett. Appl. Microbiol.* **2022**, *75*, 607.
- [199] C. Zeng, Y. Liu, Z. Ding, H. Xia, S. Guo, *J. Mol. Liq.* **2021**, *338*, 116950.
- [200] H. J. Park, M. R. Prausnitz, *AIChE J.* **2015**, *61*, 2732.
- [201] C. V. Pereira, J. M. Silva, L. Rodrigues, R. L. Reis, A. Paiva, A. R. C. Duarte, A. Matias, *Sci. Rep.* **2019**, *9*, 14926.
- [202] W. A. Ahmed Arafa, *R. Soc. Open Sci.* **2019**, *6*, 182046.
- [203] C. Lu, J. Cao, N. Wang, E. Su, *MedChemComm* **2016**, *7*, 955.
- [204] B. K. Hwang, S. W. Lim, B. S. Kim, J. Y. Lee, S. S. Moon, *Appl. Environ. Microbiol.* **2001**, *67*, 3739.
- [205] A. F. Sánchez-Maldonado, A. Schieber, M. G. Gänzle, *J. Appl. Microbiol.* **2011**, *111*, 1176.
- [206] N. Schultheiss, A. Newman, *Cryst. Growth Des.* **2009**, *9*, 2950.
- [207] Y. H. Choi, J. van Spronsen, Y. Dai, M. Verberne, F. Hollmann, I. W. C. E. Arends, G.-J. Witkamp, R. Verpoorte, *Plant Physiol.* **2011**, *156*, 1701.
- [208] Y. Dai, J. van Spronsen, G.-J. Witkamp, R. Verpoorte, Y. H. Choi, *Anal. Chim. Acta* **2013**, *766*, 61.
- [209] R. E. Hubbard, M. Kamran Haider, in *Encycl. Life Sci.*, John Wiley & Sons, Ltd, **2010**.
- [210] T. E. Creighton, *BioEssays* **1988**, *8*, 57.
- [211] R. Esquembre, J. M. Sanz, J. G. Wall, F. del Monte, C. R. Mateo, M. L. Ferrer, *Phys. Chem. Chem. Phys.* **2013**, *15*, 11248.
- [212] E. Su, A. M. Klibanov, *Appl. Biochem. Biotechnol.* **2015**, *177*, 753.
- [213] A. Sanchez-Fernandez, K. J. Edler, T. Arnold, D. Alba Venero, A. J. Jackson, *Phys. Chem. Chem. Phys.* **2017**, *19*, 8667.
- [214] P. Angsantikul, K. Peng, A. M. Curreri, Y. Chua, K. Z. Chen, J. Ehondor, S. Mitragotri, *Adv. Funct. Mater.* **2021**, *31*, 2002912.
- [215] E. Cruz, V. Kayser, *Biol. Targets Ther.* **2019**, *Volume 13*, 33.
- [216] K. Marhelava, Z. Pilch, M. Bajor, A. Graczyk-Jarzynka, R. Zagozdzon, *Cancers* **2019**, *11*, 1756.
- [217] G. P. Adams, L. M. Weiner, *Nat. Biotechnol.* **2005**, *23*, 1147.
- [218] L. Senolt, *F1000Research* **2019**, *8*, 1549.
- [219] J. D. Isaacs, G. Hale, S. P. Cobbold, H. Waldmann, R. A. Watts, B. L. Hazleman, M. T. Keogan, *The Lancet* **1992**, *340*, 748.

- [220] N. Yadav, P. Venkatesu, *Phys. Chem. Chem. Phys.* **2022**, *24*, 13474.
- [221] I. Juneidi, M. Hayyan, M. A. Hashim, A. Hayyan, *Biochem. Eng. J.* **2017**, *117*, 129.
- [222] M. L. Toledo, M. M. Pereira, M. G. Freire, J. P. A. Silva, J. A. P. Coutinho, A. P. M. Tavares, *ACS Sustain. Chem. Eng.* **2019**, *7*, 11806.
- [223] H. Sun, R. Xin, D. Qu, F. Yao, *J. Biotechnol.* **2020**, *323*, 264.
- [224] N. Yadav, K. Bhakuni, M. Bisht, I. Bahadur, P. Venkatesu, *ACS Sustain. Chem. Eng.* **2020**, *8*, 10151.
- [225] B. Olivares, F. Martínez, L. Rivas, C. Calderón, J. M. Munita, P. R. Campodonico, *Sci. Rep.* **2018**, *8*, 14900.
- [226] J. Norgard, G. L. Best, in *Natl. Assoc. Broadcast. Eng. Handb.*, Routledge, **2017**.
- [227] R.-J. M. van Geuns, P. A. Wielopolski, H. G. de Bruin, B. J. Rensing, M. Oudkerk, P. J. de Feyter, **n.d.**
- [228] R. Chandra, I. Balasingham, H. Zhou, R. M. Narayanan, in *Med. Image Anal. Inform.*, CRC Press, **2017**.
- [229] C. Li, D. M. M.-P. Schreurs, in *Princ. Appl. RFMicrowave Healthc. Biosensing* (Eds: C. Li, M.-R. Tofighi, D. Schreurs, T.-S. J. Horng), Academic Press, **2017**, pp. 1–52.
- [230] D. J. Panagopoulos, **n.d.**
- [231] D. E. Clark, D. C. Folz, J. K. West, *Mater. Sci. Eng. A* **2000**, *287*, 153.
- [232] S. Semenov, *Philos. Trans. R. Soc. Math. Phys. Eng. Sci.* **2009**, *367*, 3021.
- [233] S. O. Nelson, *Res. Agric. Eng.* **2008**, *54*, 104.
- [234] Jung-Mu Kim, Donghoon Oh, Jeonghoon Yoon, Sungjoon Cho, Namgon Kim, Jeiwon Cho, Youngwoo Kwon, Changyul Cheon, Yong-Kweon Kim, *IEEE Trans. Microw. Theory Tech.* **2005**, *53*, 3415.
- [235] J.-M. Kim, D. H. Oh, C.-W. Baek, J.-W. Cho, Y. Kwon, C. Cheon, Y.-K. Kim, *J. Micromechanics Microengineering* **2005**, *16*, 173.
- [236] K. Haddadi, *Mes. Tests Électroniques* **2016**, DOI 10.51257/a-v1-r1117.
- [237] S. O. Nelson, A. W. Kraszewski, *Dry. Technol.* **1990**, *8*, 1123.
- [238] A. k. Datta, V. Rakesh, *Compr. Rev. Food Sci. Food Saf.* **2013**, *12*, 24.
- [239] B. Curnutte, *J. Food Prot.* **1980**, *43*, 618.
- [240] B. Reeja-Jayan, K. L. Harrison, K. Yang, C.-L. Wang, A. E. Yilmaz, A. Manthiram, *Sci. Rep.* **2012**, *2*, 1003.
- [241] S. Padmanabhan, P. Kirby, J. Daniel, L. Dunleavy, in *61st ARFTG Conf. Dig. Spring 2003*, IEEE, Philadelphia, PA, USA, **2003**, pp. 5–10.
- [242] A. Rumiantsev, N. Ridler, *IEEE Microw. Mag.* **2008**, *9*, 86.
- [243] R. E. Collin, **n.d.**
- [244] A. V. Samoilenko, *Bull. Exp. Biol. Med.* **1996**, *121*, 341.
- [245] D. Kajfez, *Dielectric Resonators*, Noble Publishing Corporation, **1998**.
- [246] In *Microw. Electron.*, John Wiley & Sons, Ltd, **2004**, pp. 288–322.
- [247] J.-S. Hong, M. J. Lancaster, **n.d.**

- [248] "QWED - your EM partner," can be found under <https://www.qwed.com.pl/>, **n.d.**
- [249] L. Wang, *Micromachines* **2023**, *14*, 1462.
- [250] K. Smith, J. Bourqui, Z. Wang, B. Besler, M. Lesiuk, M. Roumeliotis, S. Quirk, P. Grendarova, J. Pinilla, S. Price, et al., *Med. Phys.* **2023**, *50*, 7118.
- [251] A. E. Bulyshev, S. Y. Semenov, A. E. Souvorov, R. H. Svenson, A. G. Nazarov, Y. E. Sizov, G. P. Tatsis, *IEEE Trans. Biomed. Eng.* **2001**, *48*, 1053.
- [252] P. M. Meaney, A. H. Golnabi, N. R. Epstein, S. D. Geimer, M. W. Fanning, J. B. Weaver, K. D. Paulsen, *Med. Phys.* **2013**, *40*, 103101.
- [253] T. M. Grzegorzczak, P. M. Meaney, P. A. Kaufman, R. M. Di Florio-Alexander, K. D. Paulsen, *IEEE Trans. Med. Imaging* **2012**, *31*, 1584.
- [254] P. M. Meaney, S. A. Pendergrass, M. W. Fanning, D. Li, K. D. Paulsen, *J. Electromagn. Waves Appl.* **2003**, *17*, 333.
- [255] T. Svebøck, *Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) Assays Using Broth Microdilution Method*, **2023**.
- [256] I. Juneidi, M. Hayyan, O. Mohd Ali, *Environ. Sci. Pollut. Res.* **2016**, *23*, 7648.
- [257] A. P. Abbott, D. Boothby, G. Capper, D. L. Davies, R. K. Rasheed, *J. Am. Chem. Soc.* **2004**, *126*, 9142.
- [258] A. H. Panhwar, M. Tuzen, T. G. Kazi, *J. AOAC Int.* **2018**, *101*, 1183.
- [259] D. Lapeña, L. Lomba, M. Artal, C. Lafuente, B. Giner, *Fluid Phase Equilibria* **2019**, *492*, 1.
- [260] F. s. Mjalli, O. u. Ahmed, *Asia-Pac. J. Chem. Eng.* **2017**, *12*, 313.
- [261] S. N. Pedro, M. S. M. Mendes, B. M. Neves, I. F. Almeida, P. Costa, I. Correia-Sá, C. Vilela, M. G. Freire, A. J. D. Silvestre, C. S. R. Freire, *Pharmaceutics* **2022**, *14*, DOI 10.3390/pharmaceutics14040827.
- [262] R. Craveiro, I. Aroso, V. Flammia, T. Carvalho, M. T. Viciosa, M. Dionísio, S. Barreiros, R. L. Reis, A. R. C. Duarte, A. Paiva, *J. Mol. Liq.* **2016**, *215*, 534.
- [263] A. Blume, W. Huebner, G. Messner, "Fourier transform infrared spectroscopy of ¹³C:O labeled phospholipids hydrogen bonding to carbonyl groups," DOI 10.1021/bi00421a038 can be found under <https://pubs.acs.org/doi/pdf/10.1021/bi00421a038>, **2002**.
- [264] Y. Maréchal, H. Chanzy, *J. Mol. Struct.* **2000**, *523*, 183.
- [265] A. J. Rybarczyk-Pirek, A. T. Dubis, S. J. Grabowski, J. Nawrot-Modranka, *Chem. Phys.* **2006**, *320*, 247.
- [266] M. Saunders, in *Princ. Appl. Therm. Anal.*, John Wiley & Sons, Ltd, **2008**, pp. 286–329.
- [267] M. Uematsu, E. U. Franck, *J. Phys. Chem. Ref. Data* **1980**, *9*, 1291.
- [268] F. X. Hassion, R. H. Cole, *J. Chem. Phys.* **1955**, *23*, 1756.
- [269] U. Kaatz, *J. Chem. Eng. Data* **1989**, *34*, 371.
- [270] E. H. Grant, R. J. Sheppard, G. P. South, *Dielectric Behaviour of Biological Molecules in Solution*, Clarendon Press, **1978**.
- [271] Clinical & Laboratory Standards Institute, *30* **2020**.
- [272] M. Hayyan, M. A. Hashim, A. Hayyan, M. A. Al-Saadi, I. M. AlNashef, M. E. S. Mirghani, O. K. Saheed, *Chemosphere* **2013**, *90*, 2193.

- [273] B.-Y. Zhao, P. Xu, F.-X. Yang, H. Wu, M.-H. Zong, W.-Y. Lou, *ACS Sustain. Chem. Eng.* **2015**, *3*, 2746.
- [274] G. A. McKay, S. Beaulieu, F. F. Arhin, A. Belley, I. Sarmiento, T. Parr Jr, G. Moeck, *J. Antimicrob. Chemother.* **2009**, *63*, 1191.
- [275] B. E. Ferro, J. van Ingen, M. Wattenberg, D. van Soolingen, J. W. Mouton, *J. Antimicrob. Chemother.* **2015**, *70*, 811.
- [276] K. Syal, *Curr. Microbiol.* **2017**, *74*, 1194.
- [277] J. Castro, Â. Lima, L. G. V. Sousa, A. S. Rosca, C. A. Muzny, N. Cerca, *Front. Cell. Infect. Microbiol.* **2022**, *11*, DOI 10.3389/fcimb.2021.795797.
- [278] L. M. Lim, N. Ly, D. Anderson, J. C. Yang, L. Macander, A. Jarkowski III, A. Forrest, J. B. Bulitta, B. T. Tsuji, *Pharmacother. J. Hum. Pharmacol. Drug Ther.* **2010**, *30*, 1279.
- [279] Y.-Y. Liu, Y. Wang, T. R. Walsh, L.-X. Yi, R. Zhang, J. Spencer, Y. Doi, G. Tian, B. Dong, X. Huang, et al., *Lancet Infect. Dis.* **2016**, *16*, 161.
- [280] L. Göpel, E. Prenger-Berninghoff, S. A. Wolf, T. Semmler, R. Bauerfeind, C. Ewers, *Appl. Microbiol.* **2024**, *4*, 70.
- [281] A. K. Al Atya, H. Abriouel, I. Kempf, E. Jouy, E. Auclair, A. Vachée, D. Drider, *Probiotics Antimicrob. Proteins* **2016**, *8*, 183.
- [282] H. Haddad Kashani, M. Schmelcher, H. Sabzalipoor, E. Seyed Hosseini, R. Moniri, *Clin. Microbiol. Rev.* **2018**, *31*, e00071.
- [283] T. Swebocki, A. M. Kocot, A. Barras, H. Arellano, L. Bonnaud, K. Haddadi, A. Fameau, S. Szunerits, M. Plotka, R. Boukherroub, *Adv. Healthc. Mater.* **2024**, 2303475.
- [284] L. A. Ibarra-Sánchez, N. El-Haddad, D. Mahmoud, M. J. Miller, L. Karam, *J. Dairy Sci.* **2020**, *103*, 2041.
- [285] K. Müller-Auffermann, F. Grijalva, F. Jacob, M. Hutzler, *J. Inst. Brew.* **2015**, *121*, 309.
- [286] S. R. Lockhart, *Fungal Genet. Biol.* **2019**, *131*, 103243.
- [287] B. A. Neville, C. d'Enfert, M.-E. Bougnoux, *FEMS Yeast Res.* **2015**, *15*, fov081.
- [288] M. Ruhnke, *Curr. Drug Targets* **2006**, *7*, 495.
- [289] F. L. Mayer, D. Wilson, B. Hube, *Virulence* **2013**, *4*, 119.
- [290] A. McCormick, J. Loeffler, F. Ebel, *Cell. Microbiol.* **2010**, *12*, 1535.

Appendices

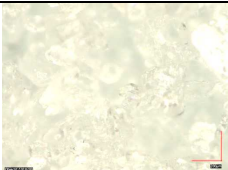
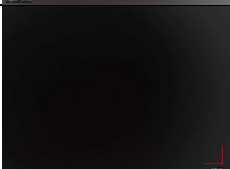
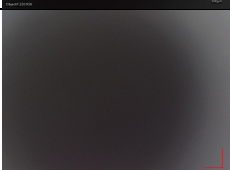
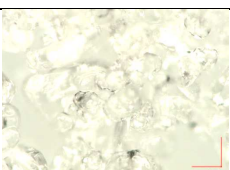
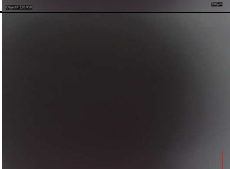
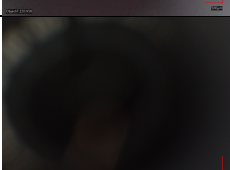
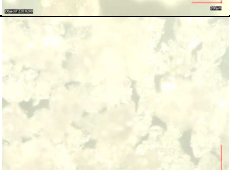
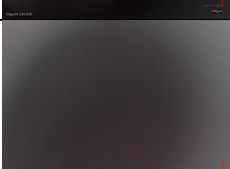
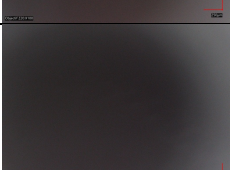
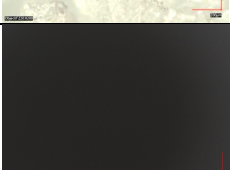
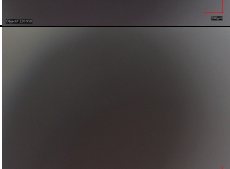
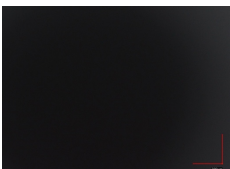
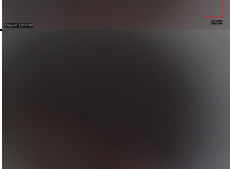
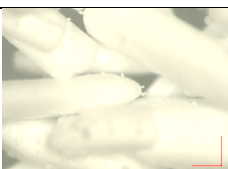
	DES		PSs
T-glycoline		TBACl	
glycoline			GlycA 
maloline			MaloA 
maline			MalA 
oxaline		ChCl	OxA 
reline			U 
ethaline			EG 
glyceline			GLY 
R-glyceline		Arg	

Figure A1. POM images of the investigated deep eutectic solvents with their parent substances.

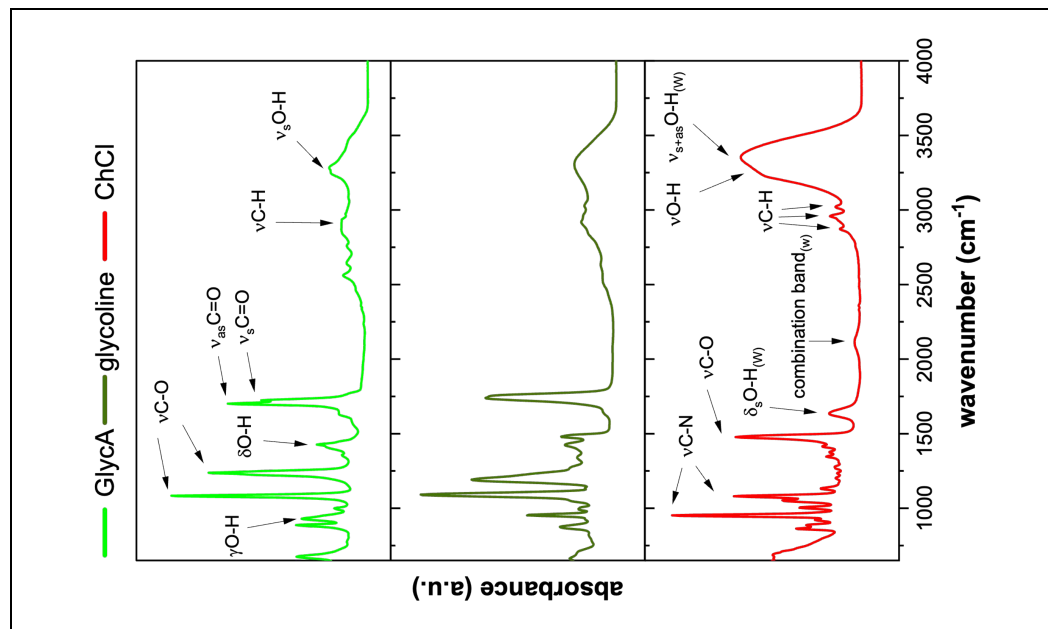


Figure A3. FTIR spectrum of T-glycoline compared to its PSs.

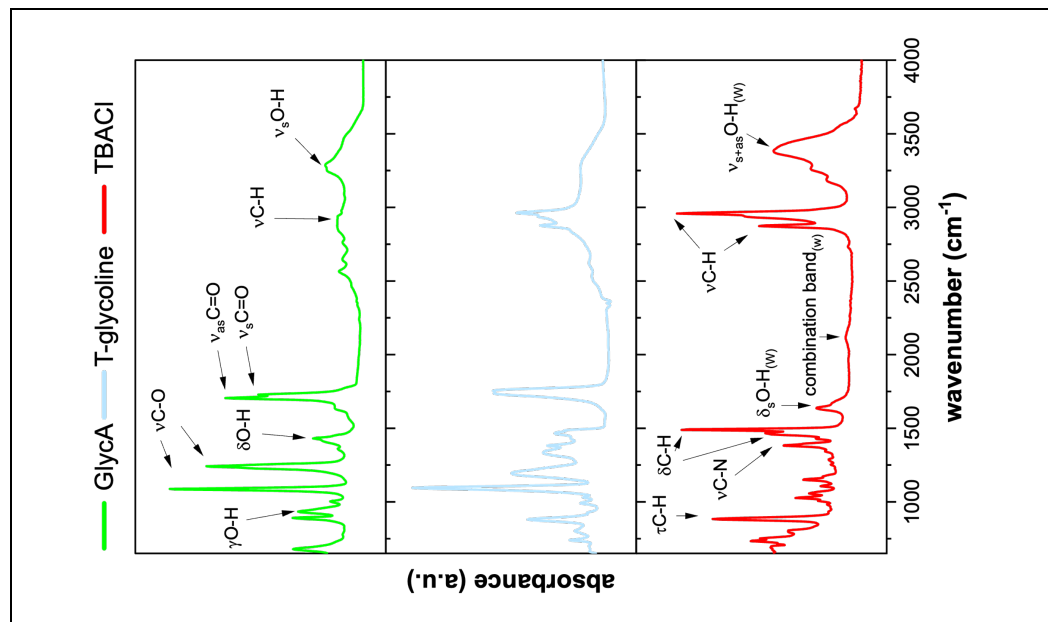


Figure A2. FTIR spectrum of glycoline compared to its PSs.

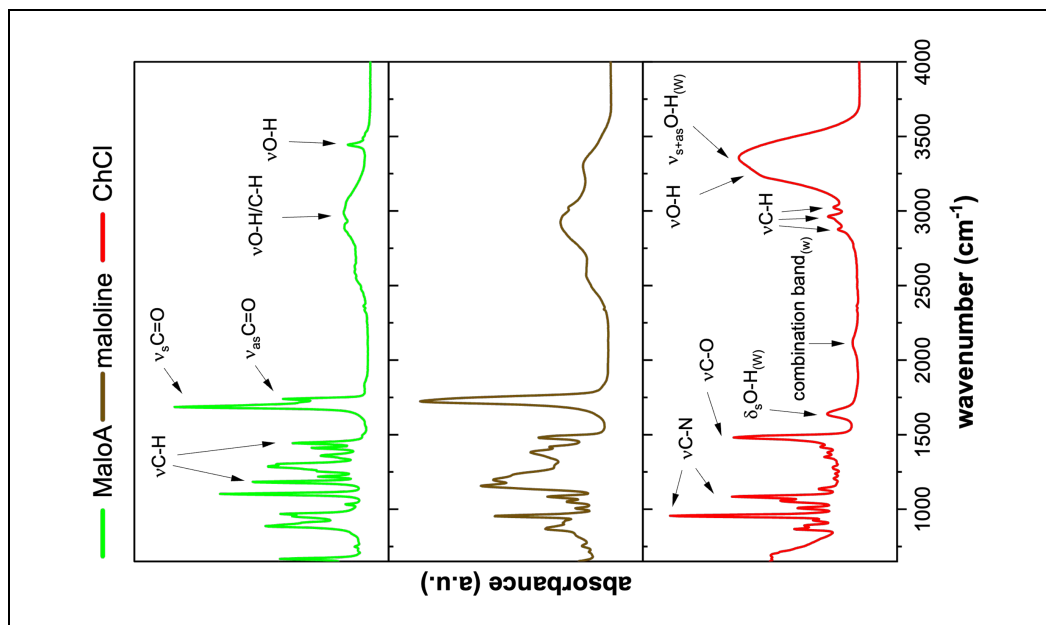


Figure A5. FTIR spectrum of maloline compared to its PSs.

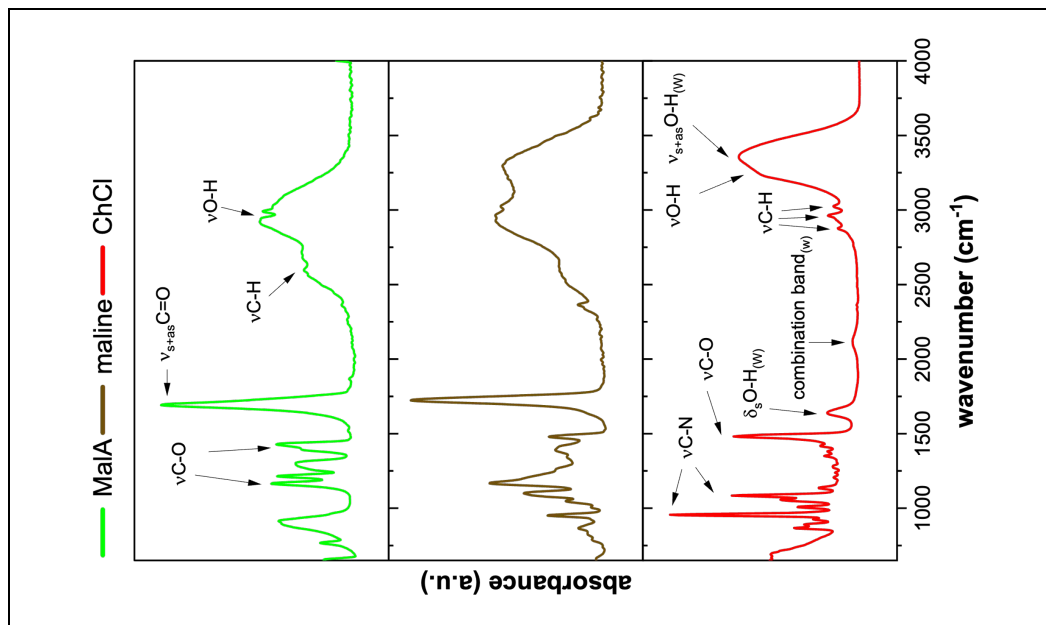


Figure A4. FTIR spectrum of maline compared to its PSs.

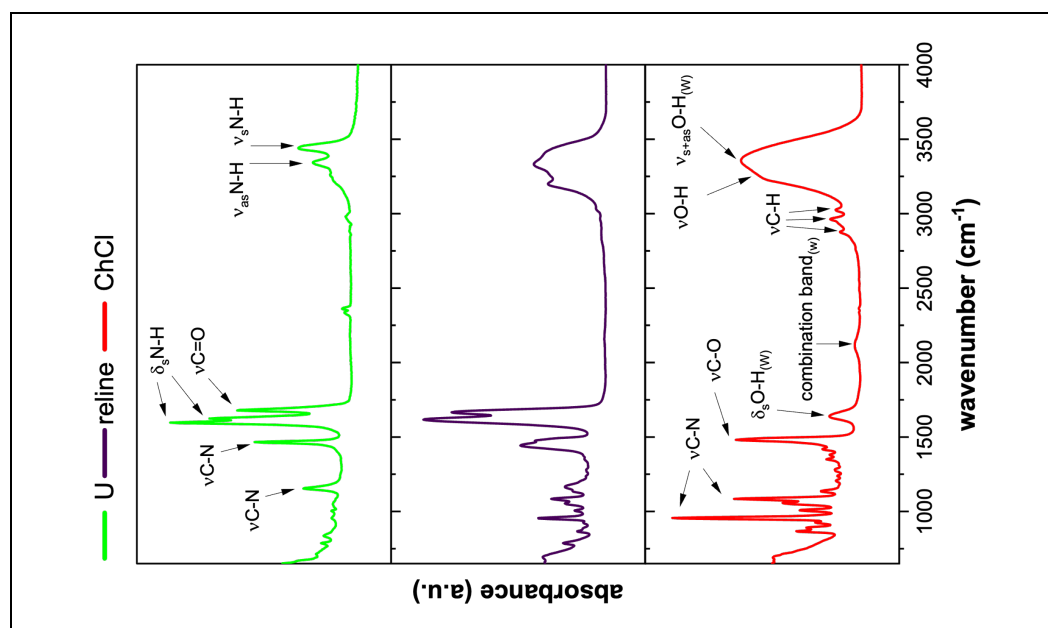


Figure A7. FTIR spectrum of reline compared to its PSs.

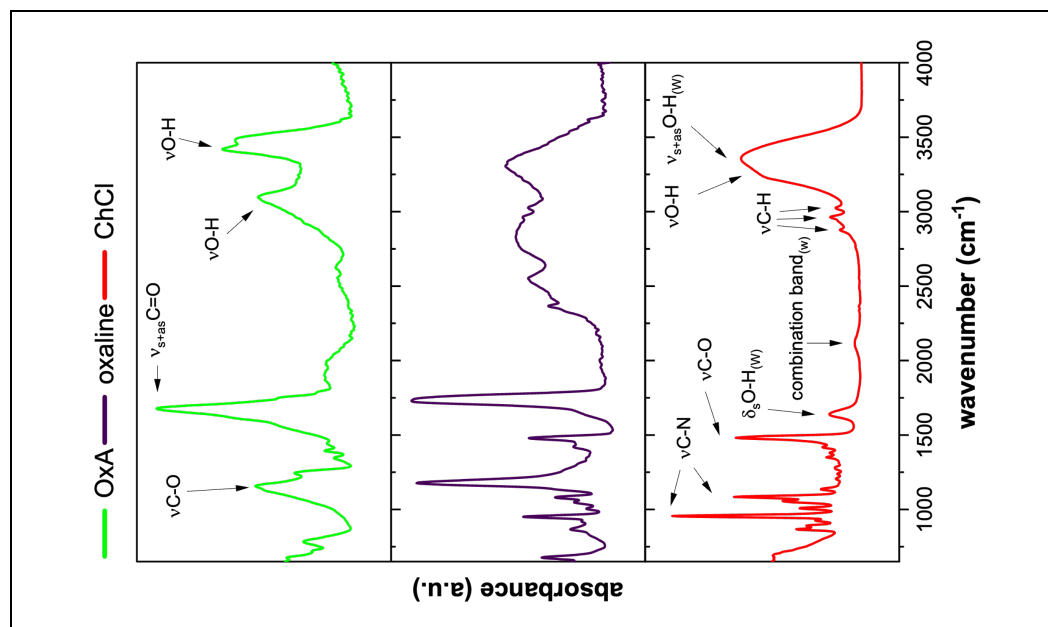


Figure A6. FTIR spectrum of oxaline compared to its PSs.

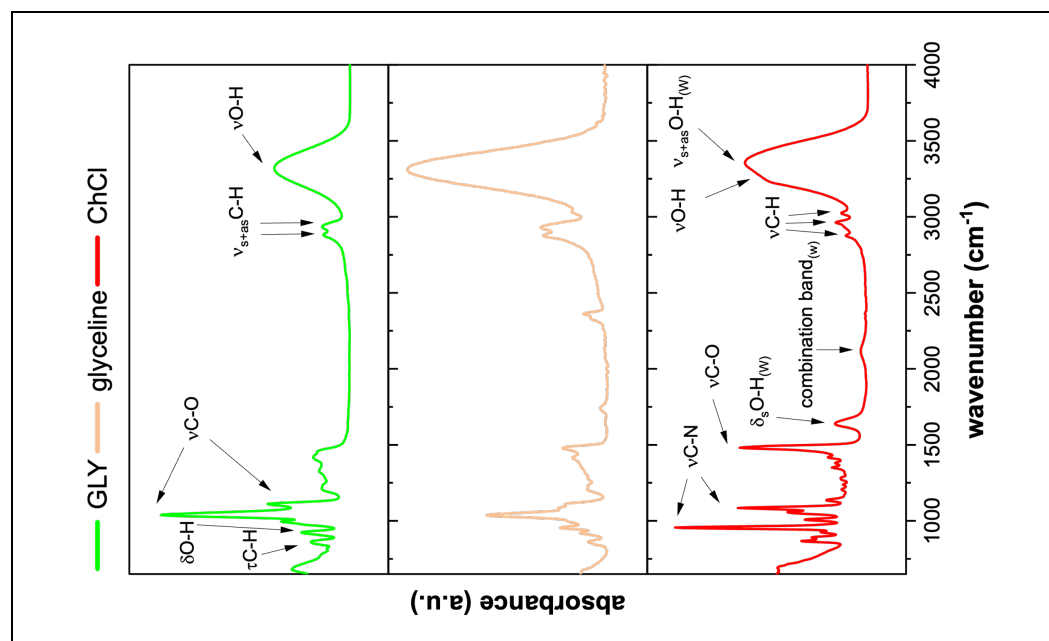


Figure A9. FTIR spectrum of glycine compared to its PSs.

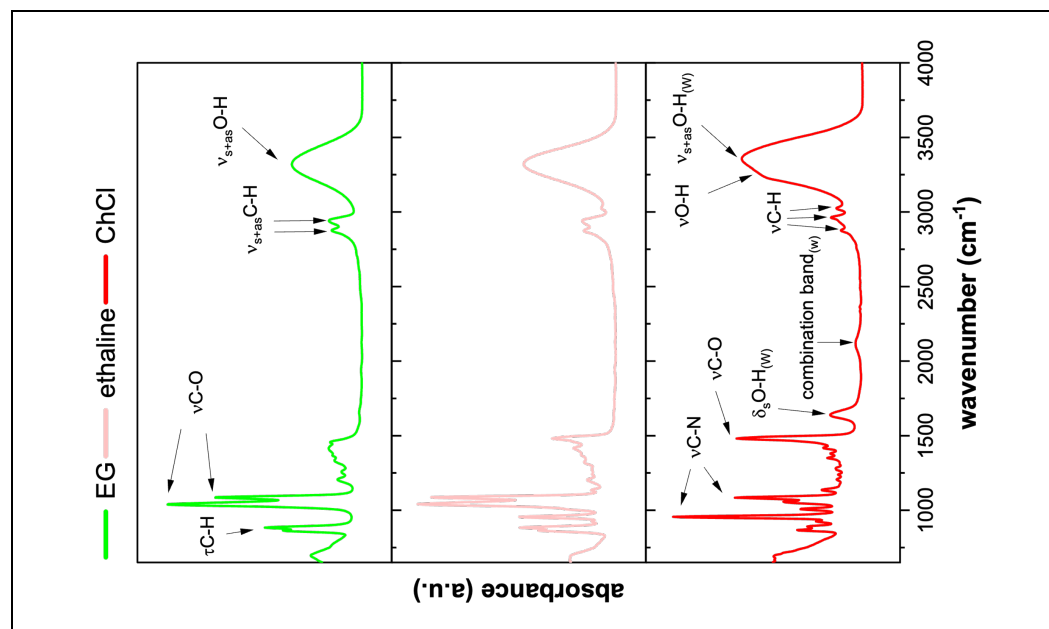


Figure A8. FTIR spectrum of ethaline compared to its PSs.

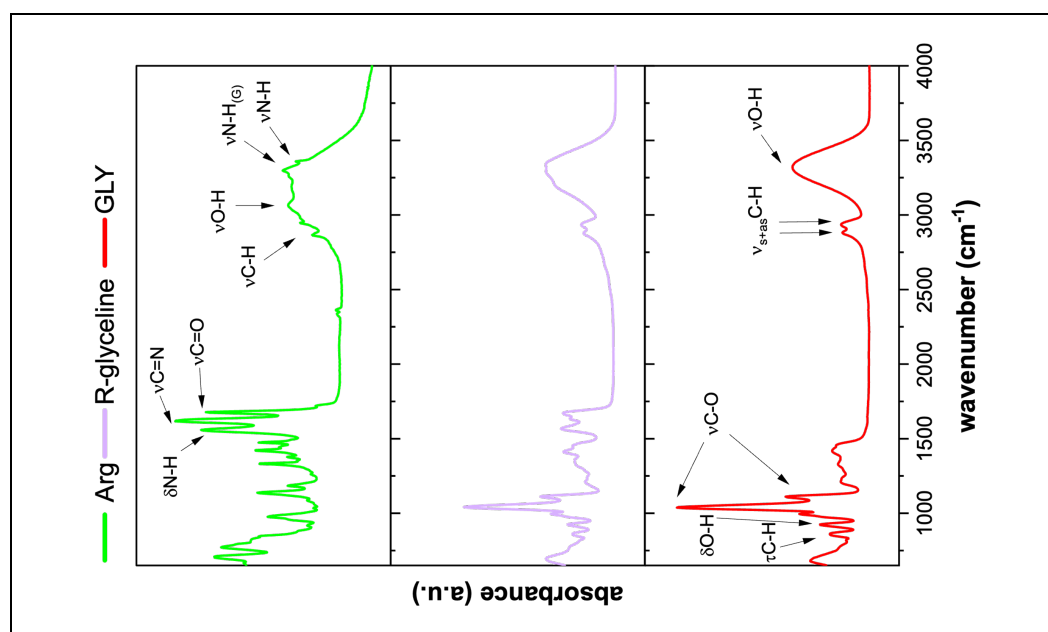
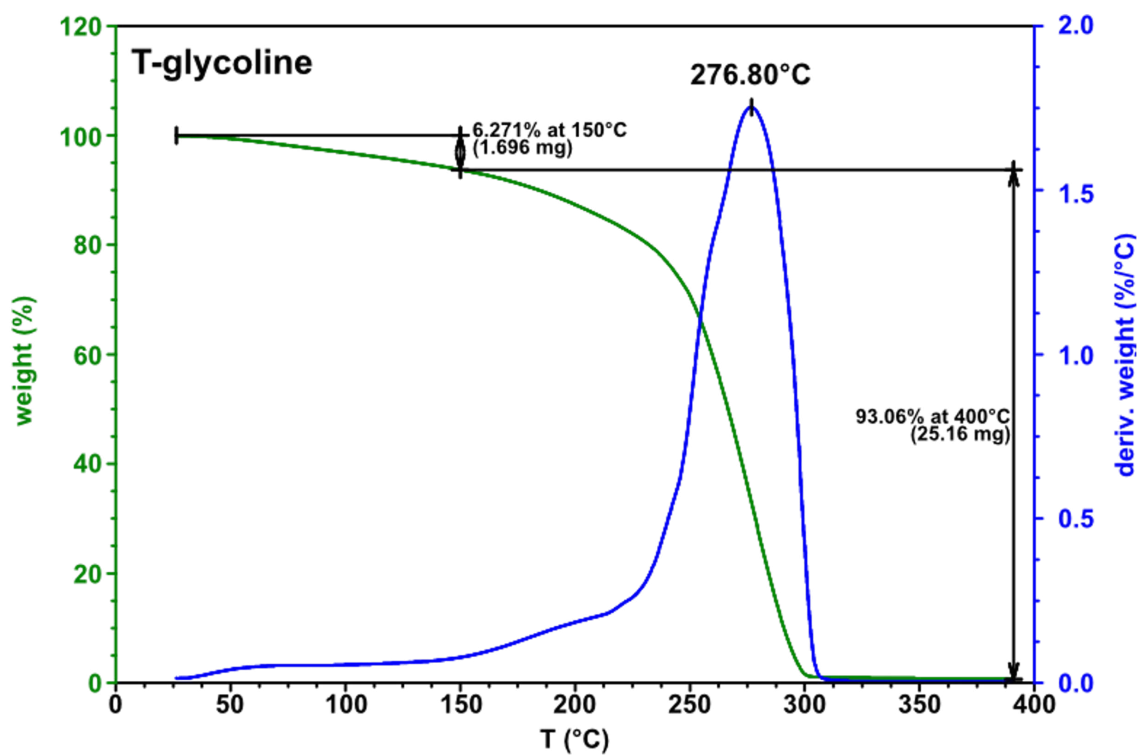
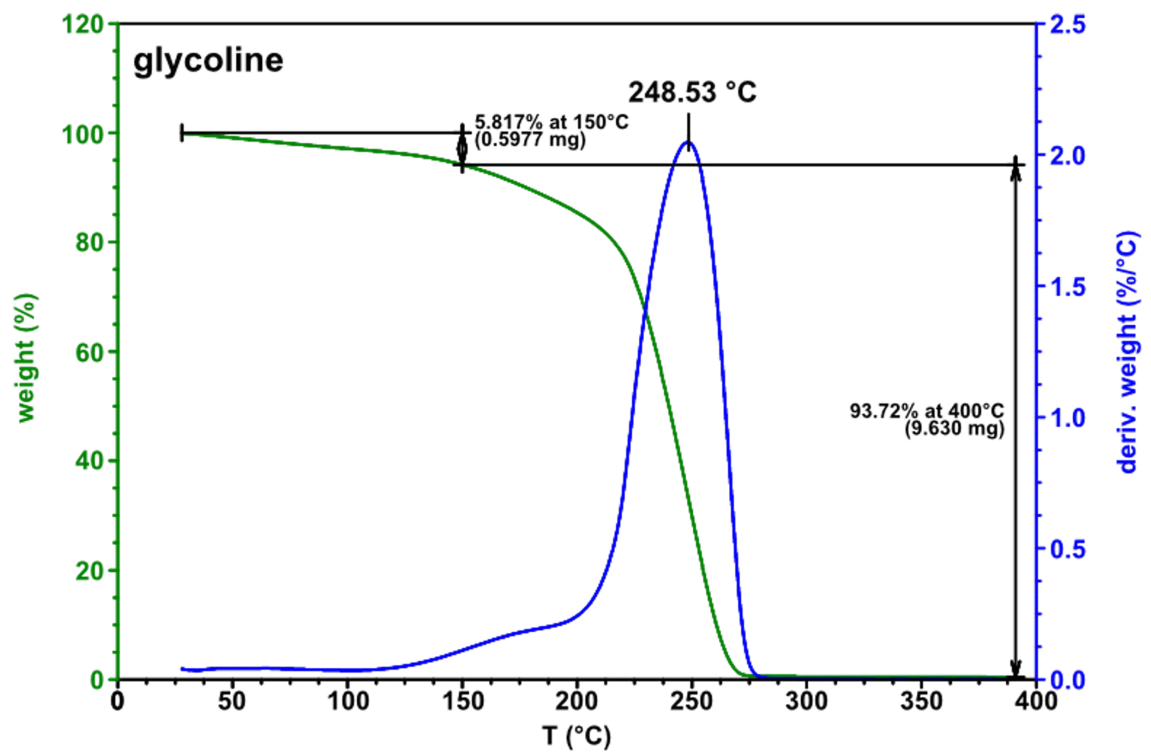
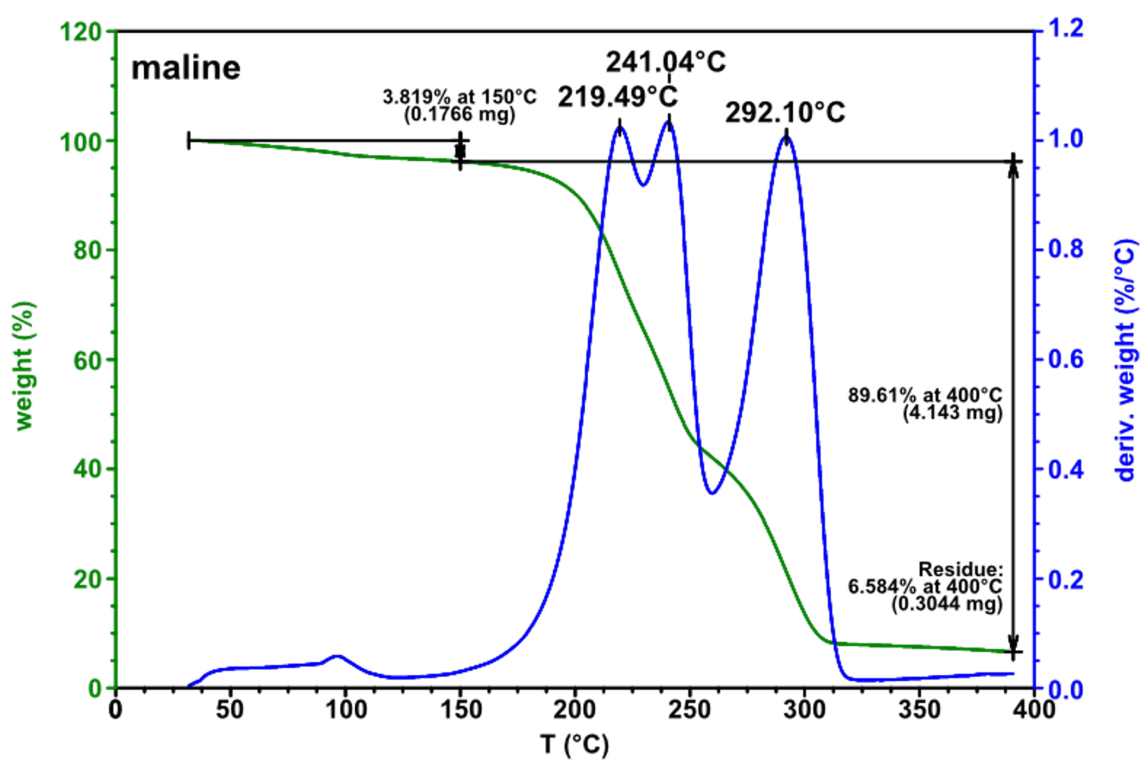
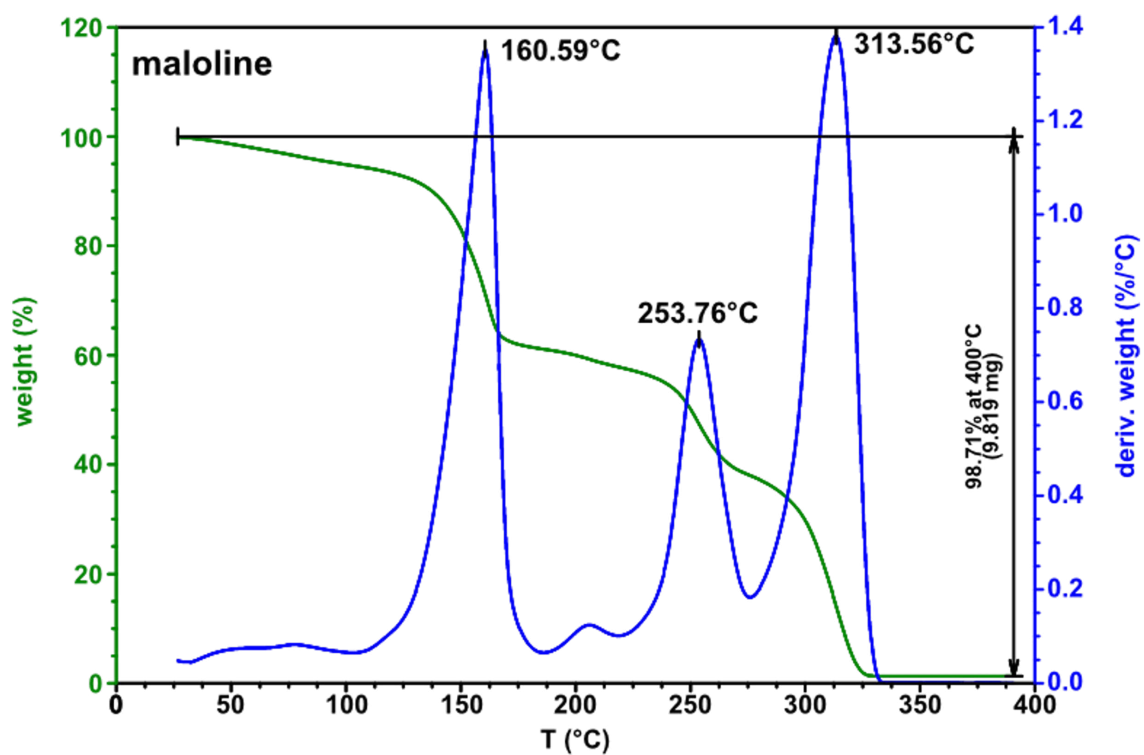
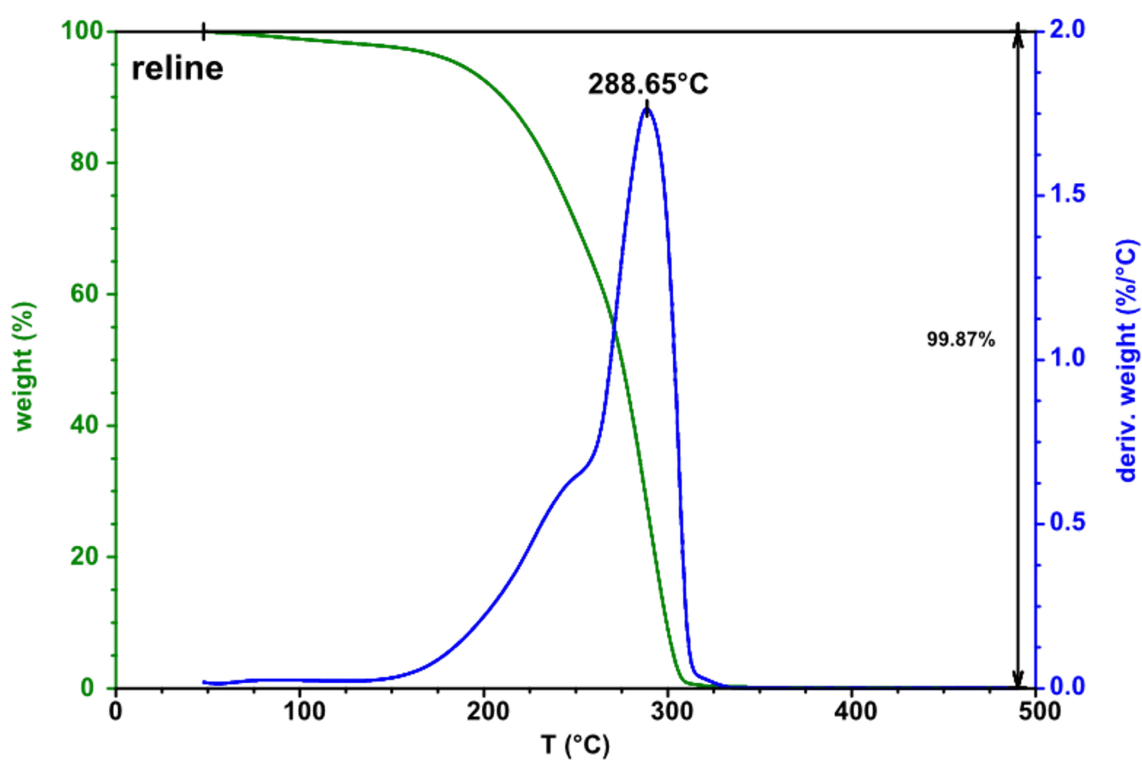
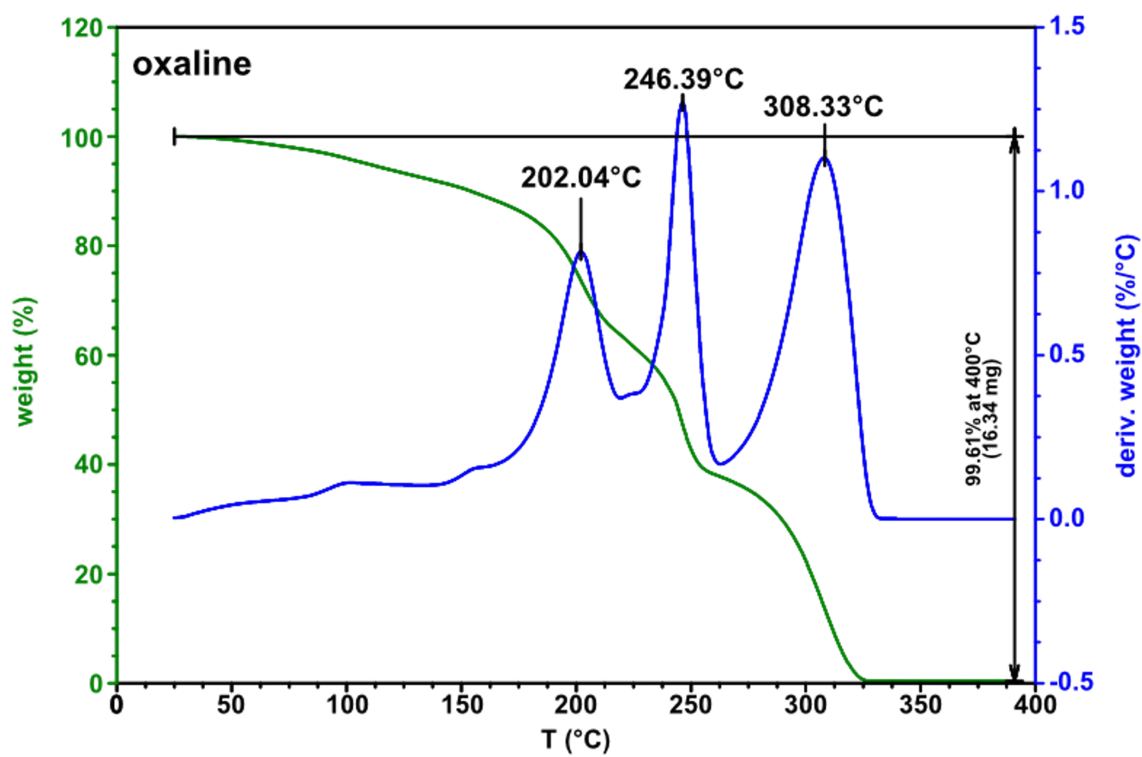
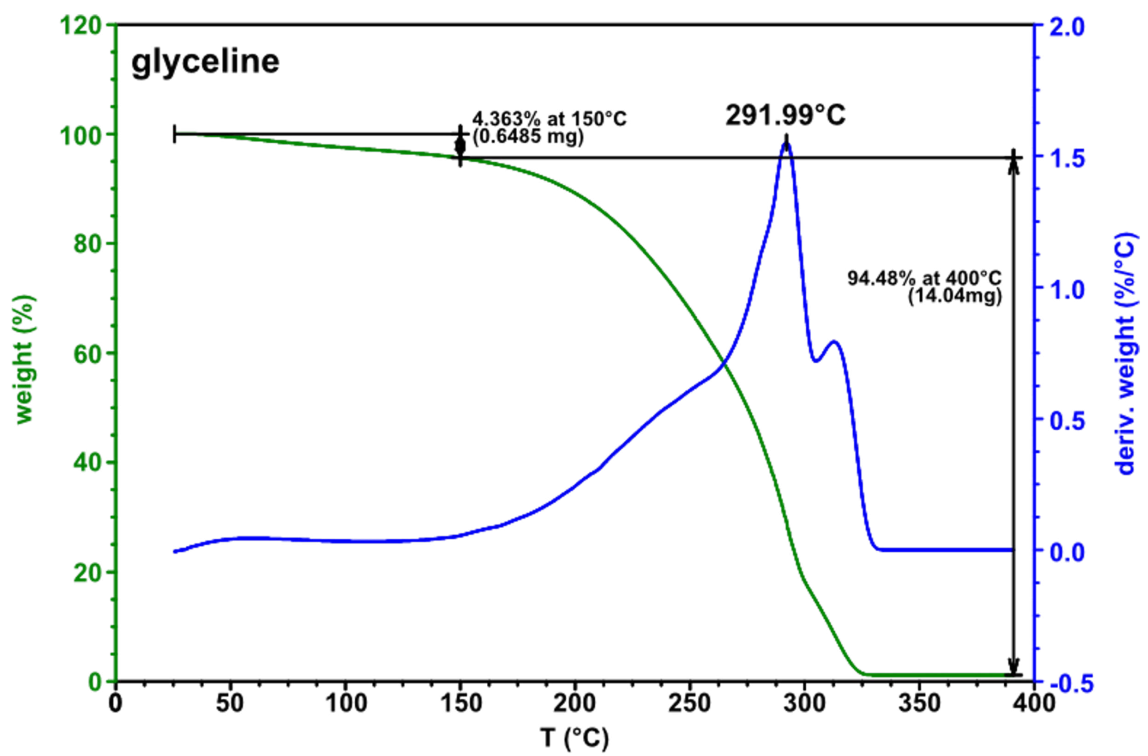
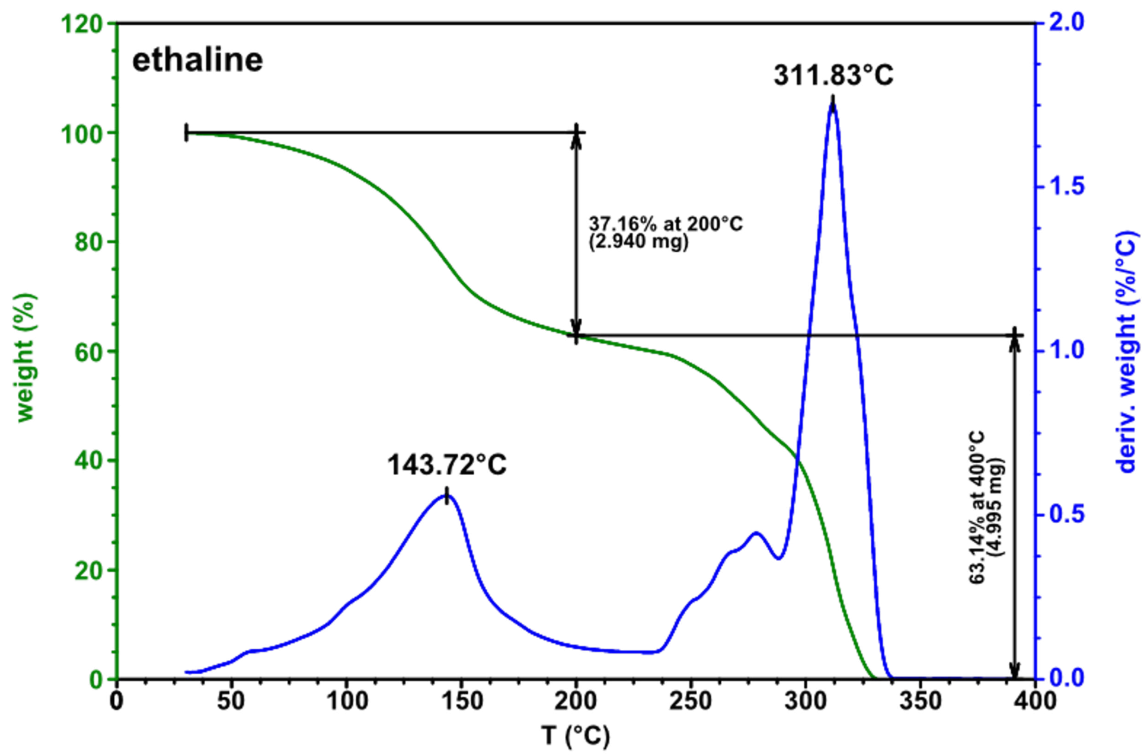


Figure A10. FTIR spectrum of R-glycine compared to its PSs.









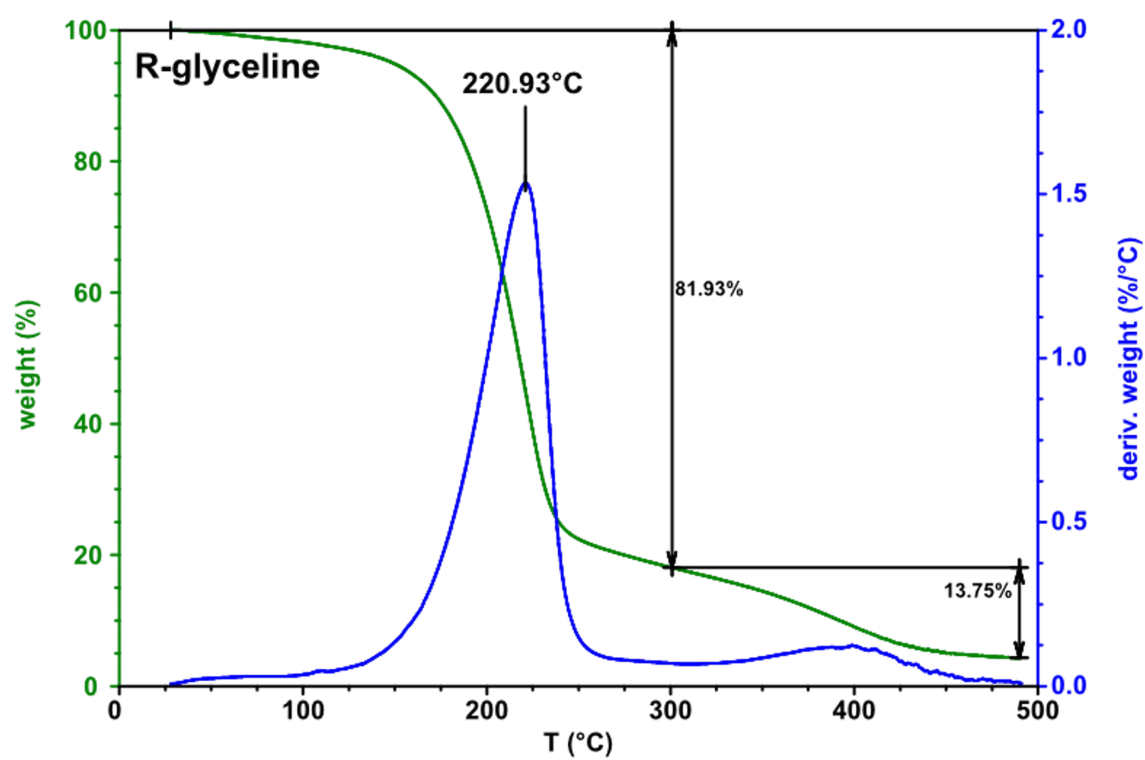


Figure A11. Thermogravimetric curves of the investigated DESs.

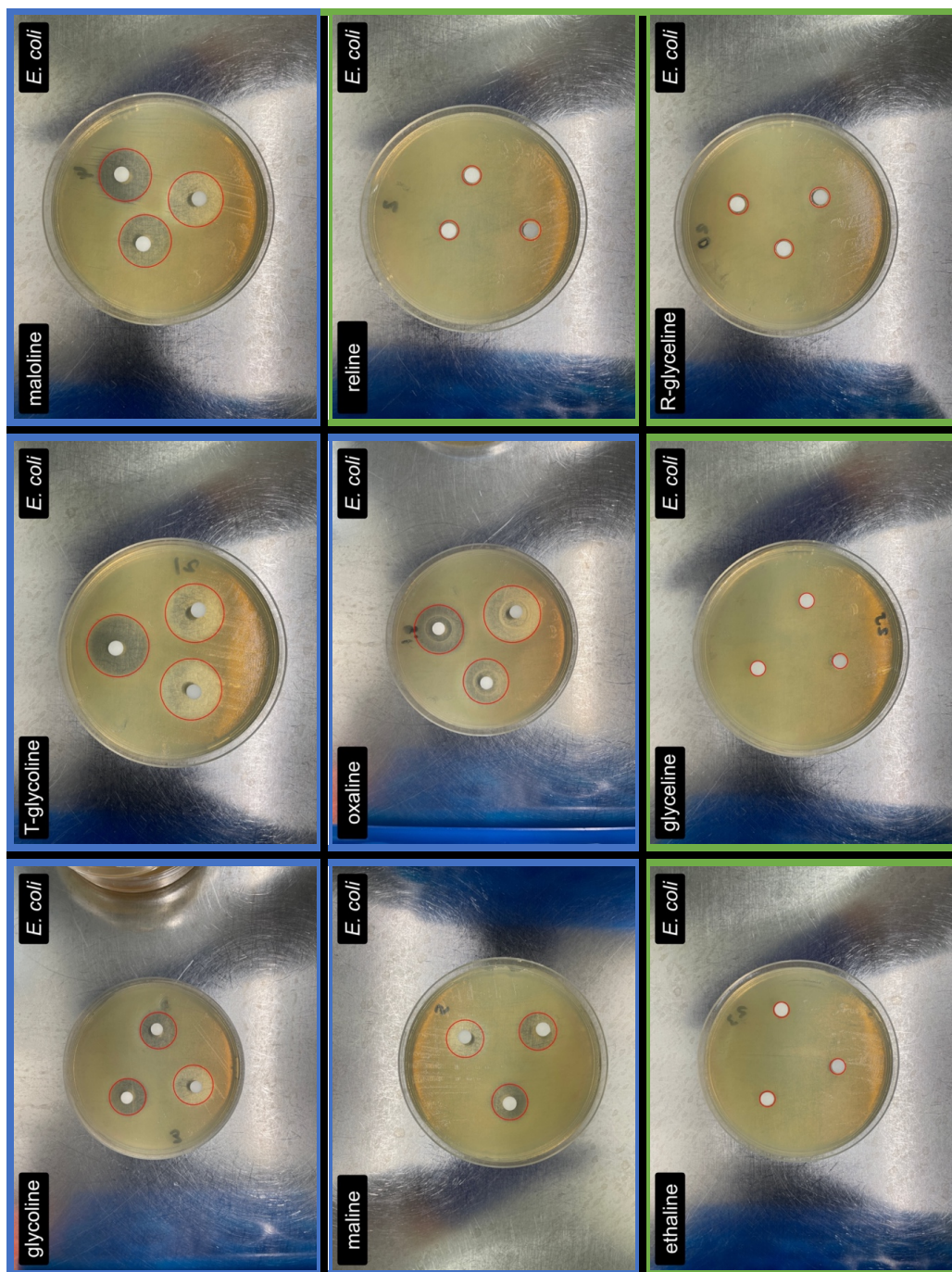


Figure A12. Table diffusion tests of the investigated DESs towards *E. coli* (n=3).

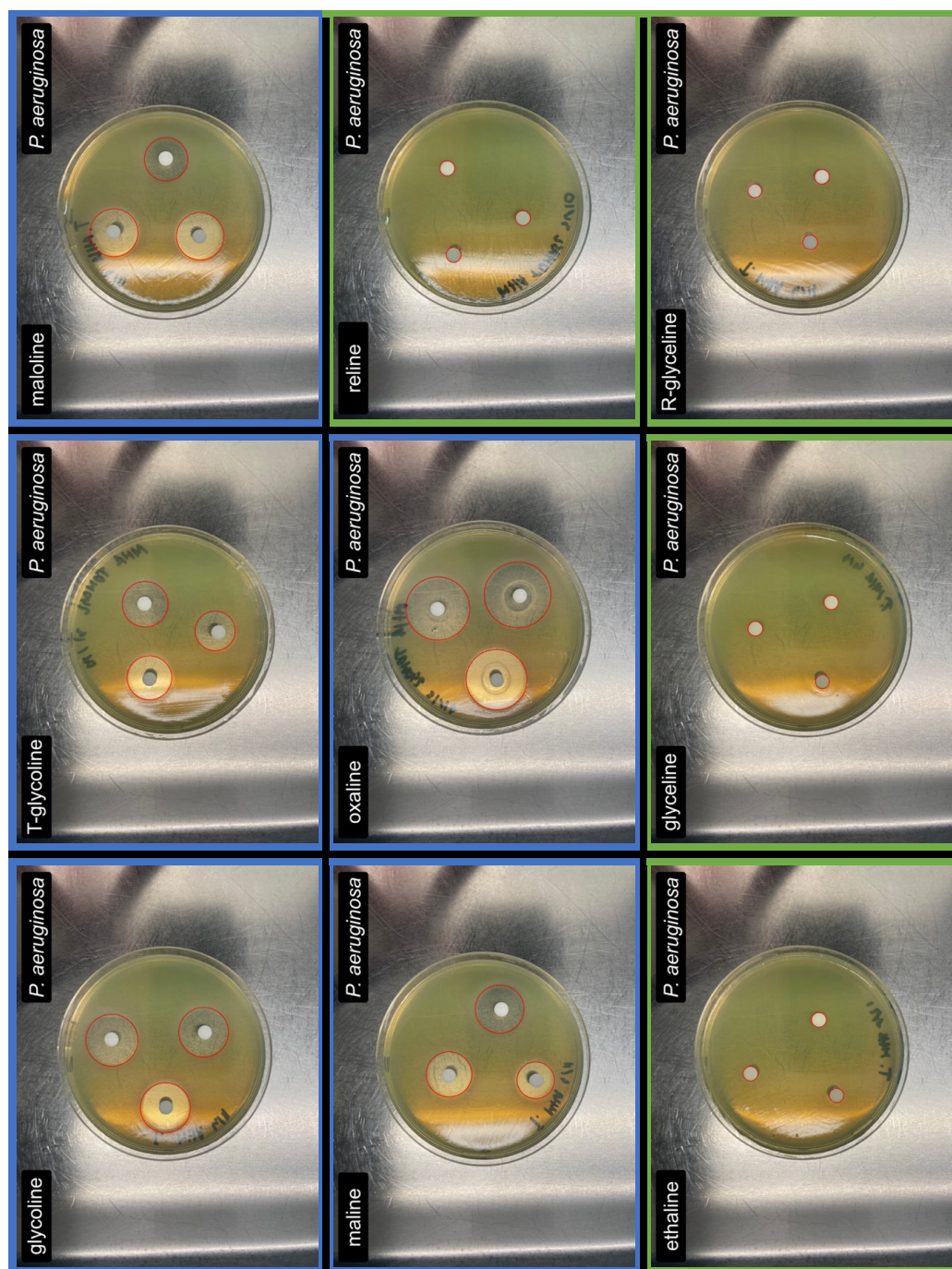


Figure A13. Disk-diffusion tests of the investigated DESs towards *P. aeruginosa* (n=3).

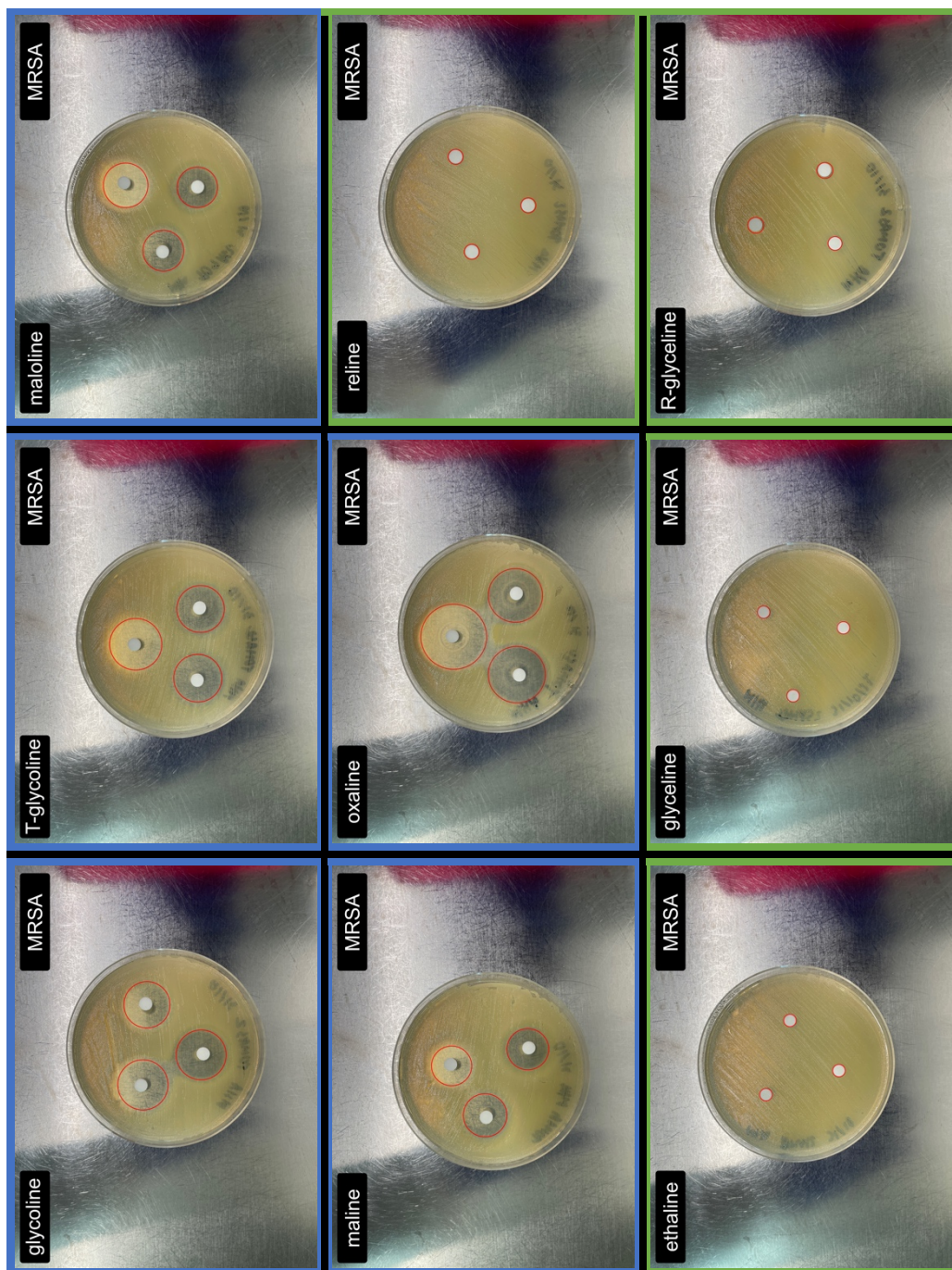


Figure A14. Disk-diffusion tests of the investigated DESs towards MRSA (n=3).

Table A1. MIC values of OA-DES against Gram-positive and Gram-negative strains.

Strain	DES				
	glycoline	T-glycoline	maloline	maline	oxaline
<i>B. cereus</i>	0.15 ± 0.01	0.18 ± 0.03	0.14 ± 0.02	0.07 ± 0.02	0.16 ± 0.01
<i>B. subtilis</i>	0.14 ± 0.01	0.15 ± 0.02	0.13 ± 0.02	0.14 ± 0.04	0.06 ± 0.01
<i>E. faecalis</i>	0.20 ± 0.01	0.14 ± 0.01	0.11 ± 0.01	0.07 ± 0.01	0.09 ± 0.01
MDRSA	0.20 ± 0.08	0.37 ± 0.02	0.20 ± 0.01	0.20 ± 0.08	0.65 ± 0.17
MRSA	0.19 ± 0.01	0.27 ± 0.03	0.21 ± 0.04	0.20 ± 0.01	0.20 ± 0.01
<i>S. epidermidis</i>	0.08 ± 0.02	0.07 ± 0.01	0.20 ± 0.09	0.11 ± 0.09	0.08 ± 0.01
<i>A. baumannii</i>	0.44 ± 0.05	0.33 ± 0.02	0.02 ± 0.01	0.04 ± 0.01	0.15 ± 0.05
CRAB	0.20 ± 0.06	0.38 ± 0.02	0.41 ± 0.02	0.37 ± 0.02	0.17 ± 0.07
<i>E. coli</i>	0.18 ± 0.01	0.18 ± 0.03	0.15 ± 0.01	0.17 ± 0.01	0.21 ± 0.01
<i>K. pneumoniae</i>	0.24 ± 0.01	0.38 ± 0.05	0.17 ± 0.02	0.36 ± 0.04	0.08 ± 0.01
<i>P. aeruginosa</i>	0.77 ± 0.09	0.20 ± 0.03	0.11 ± 0.17	0.13 ± 0.01	0.08 ± 0.01
<i>P. fluorescens</i>	0.06 ± 0.02	0.39 ± 0.03	0.36 ± 0.03	0.41 ± 0.08	0.36 ± 0.04

Table A2. MIC values of conventional DES against Gram-positive and Gram-negative strains.

Strain	DES			
	glyceline	R-glyceline	ethaline	reline
<i>B. cereus</i>	-	4.06 ± 0.40	-	-
<i>B. subtilis</i>	-	5.02 ± 2.51	-	12.97 ± 2.74
<i>E. faecalis</i>	-	-	-	-
MDRSA	22.93 ± 1.98	6.81 ± 1.94	-	-
MRSA	-	2.71 ± 0.26	-	-
<i>S. epidermidis</i>	-	0.68 ± 0.11	-	-
<i>A. baumannii</i>	13.12 ± 2.15	3.30 ± 0.28	15.54 ± 2.65	7.53 ± 0.74
CRAB	10.22 ± 1.01	4.29 ± 0.37	11.52 ± 1.59	5.22 ± 0.71
<i>E. coli</i>	7.27 ± 0.48	1.69 ± 0.08	6.96 ± 0.39	5.63 ± 0.62
<i>K. pneumoniae</i>	-	1.08 ± 0.04	-	5.02 ± 0.42
<i>P. aeruginosa</i>	12.85 ± 1.09	8.08 ± 0.93	12.22 ± 0.81	5.58 ± 0.15
<i>P. fluorescens</i>	14.93 ± 3.98	-	-	13.45 ± 1.23

"-" indicates that MIC value have not been observed in the tested range

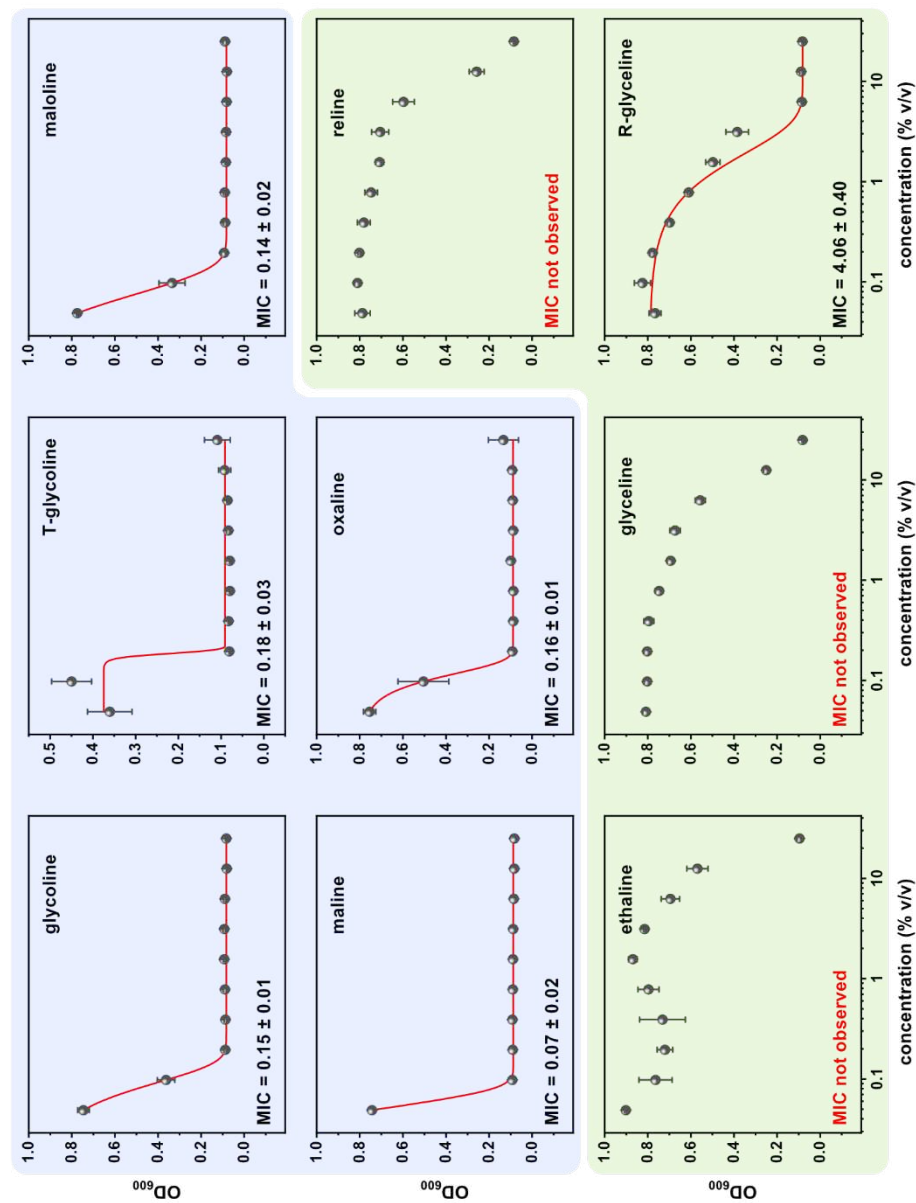


Figure A15. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against *B. cereus* (red line indicates Gompertz model fitting, $n=3$).

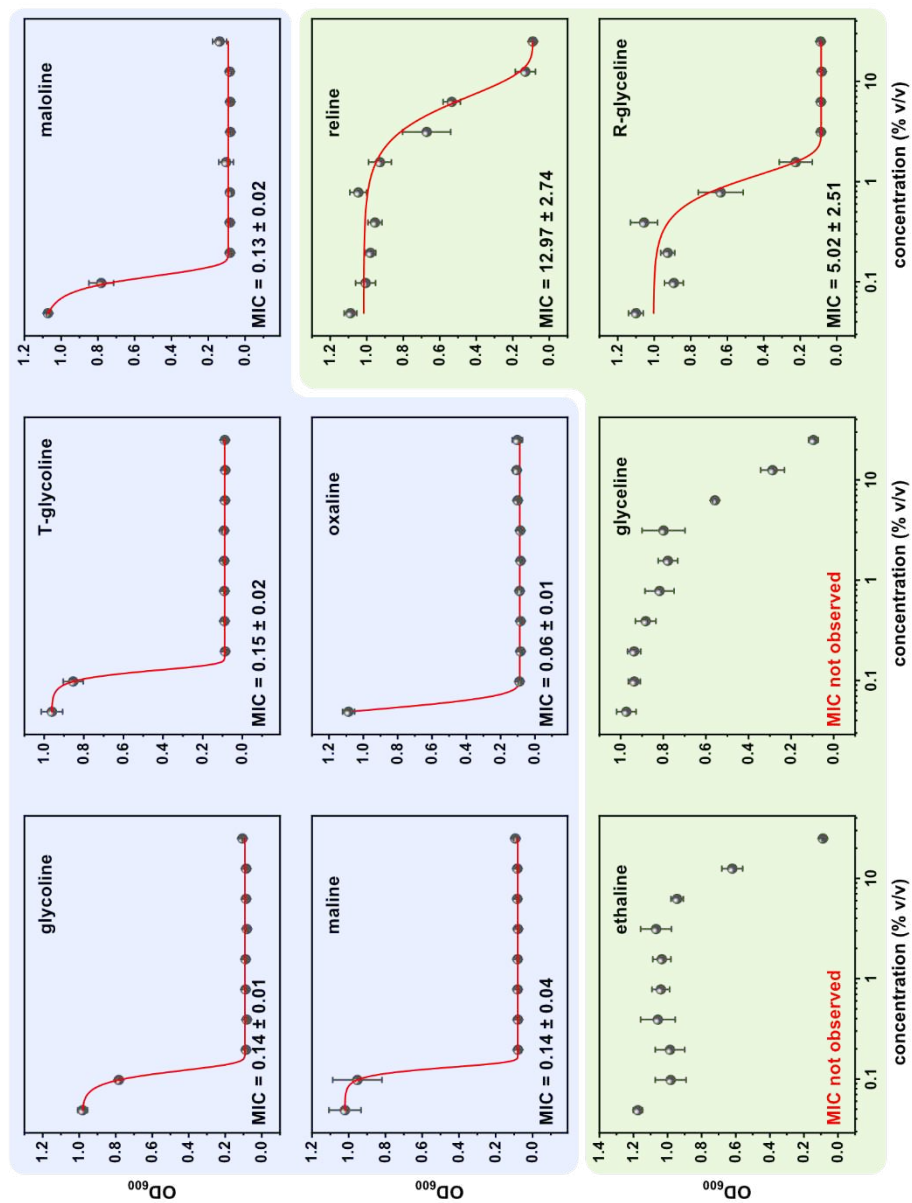


Figure A16. MIC assessment of the investigated DESs (0A-DESs (blue) and conventional DES (green)) against *B. subtilis* (red line indicates Gompertz model fitting, n=3).

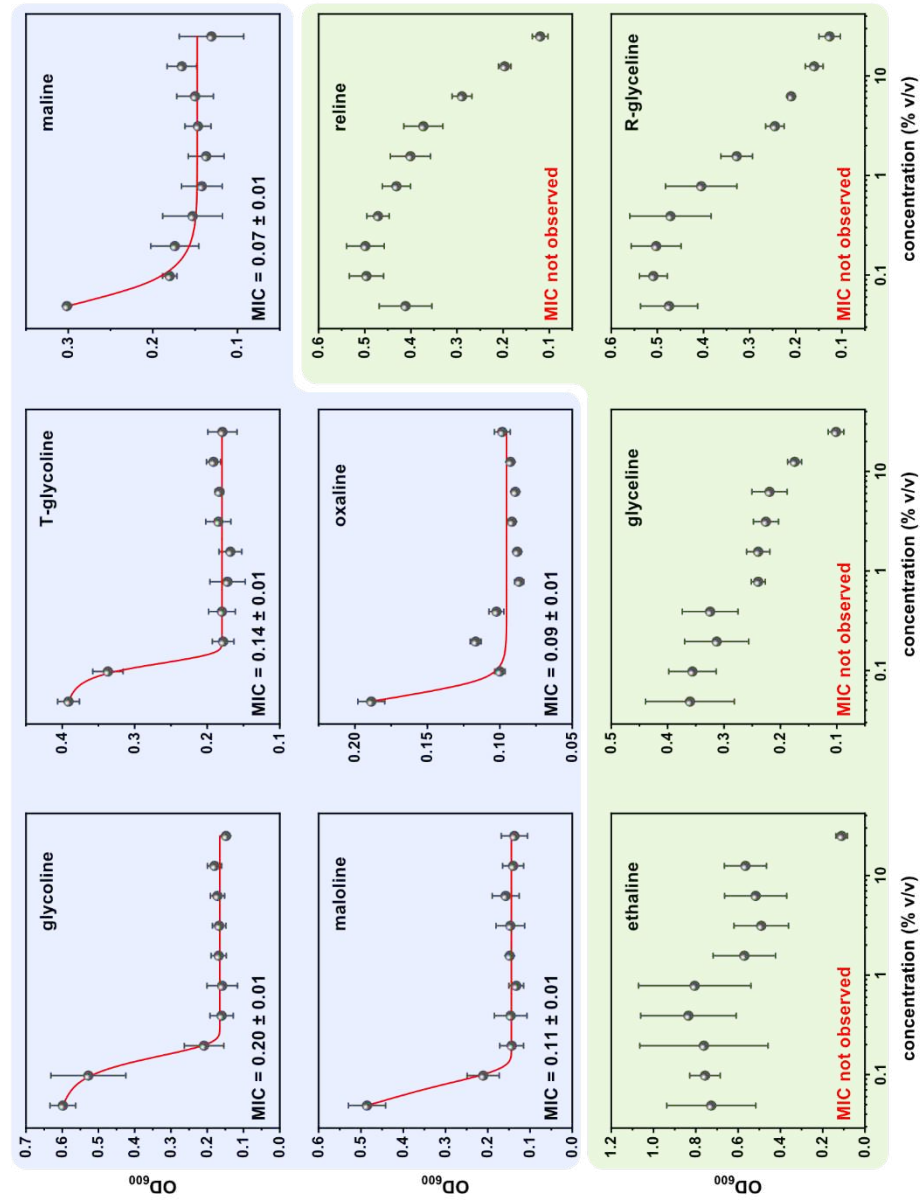


Figure A17. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against *E. faecalis* (red line indicates Gompertz model fitting, n=3).

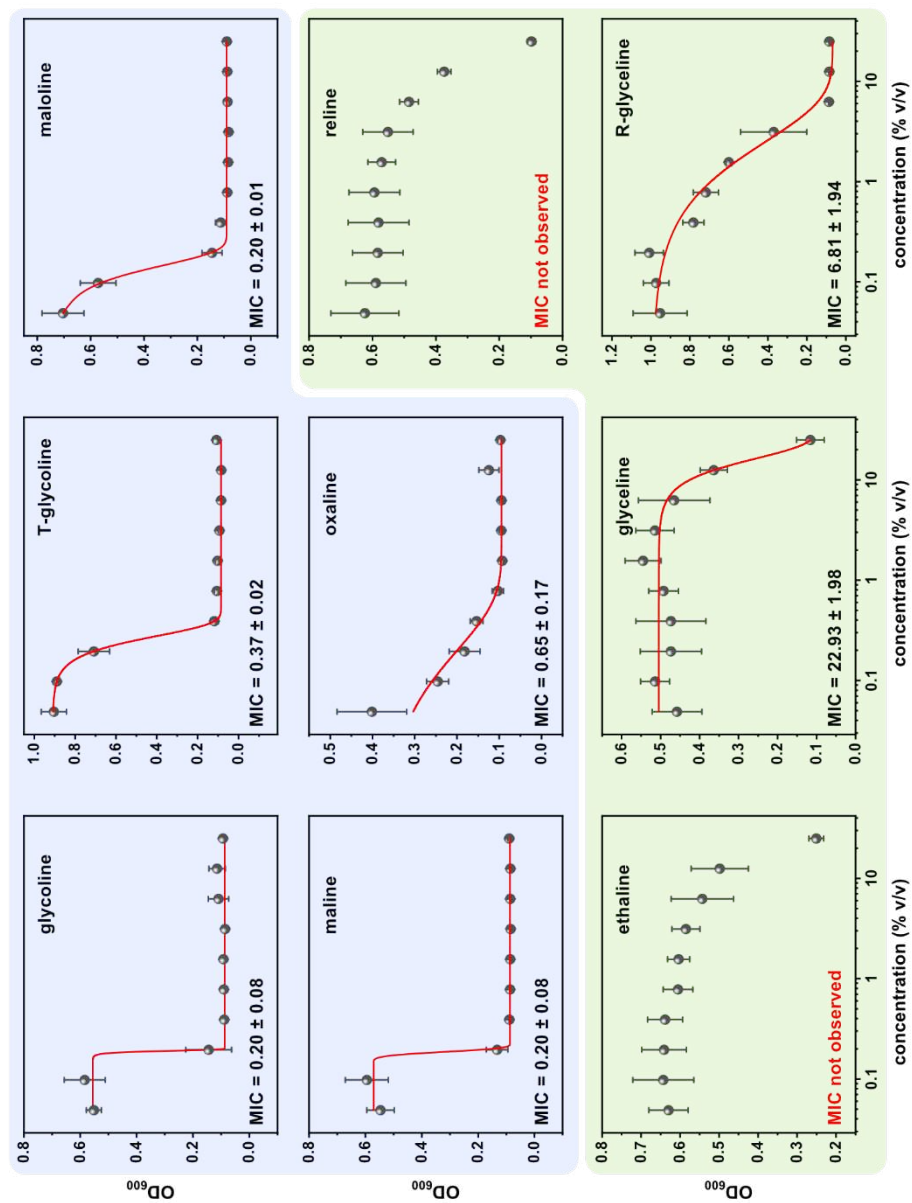


Figure A18. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against MDRSA (red line indicates Gompertz model fitting, n=3).

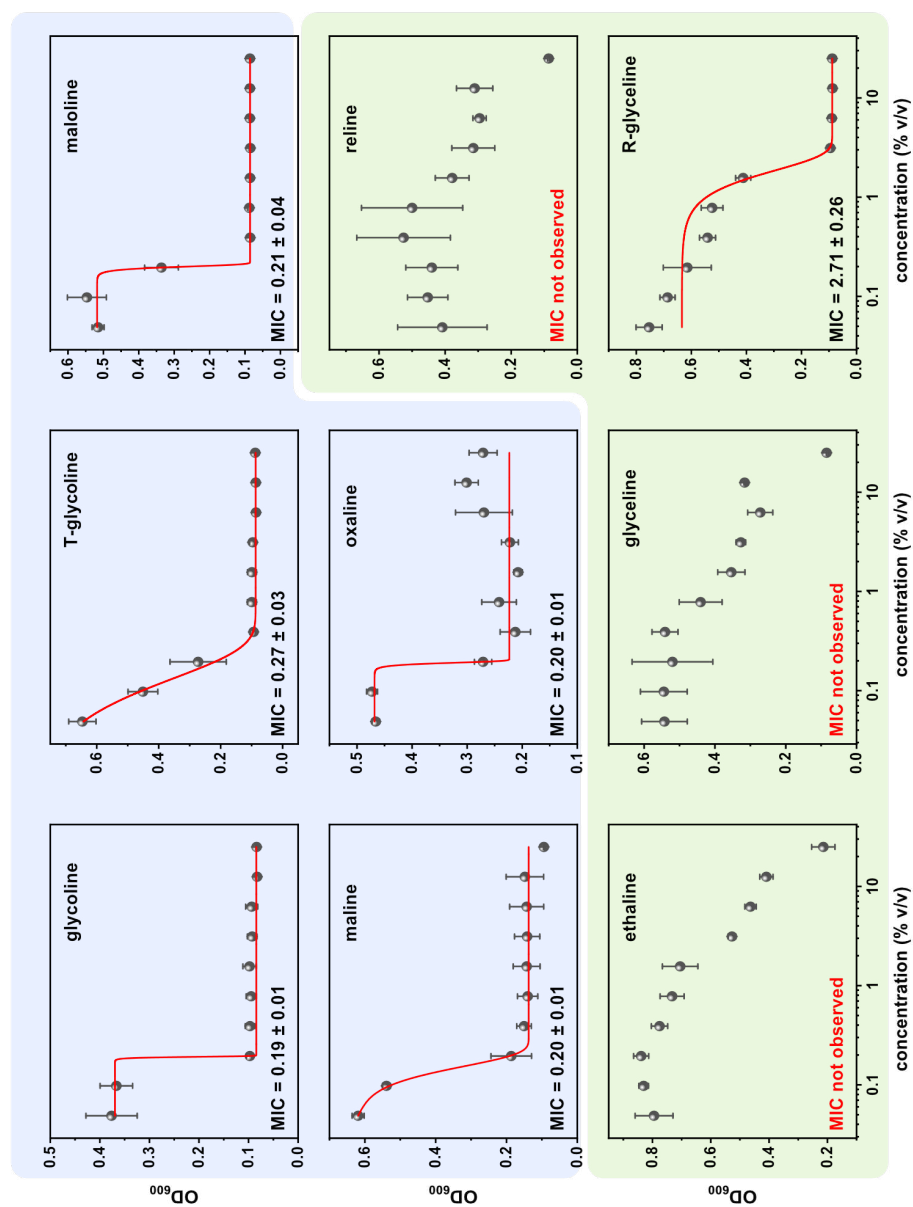


Figure A19. MIC assessment of the investigated DESs (OA-DESs [blue] and conventional DES [green]) against MRSA (red line indicates Gompertz model fitting, n=3).

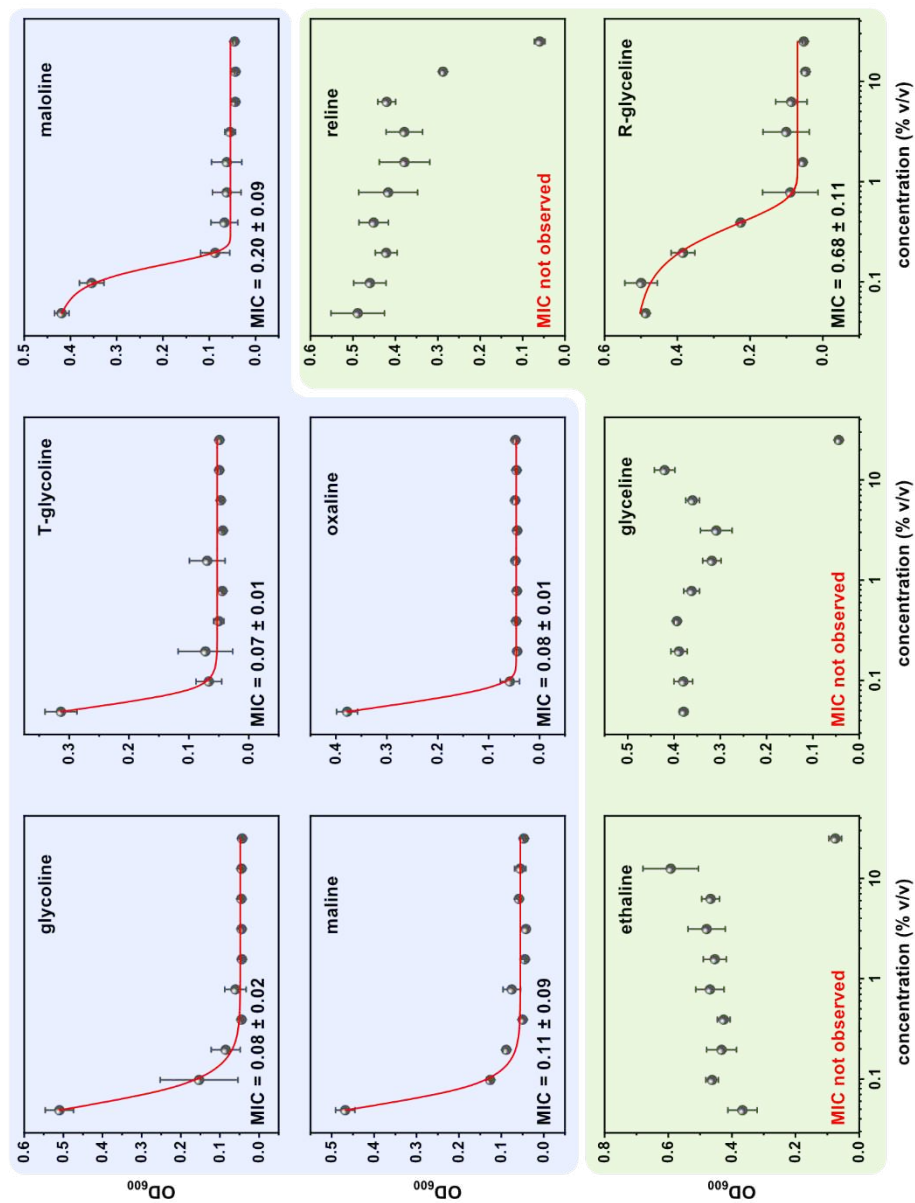


Figure A20. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against *S. epidermidis* (red line indicates Gompertz model fitting, n=3).

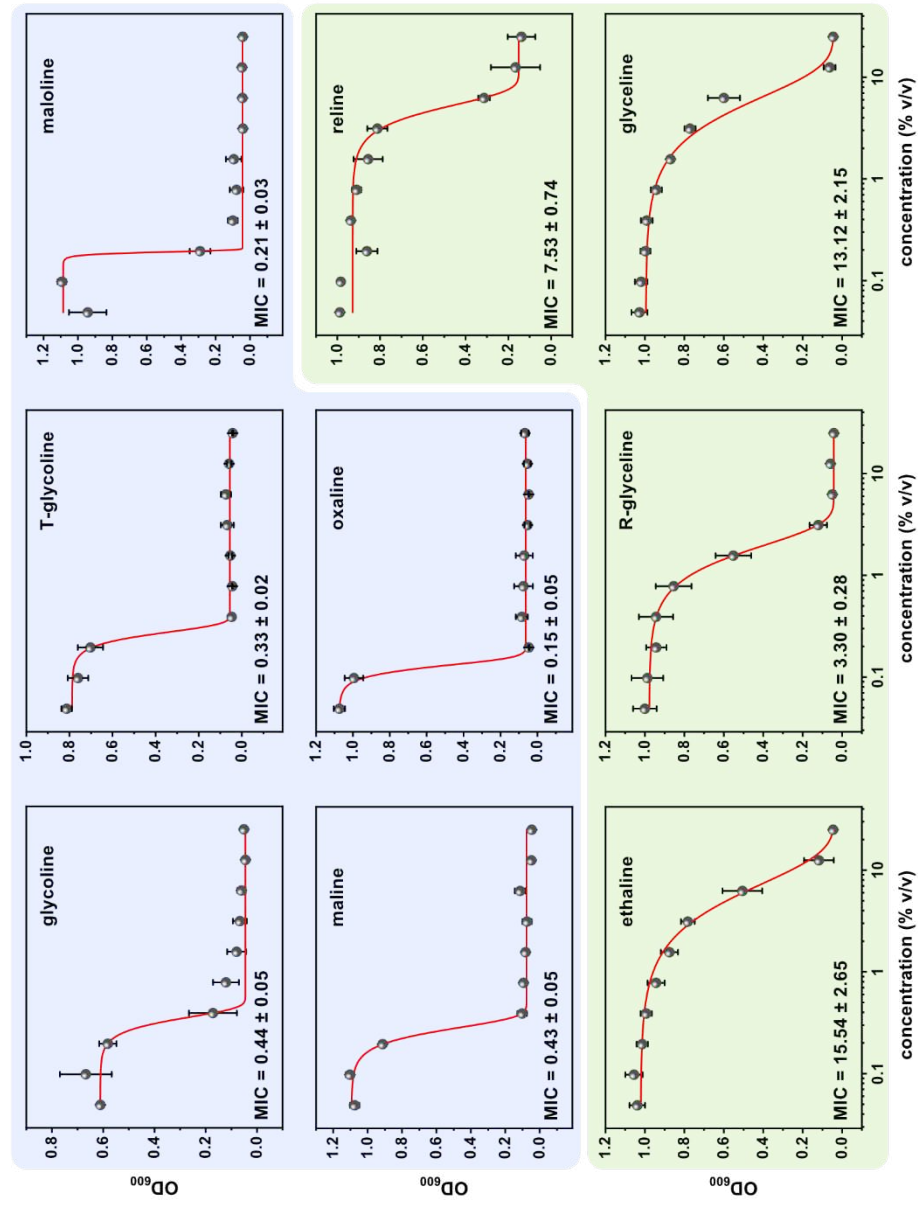


Figure A21. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against *A. baumannii* (red line indicates Gompertz model fitting, $n=3$).

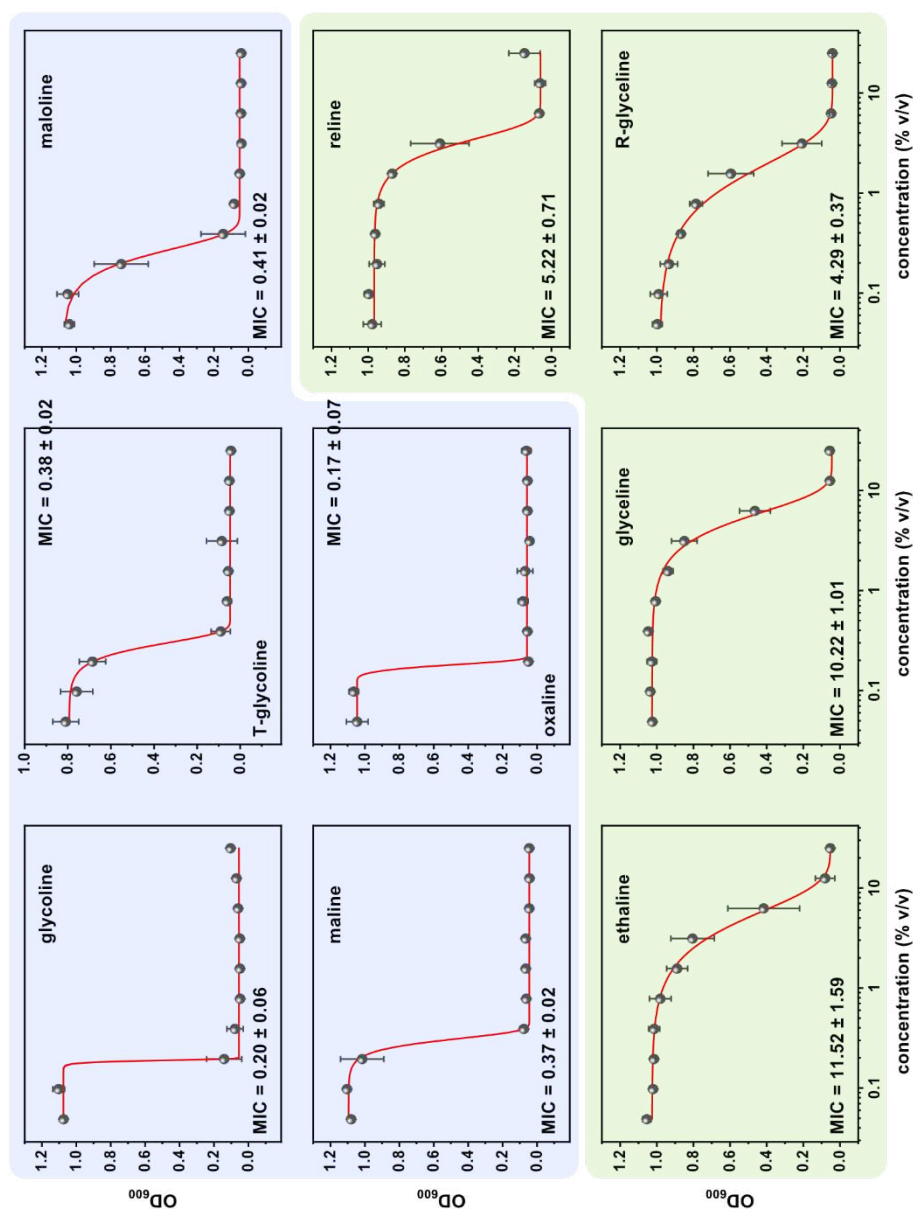


Figure A22. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against CRAB (red line indicates Gompertz model fitting, $n=3$).

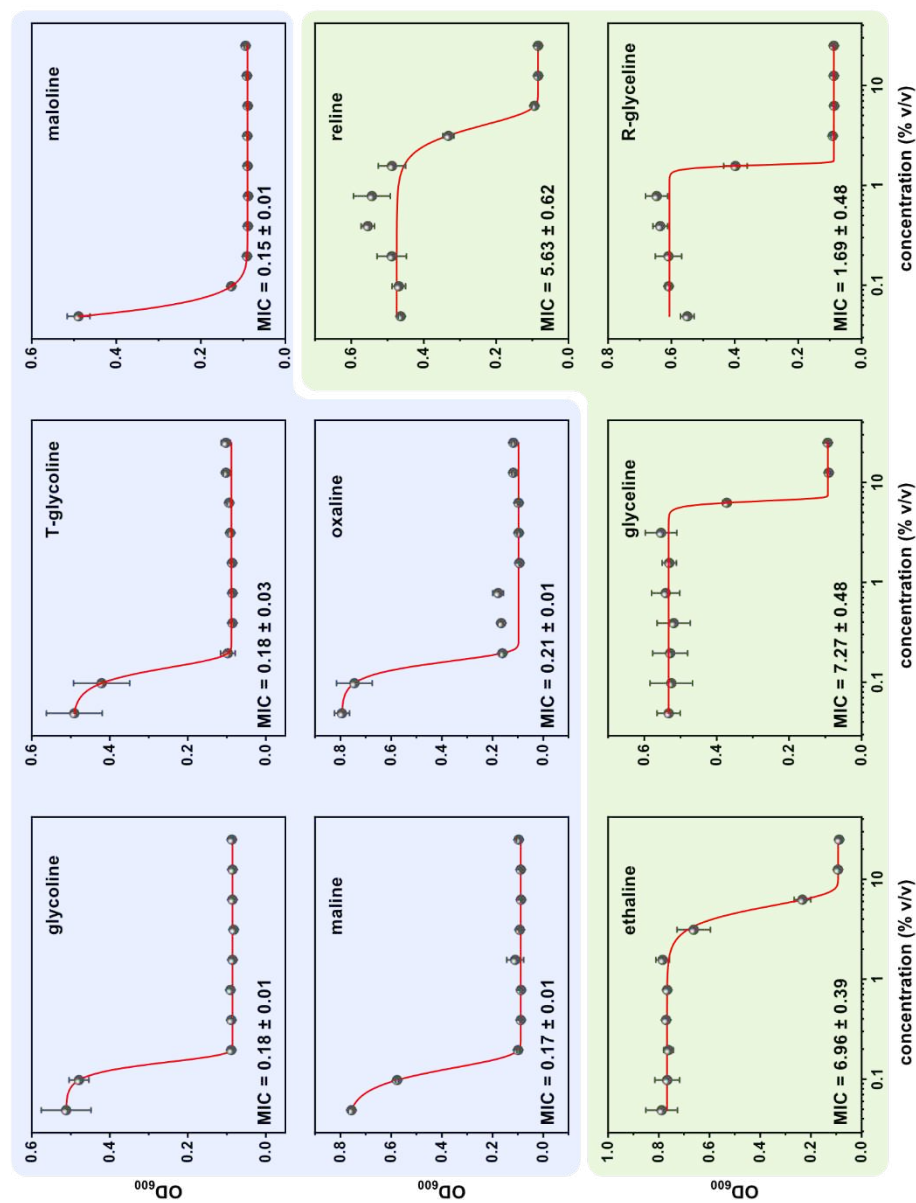


Figure A23. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against *E. coli* (red line indicates Gompertz model fitting, $n=3$).

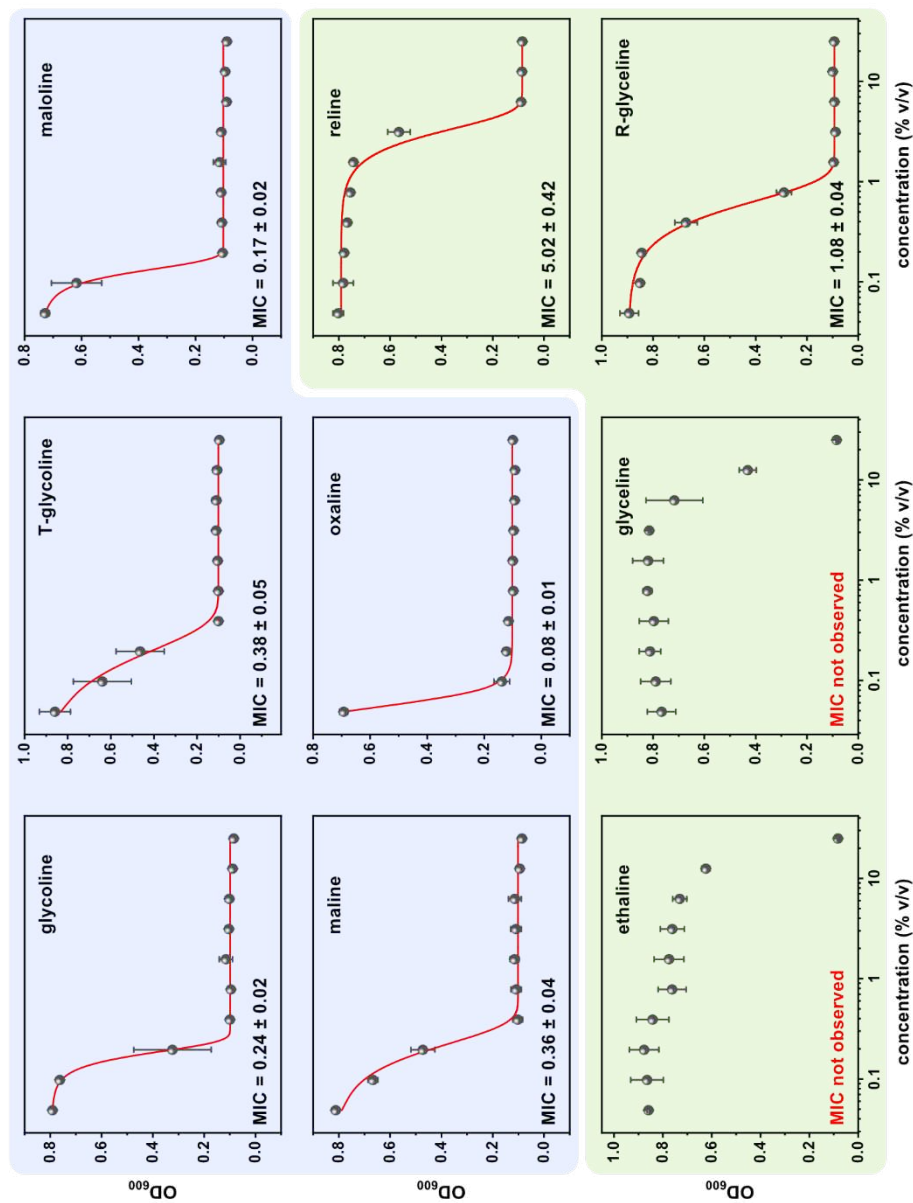


Figure A24. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against *K. pneumoniae* (red line indicates Gompertz model fitting, n=3).

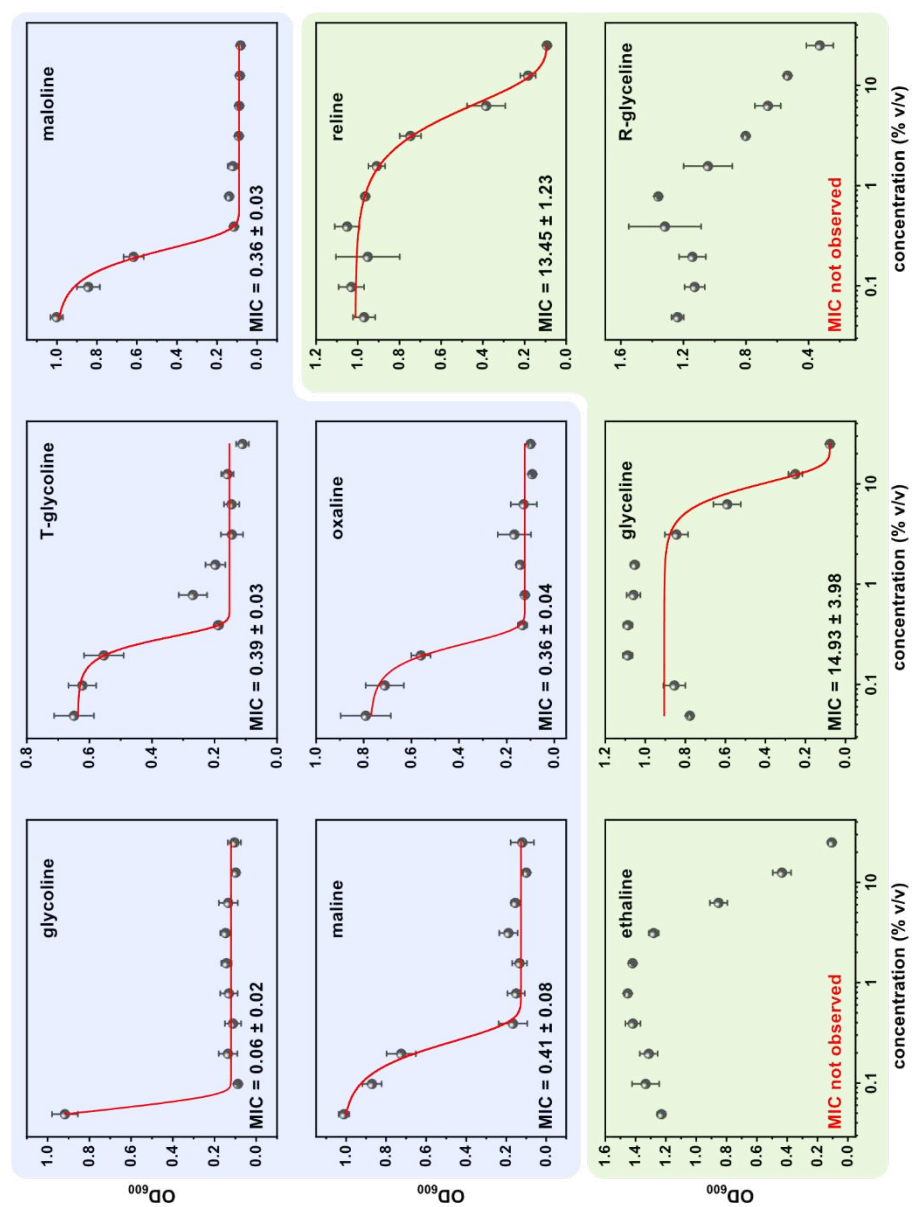


Figure A25. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against *P. fluorescens* (red line indicates Gompertz model fitting, $n=3$).

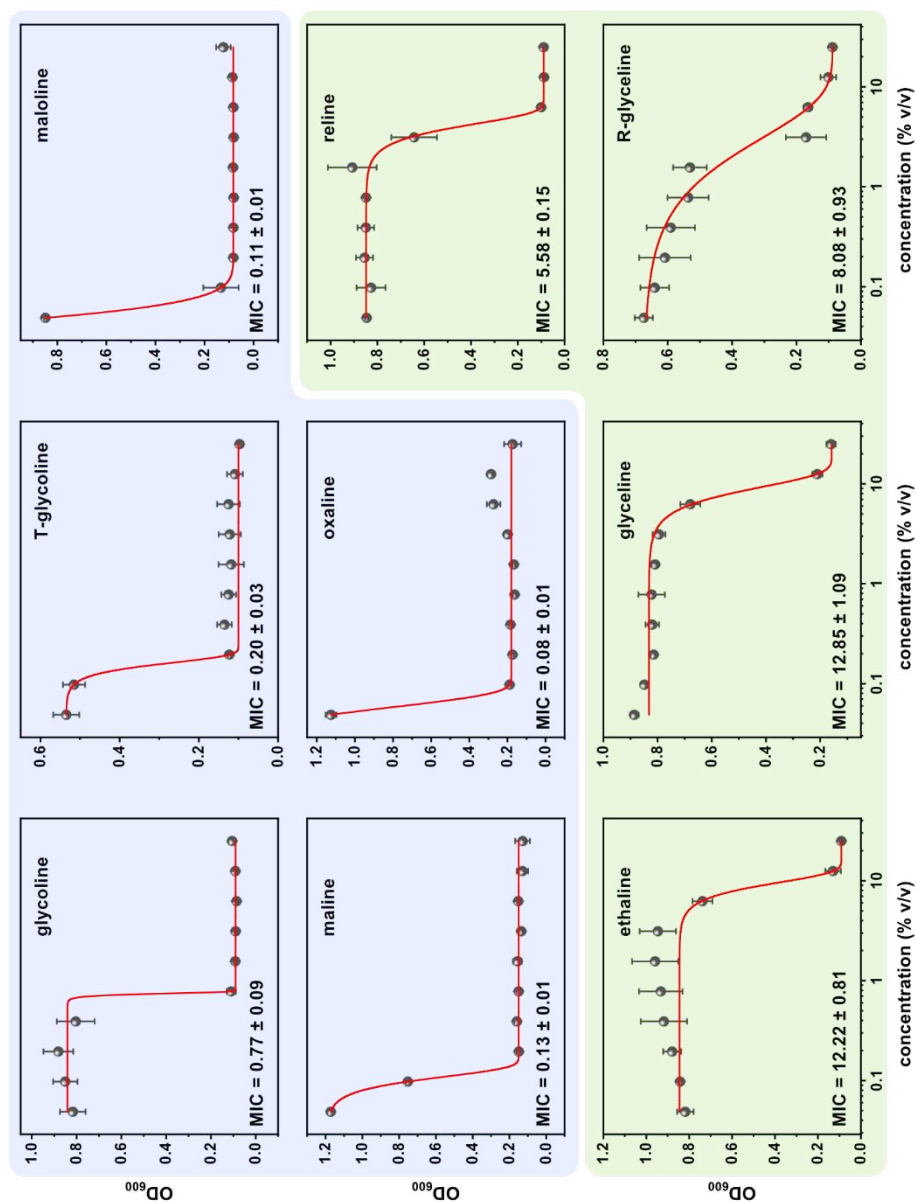
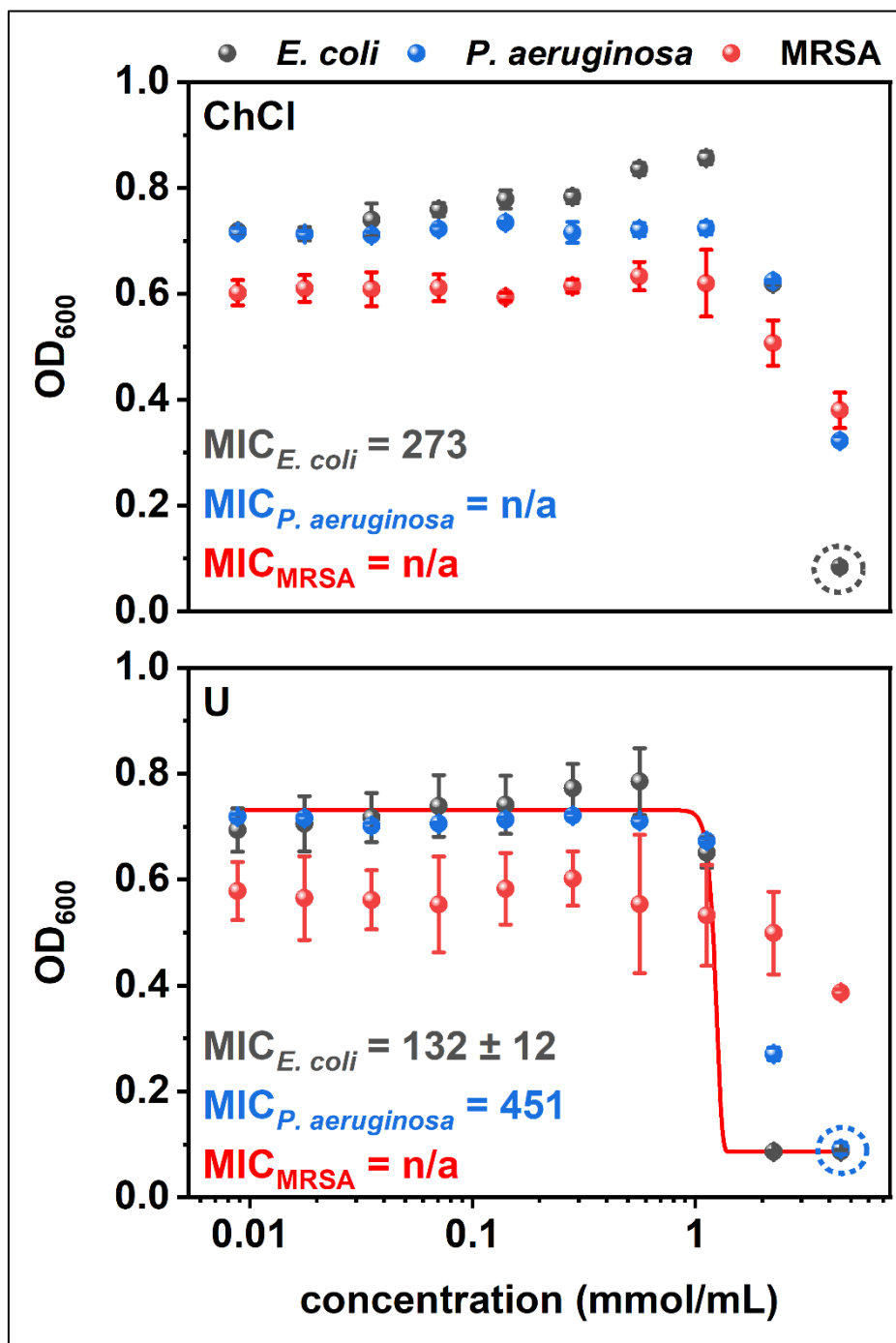
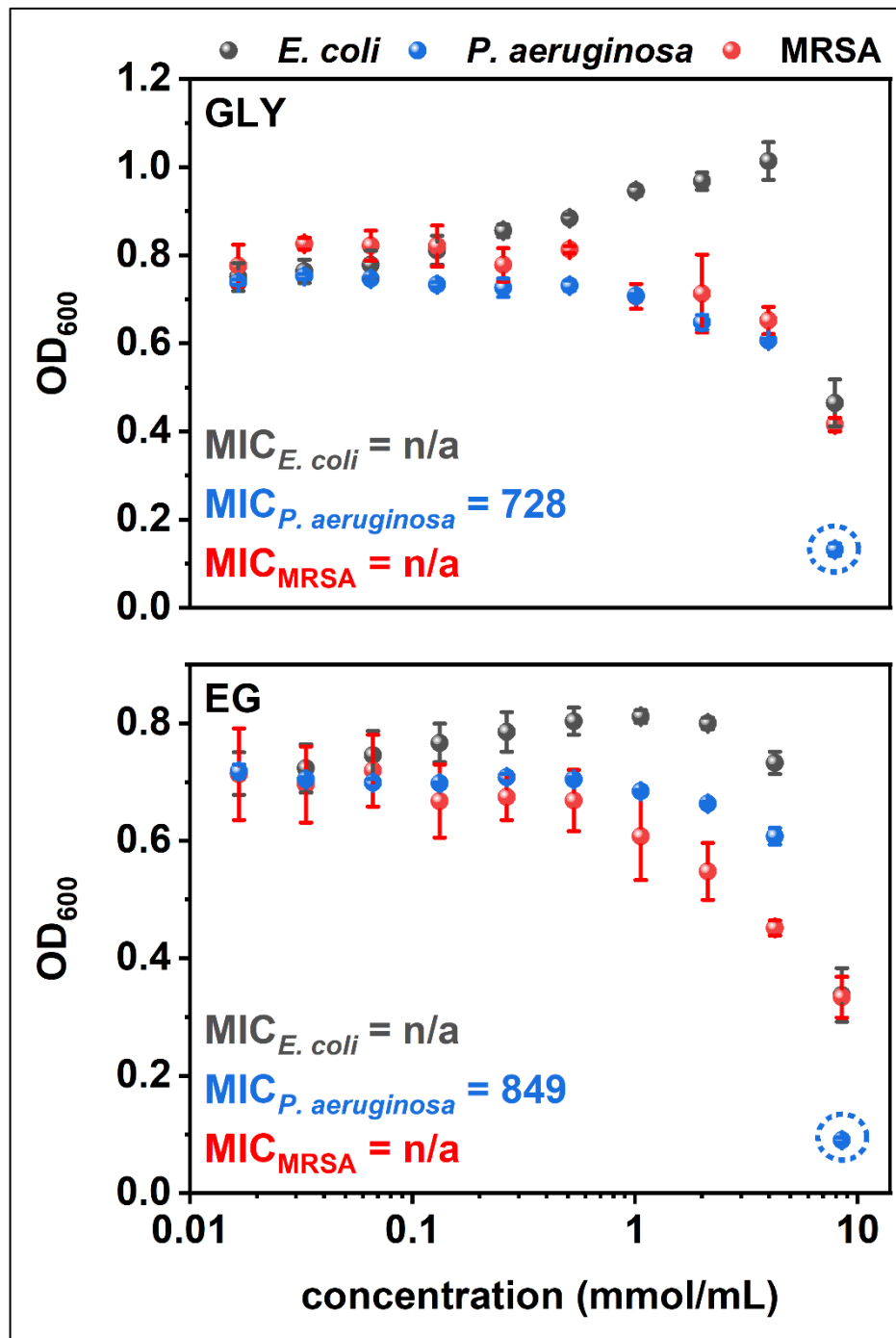
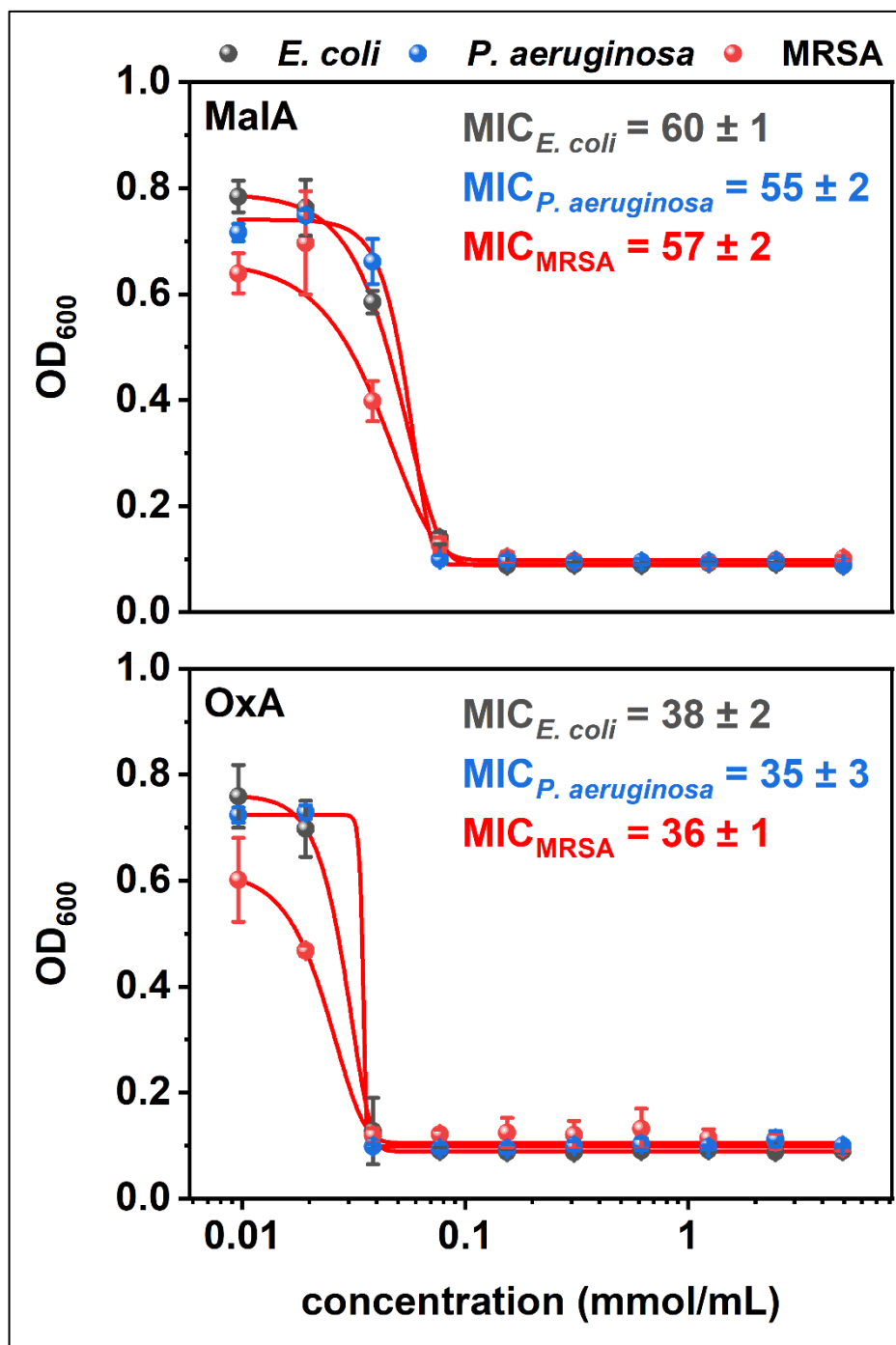


Figure A26. MIC assessment of the investigated DESs (OA-DESs (blue) and conventional DES (green)) against *P. aeruginosa* (red line indicates Gompertz model fitting, $n=3$).







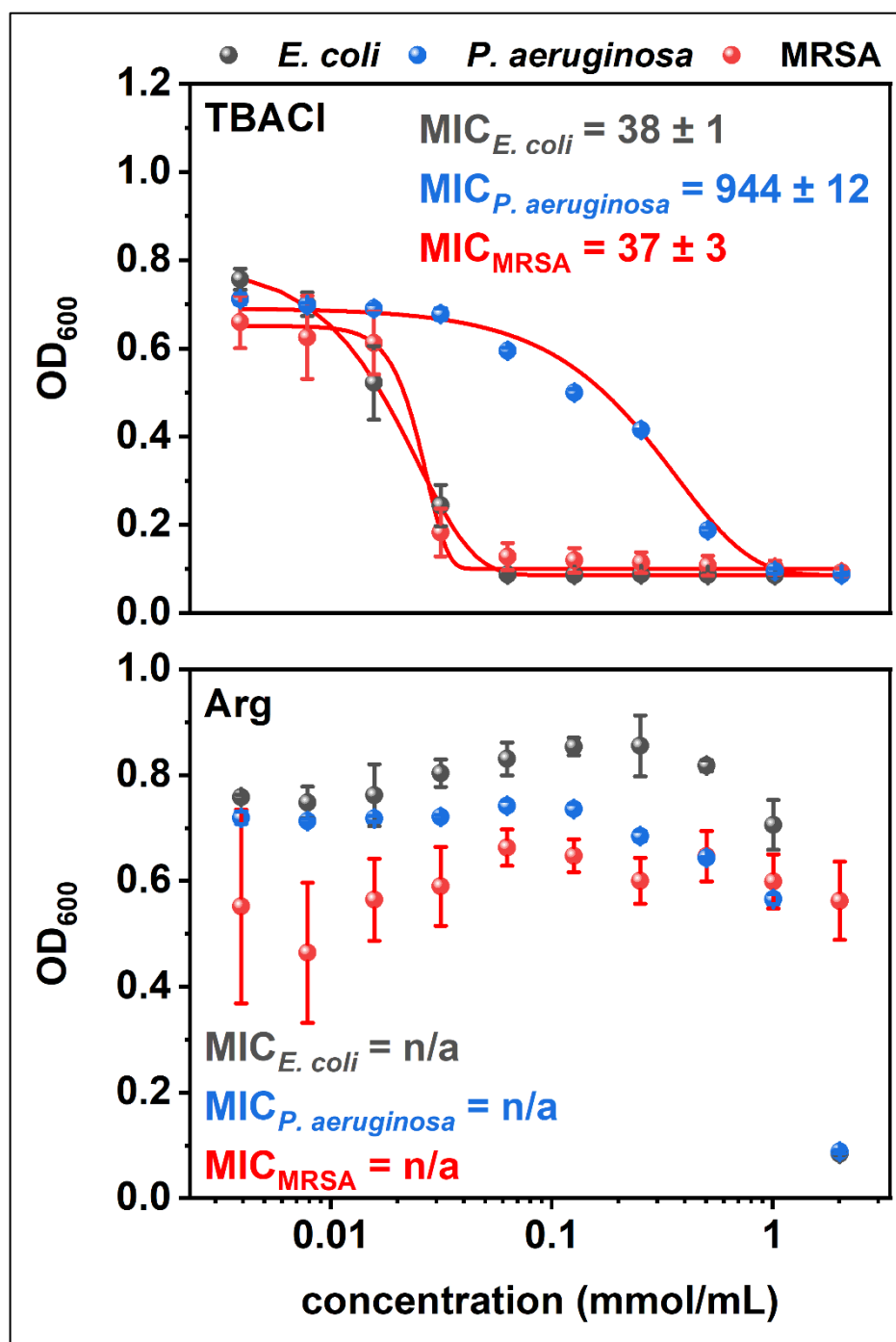


Figure A27. MIC assessment of the PSs of the investigated DESs against *E. coli*, *P. aeruginosa*, and MRSA (colored dotted circles indicate visual assessment of respective strains, red line indicates Gompertz model fitting, n=3).

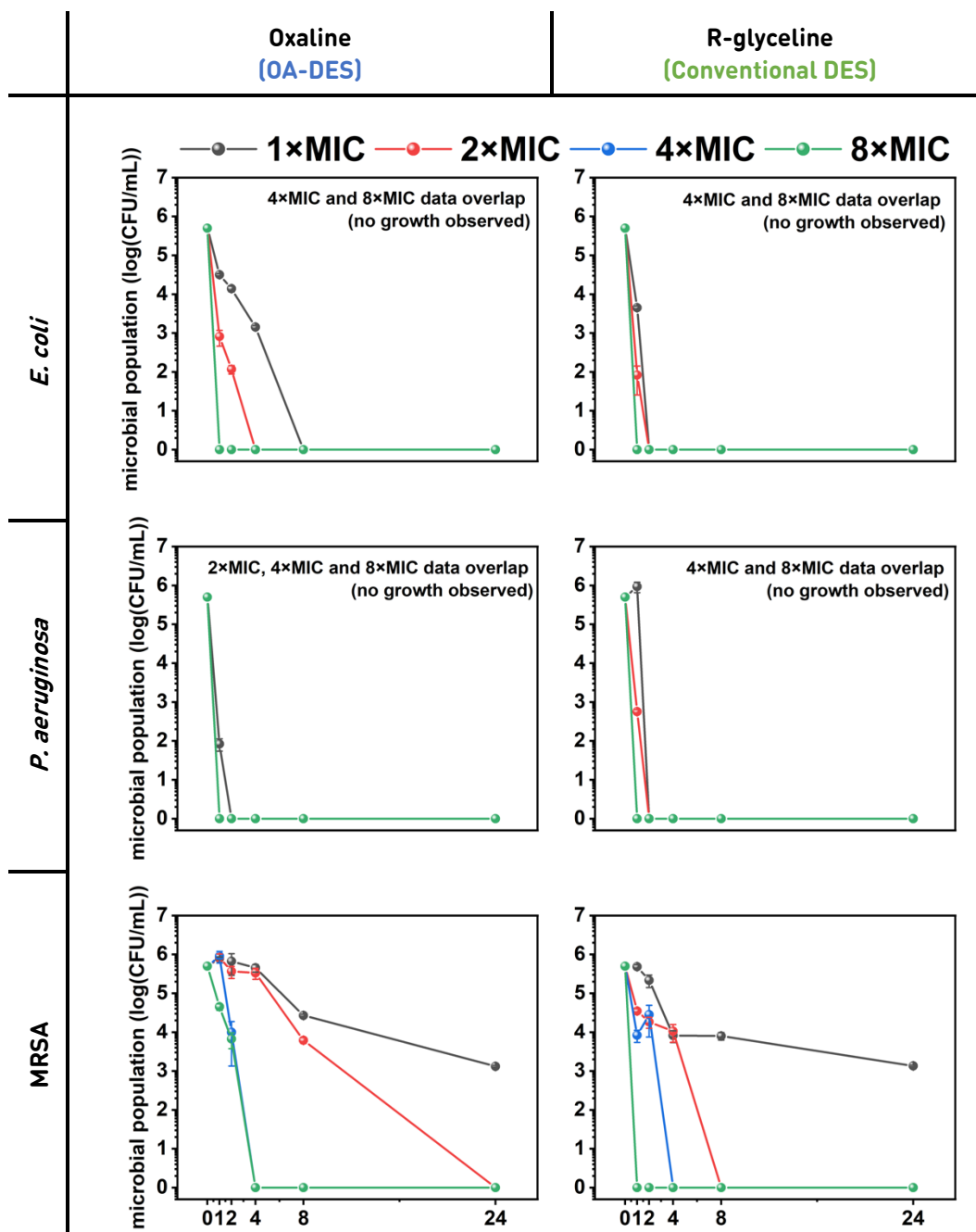


Figure A28. TK assay of most effective representatives of both types of the investigated DESs (n=3).

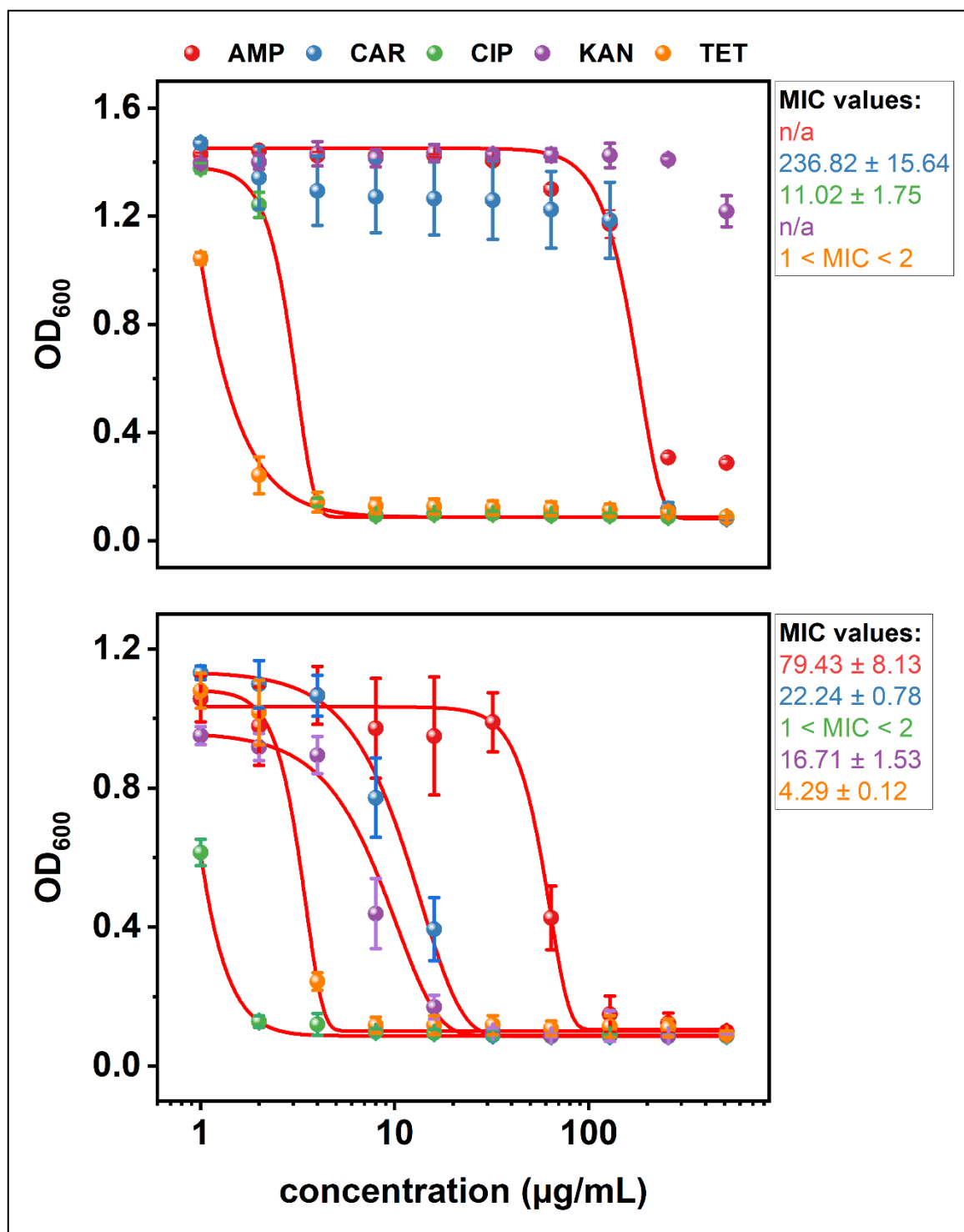


Figure A29. MIC assessments of the investigated antibiotics against *E. coli* K-12 and MRSA (n=3).

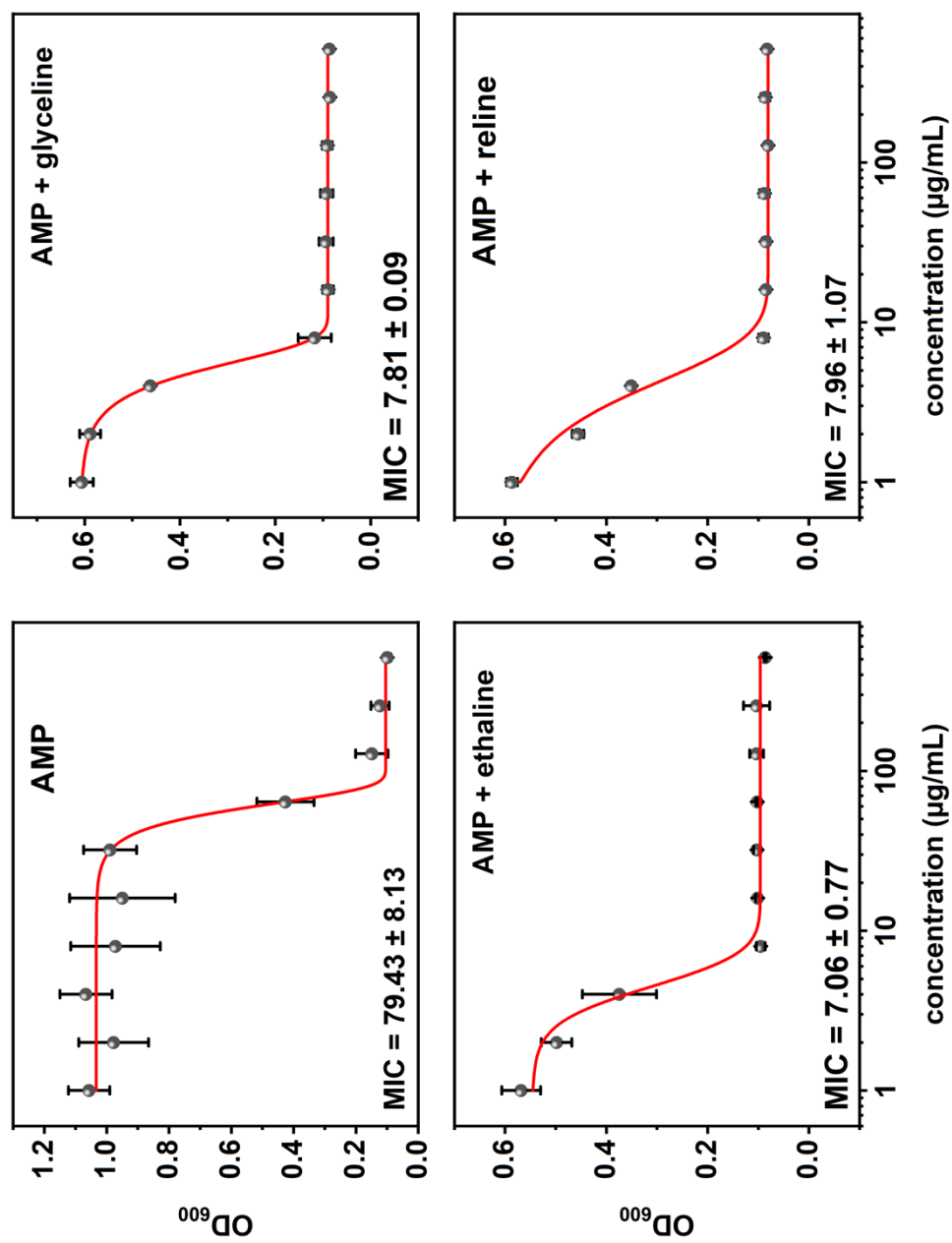


Figure A30. MIC assessments of ampicillin against *E. coli* K-12 in the presence of DESs in their sub-MIC concentrations.

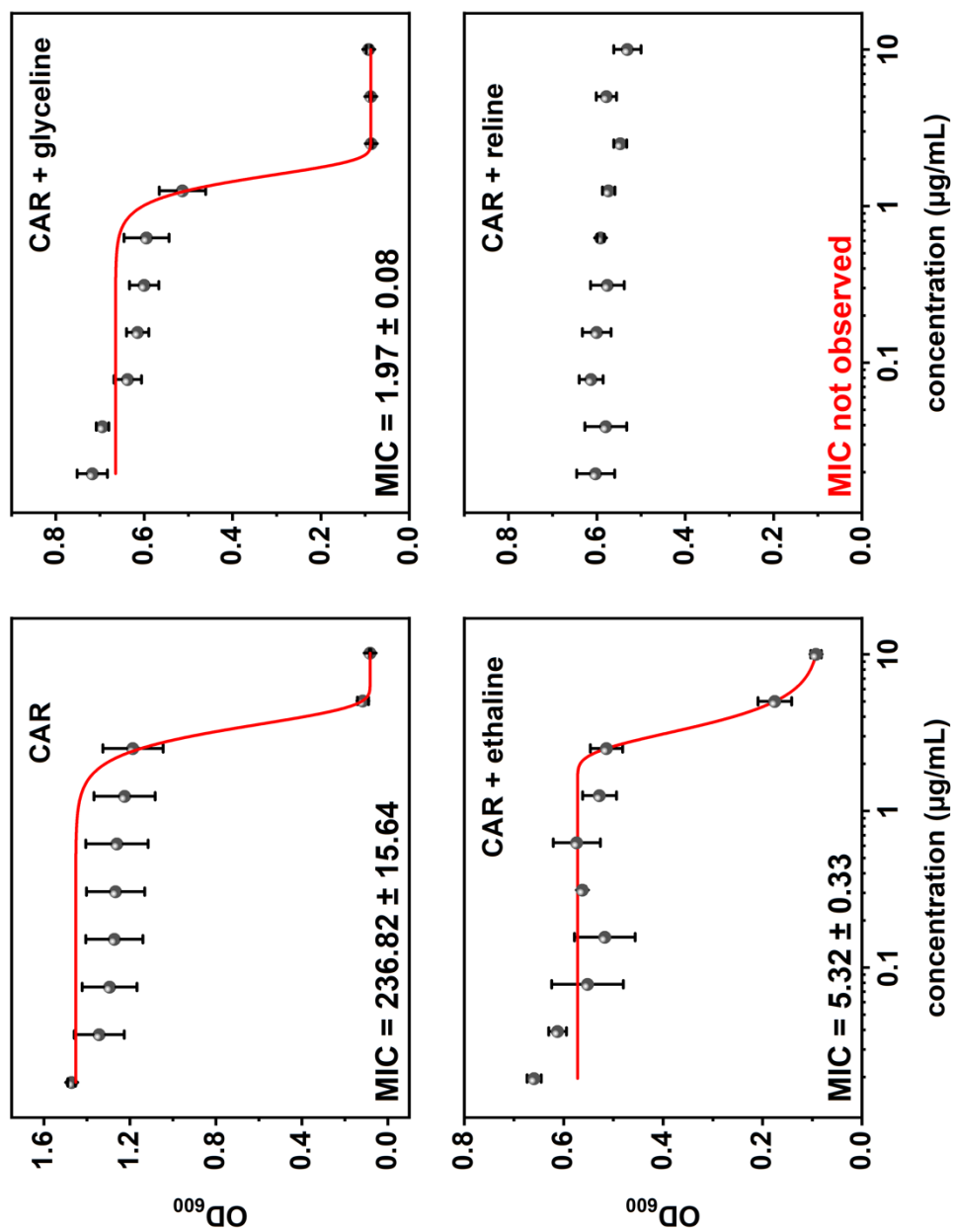


Figure A31. MIC assessments of ampicillin against MRSA in the presence of DESs in their sub-MIC concentrations.

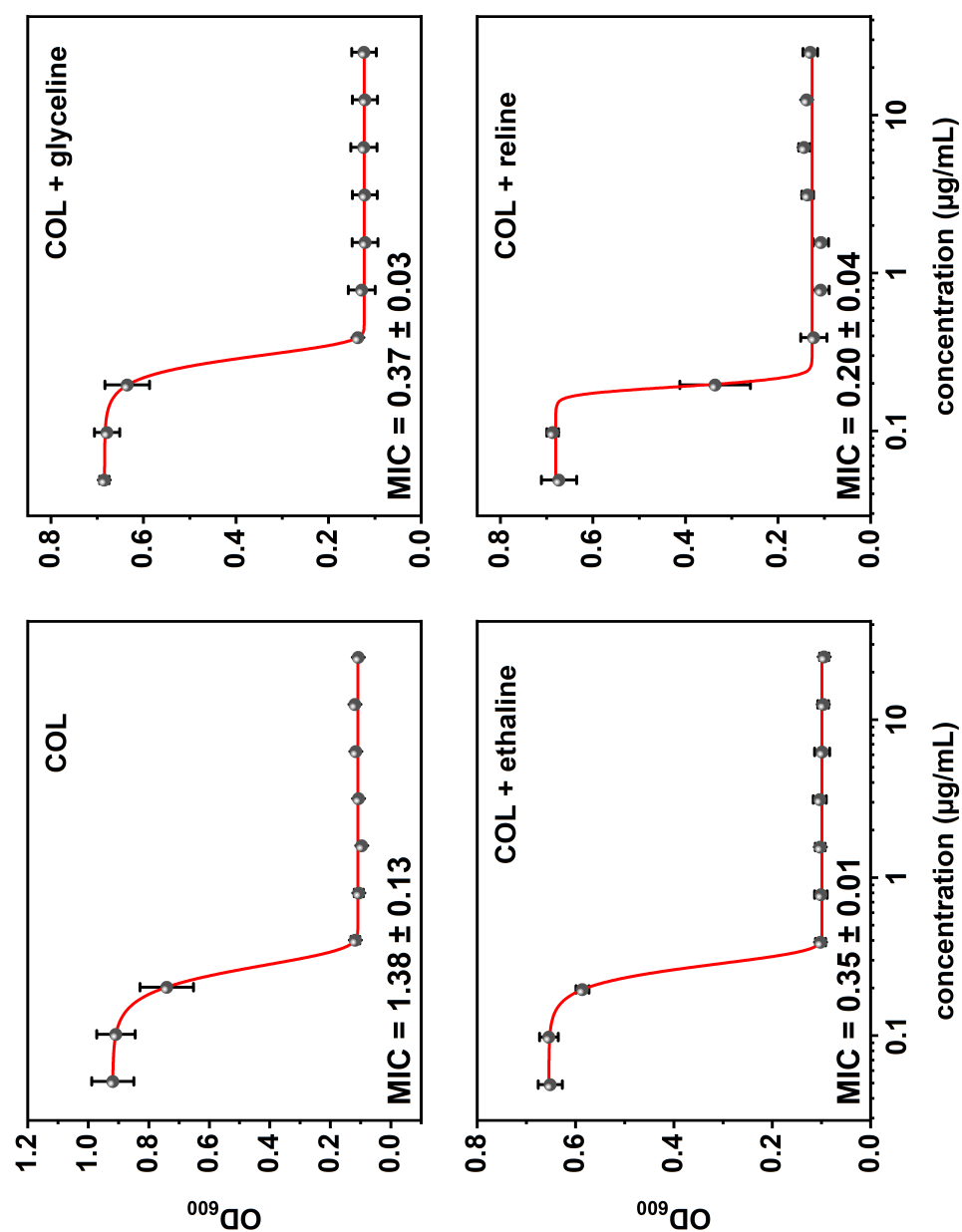


Figure A32. MIC assessments of colistin against *E. coli* K-12 in the presence of DESs in their sub-MIC concentrations.

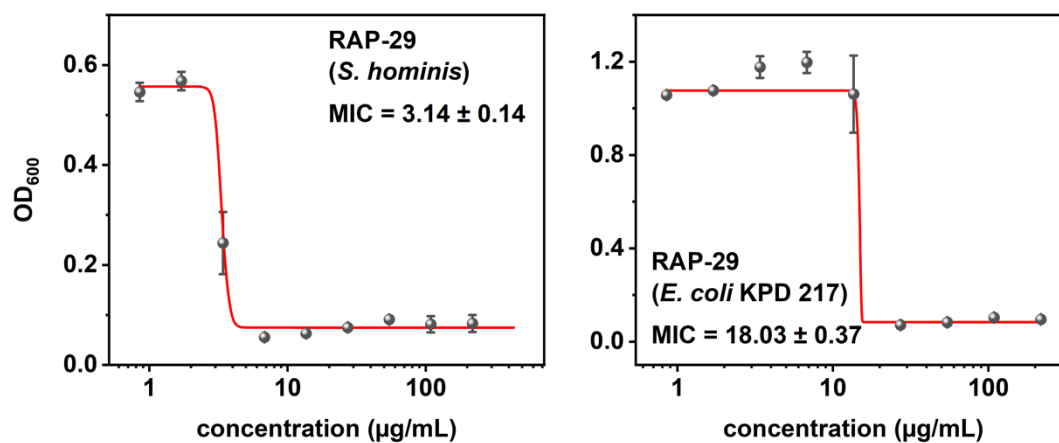


Figure A33. MIC assessments of RAP-29 against *S. hominis* and *E. coli* KPD 217 (red line indicates Gompertz model fitting; n=3).

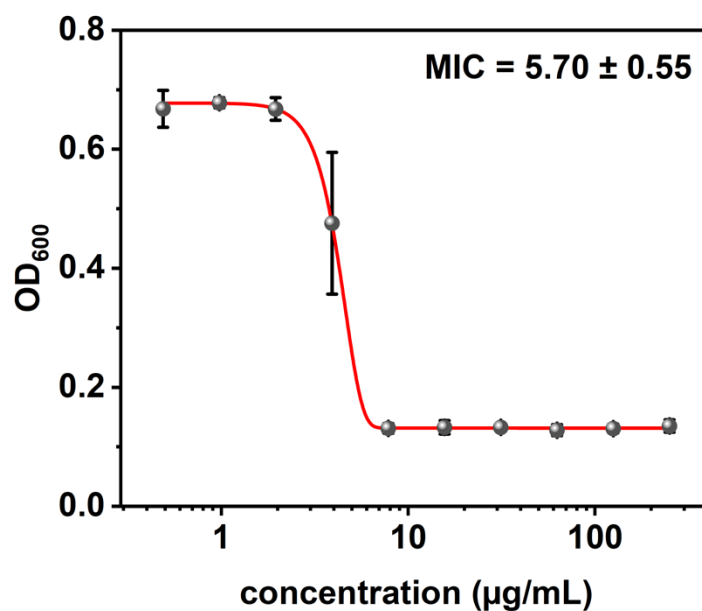
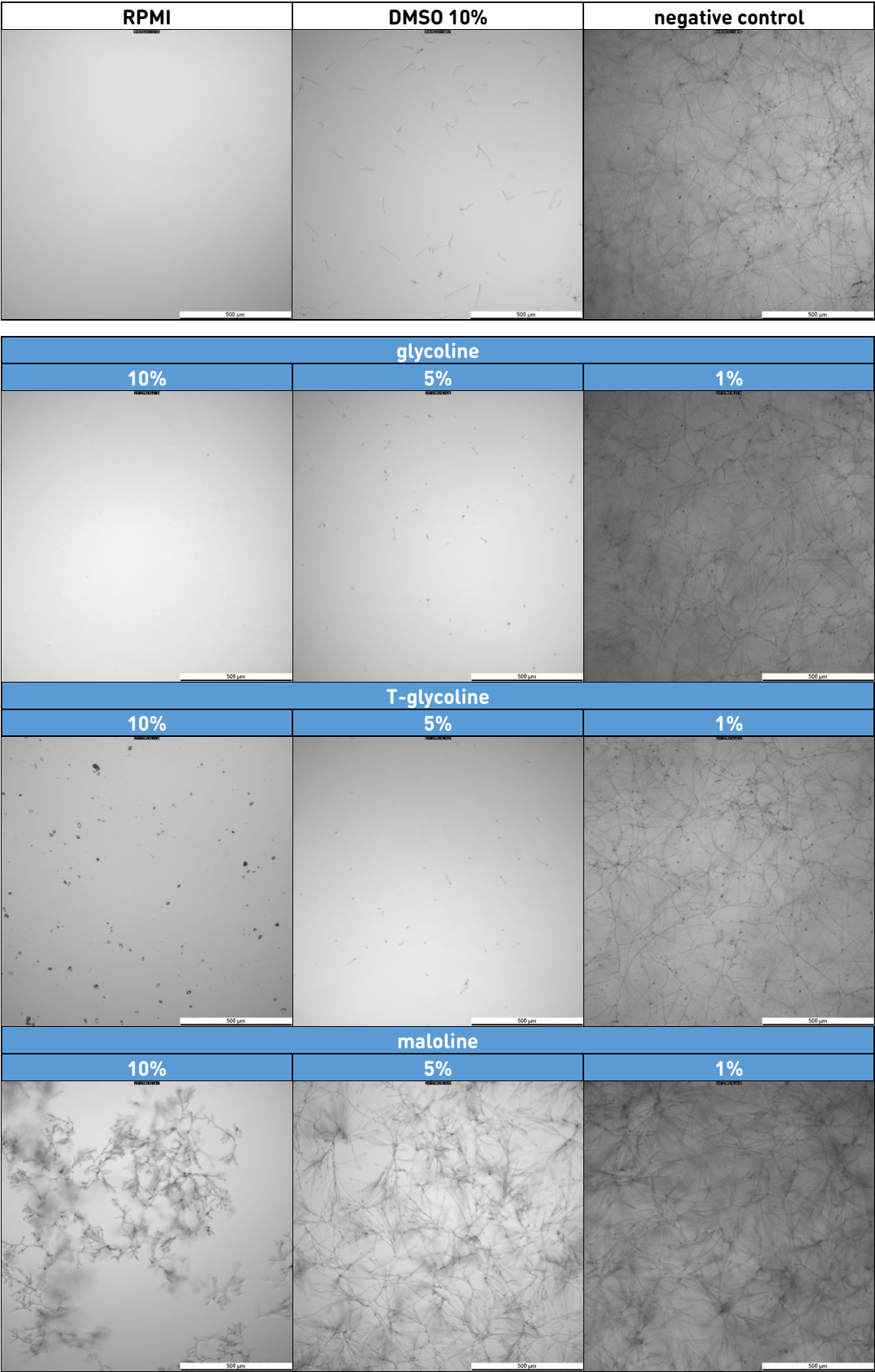
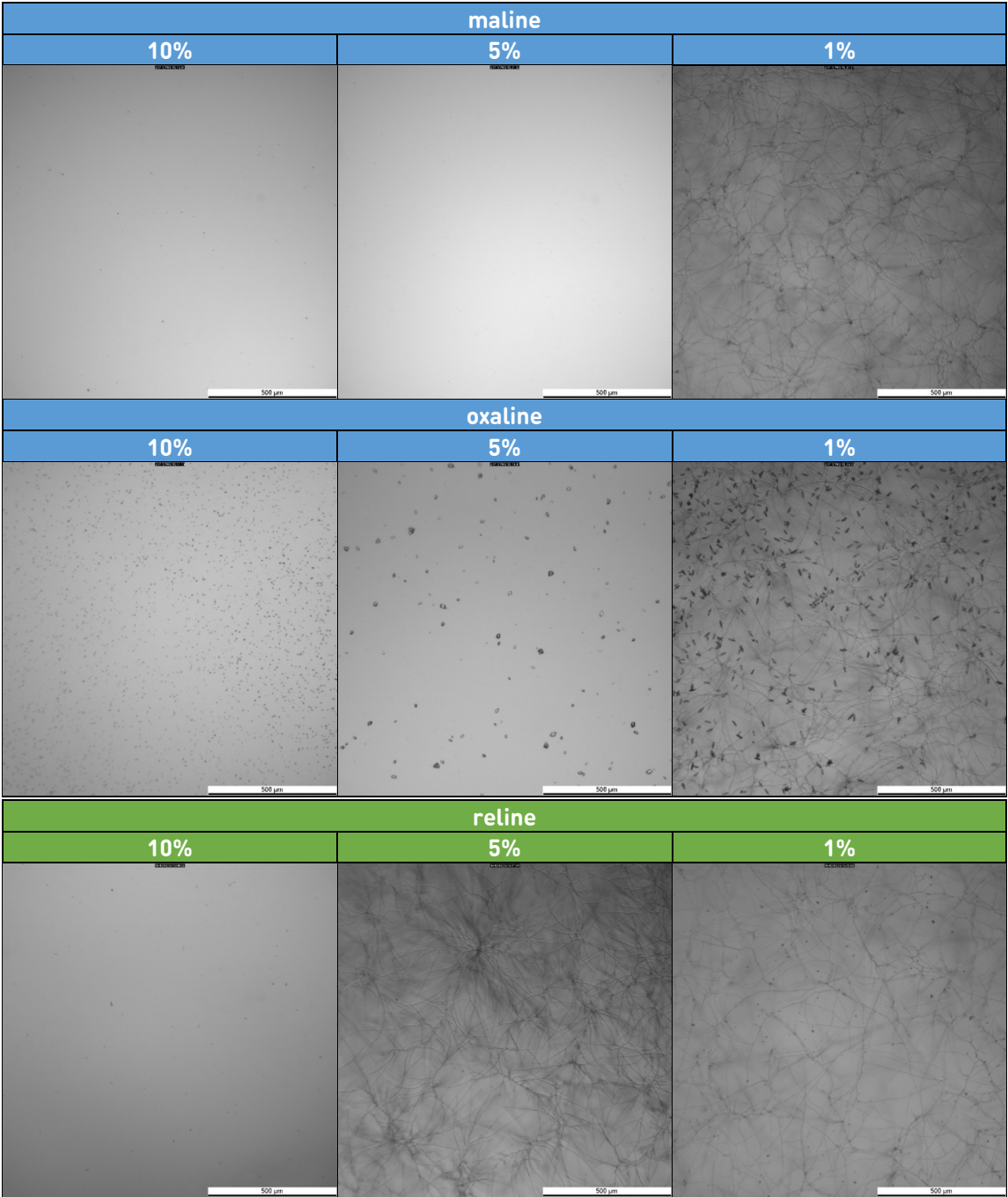


Figure A34. MIC assessments of nisin against MRSA (red line indicates Gompertz model fitting; n=3).





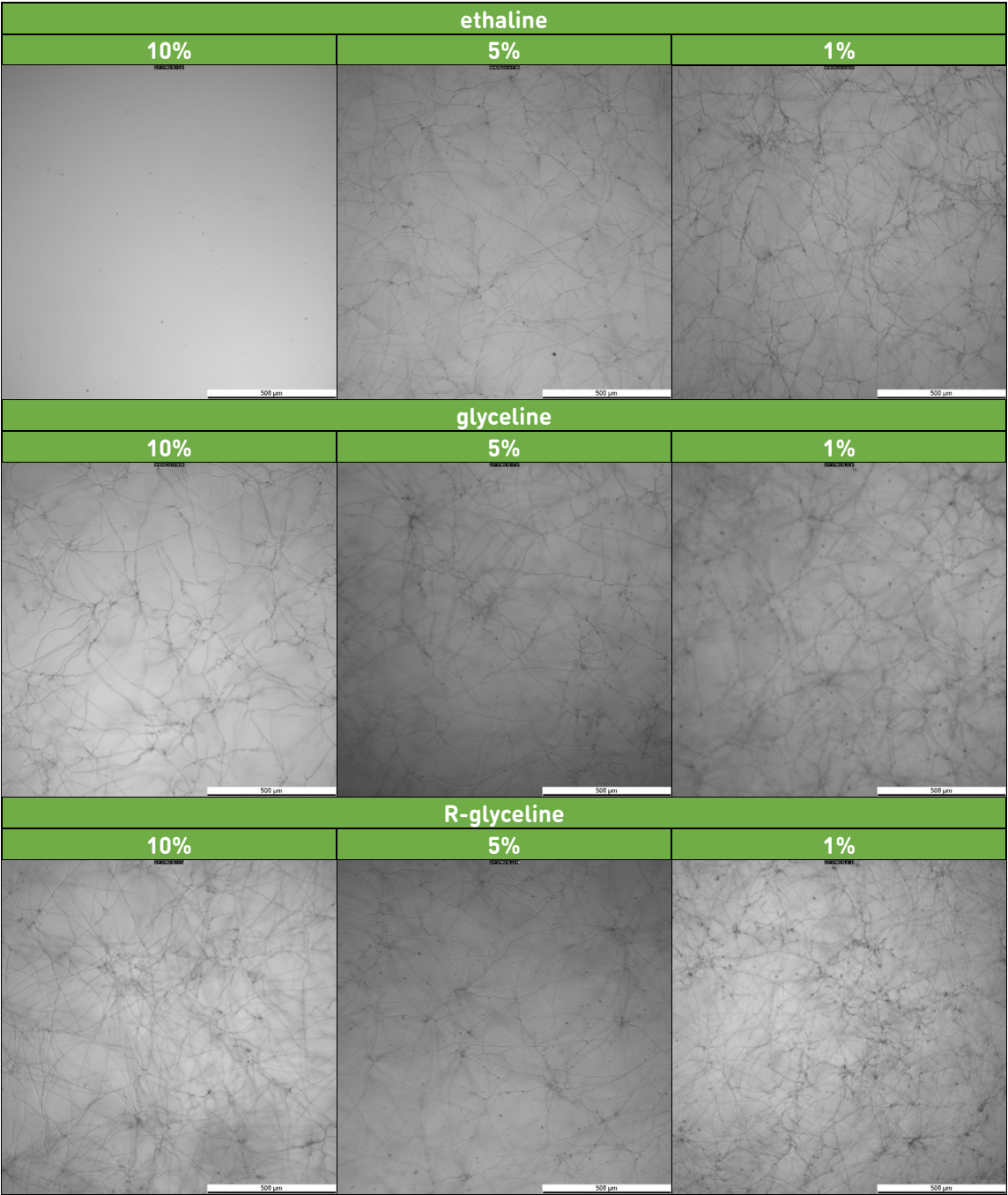
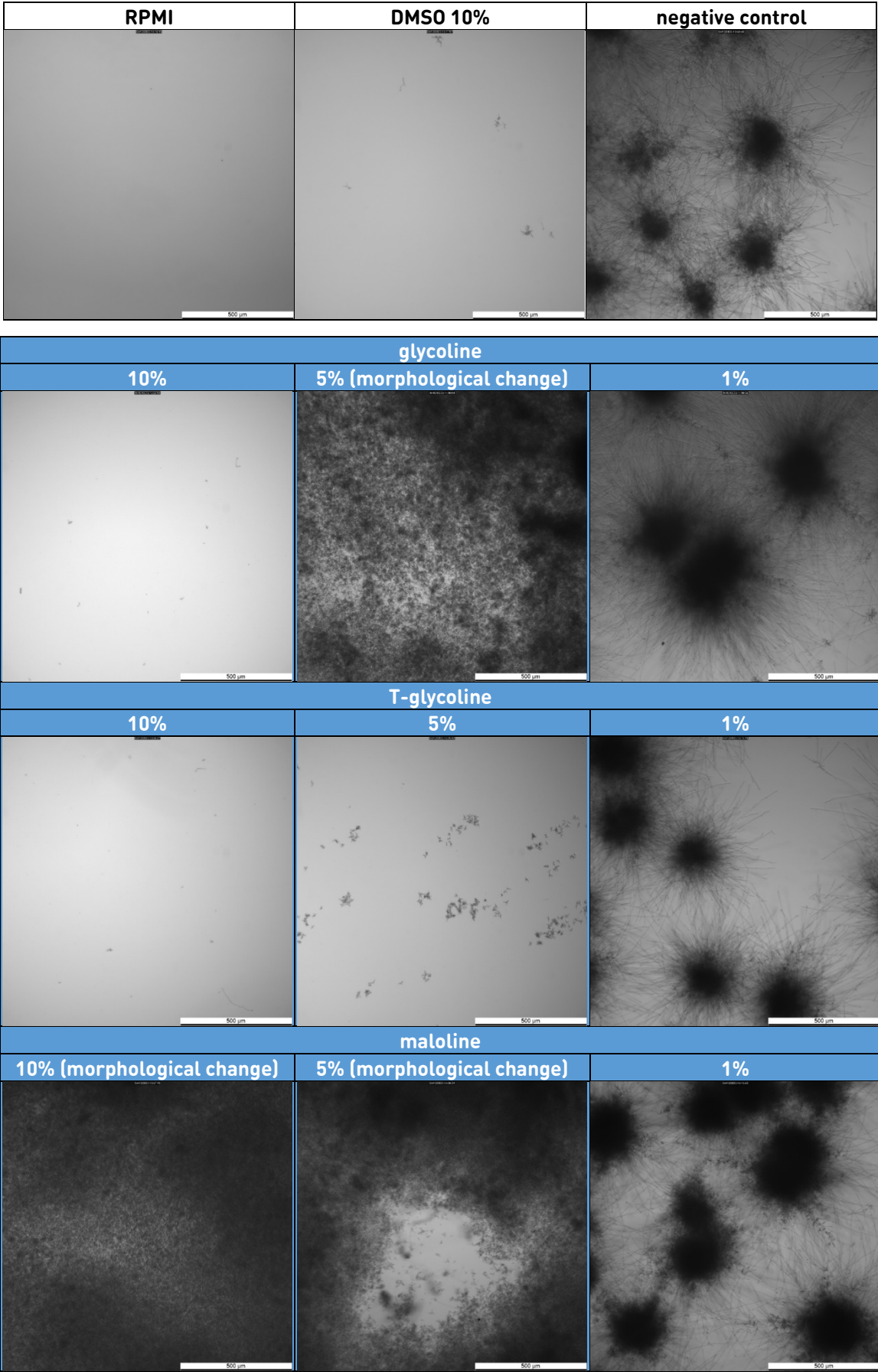
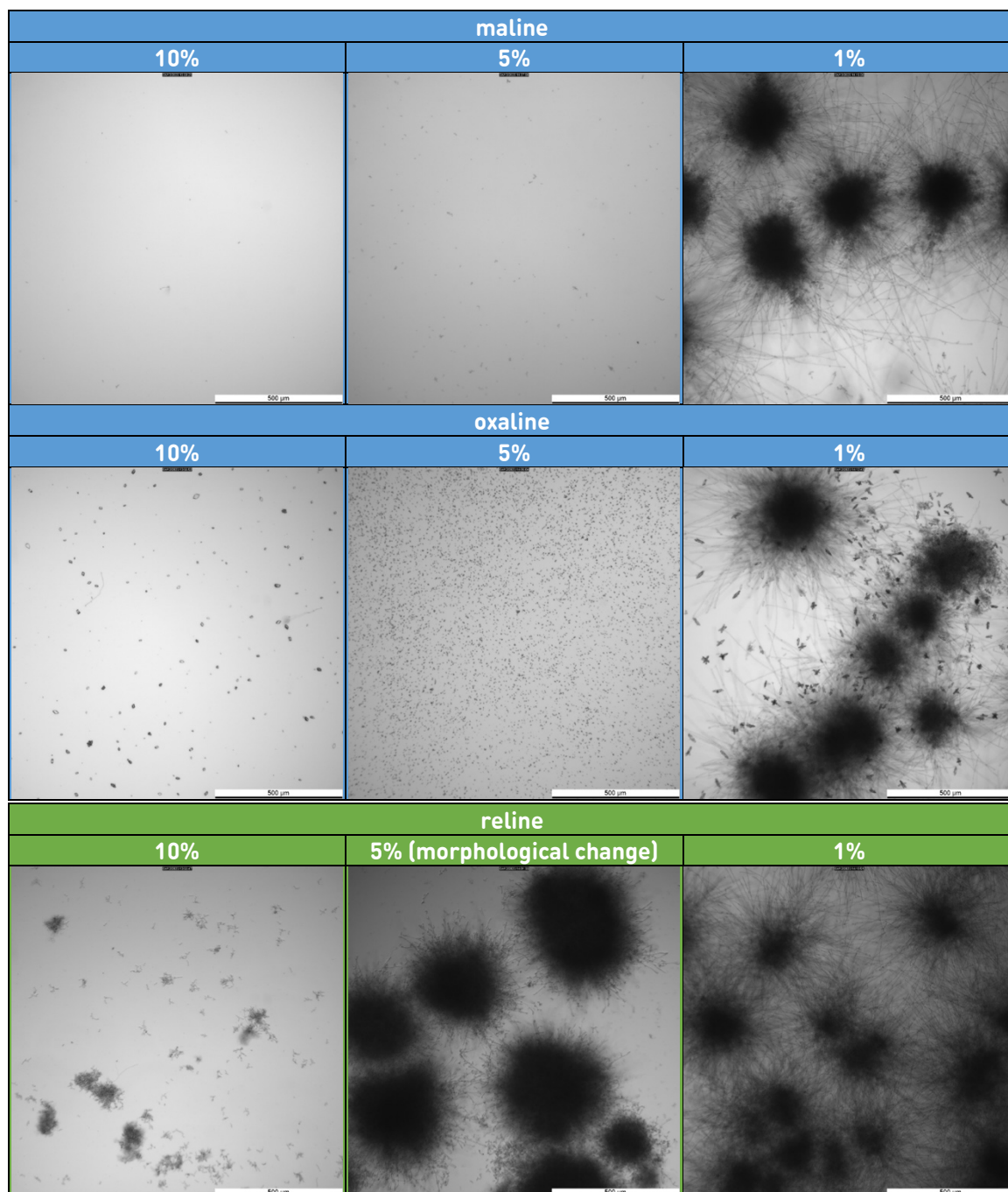


Figure A35. Optical microscopy images of *A. fumigatus* in the presence of the investigated DESs.





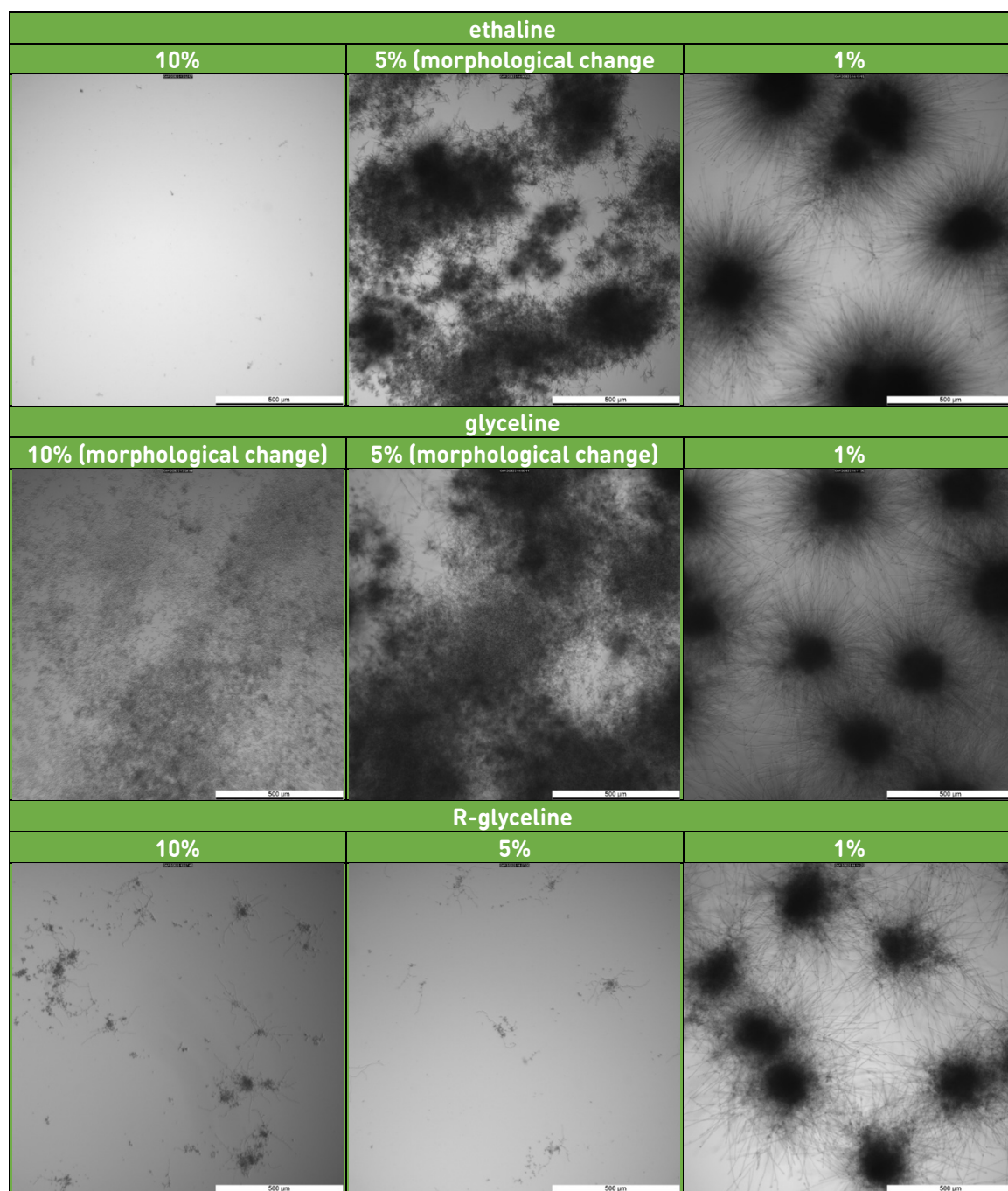
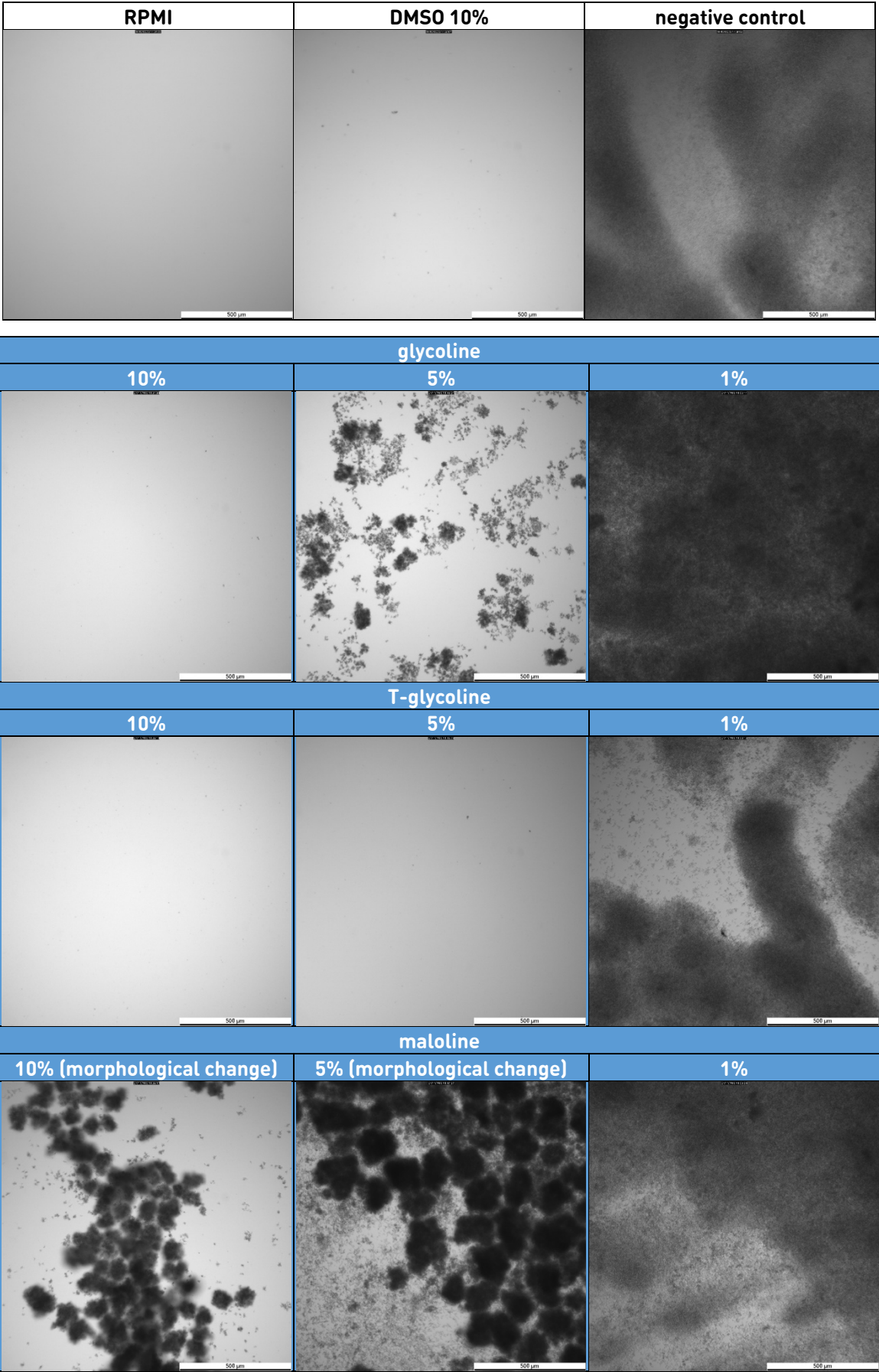
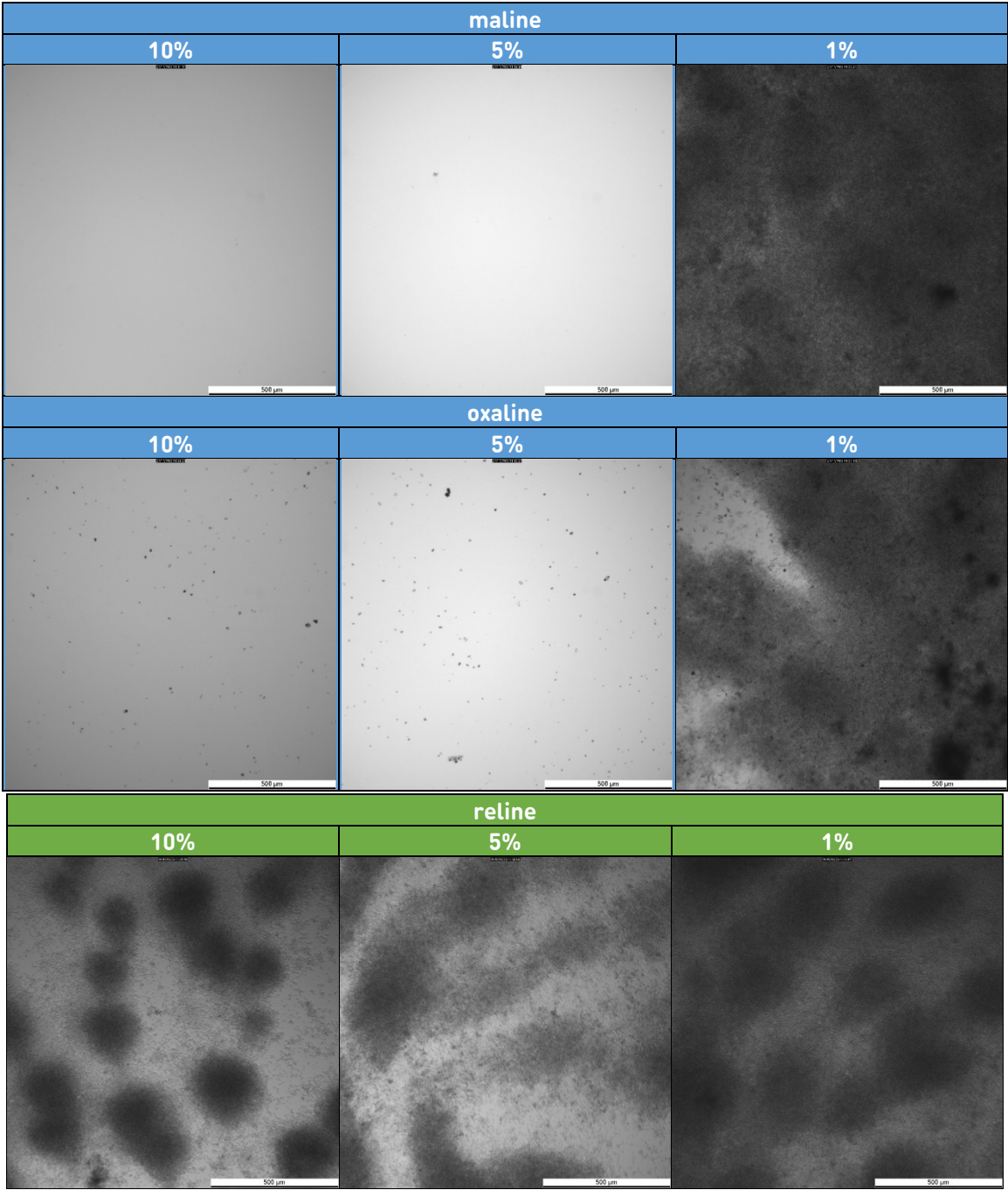


Figure A36. Optical microscopy images of *C. albicans* in the presence of the investigated DESs.





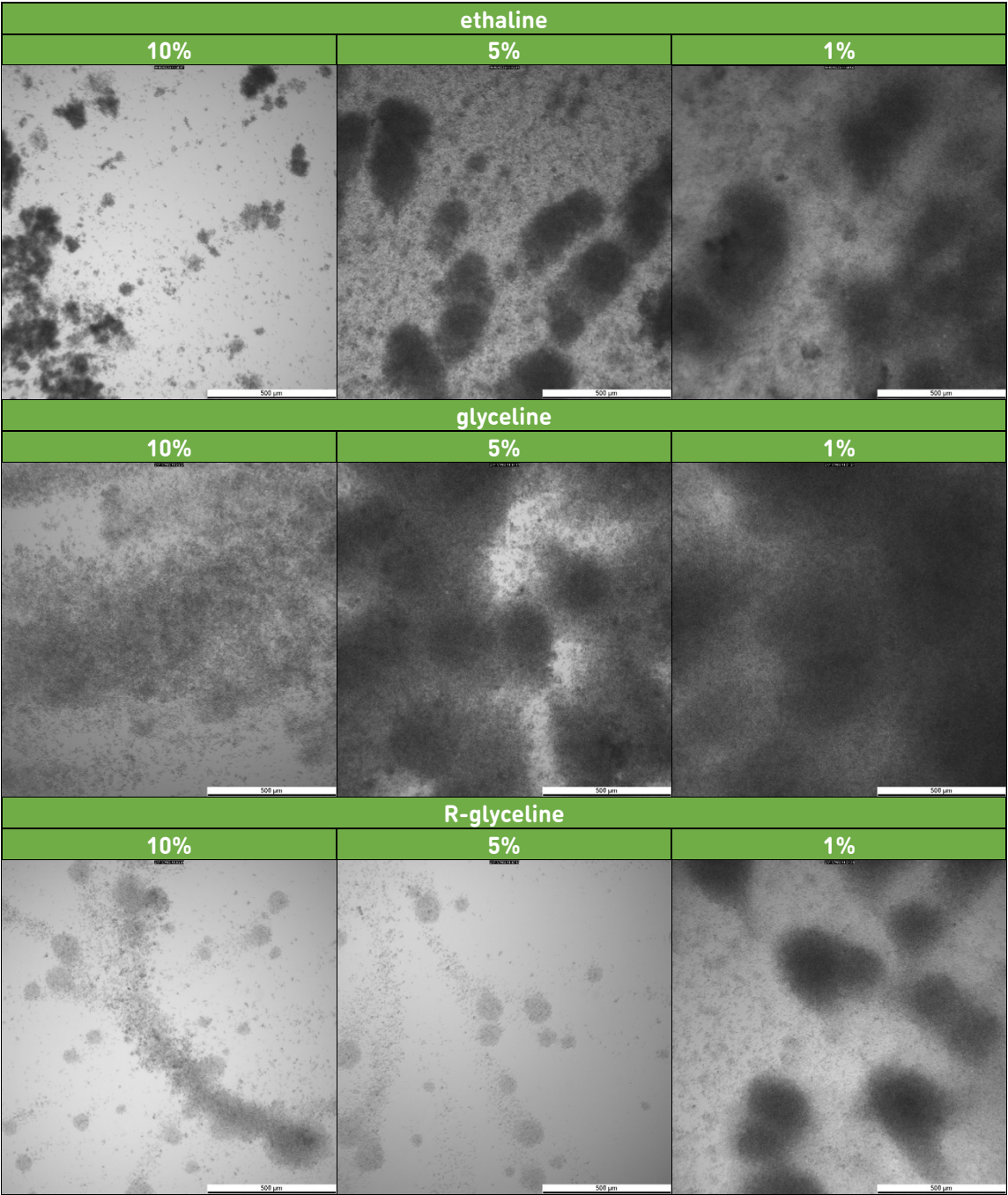


Figure A37. Optical microscopy images of *C. auris* in the presence of the investigated DESs.