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BIOFILM DISINFECTION WITH CHOLINE-BASED EUTECTIC SOLVENTS

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"A woman is like a tea bag – you never know how strong she is until she gets in hot water"

Eleanor Roosevelt

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Abstract

Infections contracted by patients within the 48 h of their stay in a hospital or other healthcare institution are known as HealthCare-Acquired Infections (HCAI). At least 20% of HCAI cases are thought to be preventable with better infection prevention and control methods, such as disinfection through the use of antimicrobial agents. However, when exposed frequently to common biocides used, bacteria might acquire resistance to them. Therefore, there is a global demand for compounds with lower toxicity for the environment and humans, that are able to improve the disinfection strategies by fighting the tolerance of microorganisms to traditional biocides. Since Natural Deep Eutectic Solvents (NADES) are based on plant-abundant primary metabolites, they have received a great deal of attention in the past few years. NADES are characterized as being mixtures of natural compounds with melting points lower than any of their individual components. In the present study, the antibiofilm activity of four choline chloride (ChCl)-based NADES, ChCl:LA (lactic acid), ChCl:CA (citric acid), ChCl:PP (1,2 – propanediol) and ChCl:UR (urea), were evaluated against single and multi-species biofilms by *Escherichia coli* and *Staphylococcus aureus*.

The potential of NADES to eradicate pre-formed biofilms was performed using a microtiter plate assay and characterized in terms of biofilm mass removal (crystal violet staining) and biofilm cells culturability. The culturability reduction assay revealed that both acidic-based NADES were significantly more effective at neutralizing bacteria than ChCl:PP and ChCl:UR.

Maximum reductions of 2.7 ± 0.5 log CFU·cm^{-2} were obtained with ChCl:CA for *E. coli* biofilms, 3.37 ± 0.31 log CFU·cm^{-2} were obtained with ChCl:LA for *S. aureus* biofilms and 2.9 ± 0.5 log CFU·cm^{-2} were obtained with ChCl:CA for the total biofilm count in multi-species biofilms. The experimental data were adjusted to the Chick-Watson model. The acidic-based NADES were appropriately adjusted, however ChCl:PP and ChCl:UR presented very low values of R^2, indicating their negligible antibiofilm properties. In single-species biofilms, there was a higher biomass removal for *E. coli* biofilms than for *S. aureus*. In multi-species biofilms, the percentages of removal were lower than in the single-species and the obtained data demonstrated the limited efficacy of NADES as biofilm disruptors. Nevertheless, this potential should be further explored, since acidic-based NADES are eco-friendly molecules with promising effects on biofilm control, specifically on the reduction of bacterial culturability.

Keywords: Antimicrobial activity, Biofilm Control, Disinfection, *E. coli*, Natural Deep Eutectic Solvents, Multi-Species Biofilms, *S. aureus*.

Resumo

As infeções contraídas pelos pacientes dentro das primeiras 48 h da sua estadia num hospital ou outra instituição de saúde são conhecidas como Infeções Adquiridas por Cuidados de Saúde (em inglês, *HealthCare-Acquired Infections,* HCAI). No mínimo 20% das HCAI são consideradas evitáveis com melhores métodos de prevenção e controlo de infeções, tais como a desinfeção através da utilização de agentes antimicrobianos. No entanto, quando expostas frequentemente aos biocidas comuns utilizados, as bactérias podem adquirir resistência aos mesmos. Assim, existe uma procura global de compostos com menor toxicidade para o ambiente e os seres humanos, que sejam capazes de melhorar as estratégias de desinfeção combatendo a tolerância dos microrganismos aos biocidas tradicionais. Uma vez que os Solventes Eutécticos Naturais Profundos (NADES) se baseiam em metabolitos primários abundantes em plantas, têm recebido grande atenção nos últimos anos. Os NADES caracterizam-se por serem misturas de compostos naturais com pontos de fusão inferiores aos de qualquer um dos seus indivíduos. Neste trabalho, foi avaliada a atividade anti-biofilme de quatro NADES à base de cloreto de colina (ChCl): ChCl:LA (ácido láctico), ChCl:CA (ácido cítrico), ChCl:PP (1,2 - propanodiol) e ChCl:UR (ureia), contra biofilmes simples e compostos de *Escherichia coli* e *Staphylococcus aureus*.

O potencial dos NADES para erradicar biofilmes foi realizado a partir de ensaios de quantificação de redução de unidades formadoras de colónias e de remoção de biofilme. O ensaio de redução de culturabilidade mostrou que ambos os NADES de base ácida selecionados têm muito mais potencial na neutralização de bactérias do que ChCl:PP e ChCl:UR.

Foi obtida uma redução máxima de 2.7 ± 0.5 log CFU·cm^{-2} com ChCl:CA para biofilmes de *E. coli*, 3.37 ± 0.31 log CFU·cm^{-2} com ChCl:LA para biofilmes de *S. aureus* e um total de 2.9 ± 0.5 log CFU·cm^{-2} com ChCl:CA para biofilmes mistos. Os valores obtidos foram ajustados ao modelo Chick-Watson. Os NADES de base ácida foram ajustados corretamente, contudo ChCl:PP e ChCl:UR apresentaram valores muito baixos de R^2, indicando propriedades anti-biofilme desprezíveis. Nos biofilmes simples, houve uma maior remoção de biomassa para biofilmes de *E. coli* do que para os de *S. aureus*. Nos biofilmes compostos, as percentagens de remoção foram inferiores às dos biofilmes simples e estes valores não demonstraram nenhuma correlação aparente entre a concentração utilizada e a percentagem de remoção de biofilme, o que se traduz numa eficácia limitada dos NADES como disruptores de biofilmes. Este potencial deve ser explorado, uma vez que os NADES de base ácida são moléculas amigas do ambiente com efeitos promissores no controlo de biofilme, especificamente na redução de cultura bacteriana.

Palavras-Chave: Atividade antimicrobiana, Controlo de Biofilmes, Desinfeção, *E. coli*, *S. aureus*, Solventes Eutécticos Naturais Profundos.

Declaration

I hereby declare, under word of honor, that this work is original and that all non-original contributions is indicated, and due reference is given to the author and source

Eva Carolina Freitas Correia

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Notation and Glossary

A_c	Absorbance for positive control	
A_X	Absorbance for exposed bacteria	
C	Biocide concentration	$mg \cdot mL^{-1}$
n	Concentration dependence of killing	
X	Count of colonies forming units after biocide exposure	$CFU \cdot cm^{-2}$
X_0	Count of colonies forming units before biocide exposure	$CFU \cdot cm^{-2}$
k_{dis}	Disinfectant rate coefficient	$(mL \cdot mg^{-1})^n \cdot min^{-1}$
t	Time	min

List of Acronyms

CDC	Center for Disease Control and Prevention
COSMO-RS	Conductor-like Screening Model for Real Solvents
DES	Deep Eutectic Solvents
ECDC	European Centre for Disease Control
EFSA	European Food Safety Authority
EPS	Extracellular Polymeric Substance
HBA	Hydrogen Bond Acceptor
HBD	Hydrogen Bond Donor
HCAI	HealthCare-Associated Infections
HLD	High-Level Disinfection
ILS	Ionic Liquids
LLD	Low-Level Disinfection
MD	Medical Devices
NADES	Natural Deep Eutectic Solvents
QACs	Quaternary Ammonium Compounds
QS	Quorum-Sensing

1 Introduction

1.1 Framing and presentation of the work

Healthcare-Associated Infections (HCAI) are infections acquired by patients within the first 48 h of their stay in a hospital or other type of healthcare facility. HCAI can occur as a direct result of healthcare interventions or as a consequence of interaction with a healthcare facility. At least 20% of HCAI cases are believed to be preventable with better strategies for infection prevention and control.

Disinfection is defined as the antimicrobial reduction of microorganisms to a level previously specified as appropriate. The disinfectant used and the method utilized are the two factors that have the most impact on the effectiveness of surface disinfection. These factors, along with regular exposure to disinfectants, may cause bacteria to develop a tolerance to them, which is a serious clinical problem since these substances are essential for avoiding hospital infections. Bacterial resistance has been developed in microorganisms by different mechanisms of action, yet this study focuses on biofilm formation as the basic survival strategy that shields organisms against external factors since 99% of all microorganisms adhere to surfaces and grow as biofilms.

The creation of new green solvents, particularly to replace the prevalent organic solvents, is one of the main topics that green chemistry focuses on and is thus of utmost importance. Natural Deep Eutectic Solvents (NADES) are recently discovered solvents that are based on abundant plant metabolites and are found in living cells. Despite being relatively new, NADES have a wide range of applications, and some of these solvents have been shown to have antibacterial properties. This study focuses on the antibacterial activity of NADES against biofilms and its potential usage as a replacement for common organic disinfectants that are known to be hazardous to human and environmental health.

1.2 Contribution of the author to the work

The work began with a search on the literature for existing NADES and the identification of those with potential antibiofilm activity. Then, NADES production method was carefully selected, as well as the actual production of selected NADES. After evaluating the stability of the NADES obtained, the assessment of its antibiofilm effect took place. For that matter, the bacterial cultures were prepared, and single and multi-species biofilms were formed. All the biofilm treatment assays were carried out by the author, as well as the calculations related to these tests. With the results obtained, the author evaluated the antimicrobial concentration dependency of biofilm killing using the Chick-Watson mathematical model. All the statistical analysis was also carried out by the author. In summary, the author performed all laboratorial work and associated results treatment and analysis, and the literature research and review, under the guidance of her supervisors.

1.3 Organization of the dissertation

This work is organized into seven chapters, including this introductory chapter.

Chapter 1: Introduction. This segment is dedicated to the description of the addressed problem and the work that was performed.

Chapter 2: Context and State of the art. This section briefly describes the definition of HCAI and explains how bacteria that have developed resistance to frequently used disinfectants may cause infections. Disinfection processes are also defined and characterized, describing the requirements needed for each type of medical equipment. Following, an overview on biofilm development and the mechanisms through which bacteria become resistant to disinfection. Lastly, this chapter provides a brief overview of eco-friendly alternatives to common disinfectants, that are known to be harmful to both the environment and our health, focusing on Natural Deep Eutectic Solvents (NADES) with the potential to control or prevent biofilm development.

Chapter 3: Materials and Methods. This chapter contains information about the reagents and materials used throughout the project, as well as an explanation of the procedures used to generate and analyze the results obtained.

Chapter 4: Results and Discussion. This chapter assesses the work's primary highlights while also allowing for more reflection on which of the work's aims were accomplished, providing the obtained results about the potential use of NADES as disinfectants able to control the biofilms pre-established on surfaces.

Chapter 5: Conclusions. This chapter assesses the work's primary highlights while also allowing for more reflection on which of the work's aims were accomplished, providing the conclusions about the potential use of NADES as disinfectants able to control the biofilms pre-established on surfaces.

Chapter 6: Assessment of work done. As stated in the preceding chapter, a reflection of the project completed is made. A summary of future work that could be developed to complete the presented results and improve conclusions is also provided.

Chapter 7: References. This last section contains all of the bibliography used throughout the work.

2 Context and State of the art

2.1 Introduction

The Center for Disease Control and Prevention (CDC) defines hospital-acquired infections (also known as Healthcare-Associated Infections (HCAI) or nosocomial infections) as infections acquired by patients within the first 48 hours of their stay in a hospital or other type of healthcare facility [1]. These HCAI can occur as a direct result of healthcare interventions such as medical or surgical treatment, as well as the contact with the materials involved in these procedures, or as a consequence from the interaction with a healthcare facility [2]. Urinary tract infections, pneumonia, surgical site infections, bloodstream infections, and gastrointestinal infections are the most prevalent forms of HCAI [3,4].

These infections continue to be a major cause of patient morbidity and mortality. The European Centre for Disease Control (ECDC) estimates that 3.8 million people in acute care hospitals in European countries contract a HCAI each year, and nearly 90 000 people in the EU die each year from the most common HCAI in health care settings [5]. According to Siani & Maillard [6], the most often observed bacteria in HCAI are *Escherichia coli* (15.9%), *Staphylococcus aureus* (12.3%), *Enterococcus* spp. (9.6%), *Pseudomonas aeruginosa* (8.9%), *Klebsiella* spp. (8.7%) and coagulase-negative staphylococci (7.5%).

At least 20% of HCAI is thought to be preventable with better strategies for infection prevention and control [5]. Considering disinfection of surfaces and materials is one of the most straightforward ways to prevent cross-infection, it is critical to pay attention to this issue. Therefore, this study focuses on traditional disinfection methods and seeks a more environmentally friendly alternative to the currently used disinfectants, as the vast majority of them are harmful to the environment and their overuse leads to bacterial resistance.

2.2 Disinfection

Disinfection is the reduction of microorganisms to a previously determined acceptable level due to antimicrobial action. This definition is purposefully broad in order to include a wide range of applications such as medical/surgical instrument cleaning, liquid/gas treatment and general surface disinfection [7]. There are two types of disinfection methods: physical or chemical. The first one involves, for example, the use of heat and/or radiation, while the second relies on the use of biocides, such as alcohols, aldehydes, and quaternary ammonium compounds [8].

In 1957, a system known as Spaulding was developed to assist health care professionals in determining the best strategy for reducing patient risks safely. This system categorizes Medical Devices (MD) based on their intended usage and the respective level of disinfection required [9]. MD are divided into three categories using this system: non-critical, semi-critical and critical. **Non-critical MD** (like environmental surfaces and ultrasound probes) presents a lower risk to patients as they only come into contact with healthy, undamaged skin and thus only require a Low-Level Disinfection (LLD). **Semi-Critical MD** (such as intracavitary ultrasound probes) contact mucous membranes or non-intact skin and therefore require High-Level Disinfection (HLD). **Critical MD** (*e.g.,* probes used in surgery or vascular ablation) comes into contact with sterile bodily cavities or tissue, posing a higher risk to patients, hence requiring them to be sterilized [10–12]. The different levels of disinfection, as well as the types of microorganisms that they affect, are shown in *Table 1*. It is important to note that sterilization is different from disinfection since sterilization is defined as a process used to make a surface or product free of viable microorganisms, including bacterial spores. A sterile device is absent of live organisms, whereas disinfected devices can only be assumed to have a safe and lower microbial counts [7].

***Table 1**: Types of microorganisms affected by the different levels of disinfection. Adapted from William et al [13].*

Disinfection Levels	*Spores*	*Non-enveloped Virus*	*Fungi*	*Mycobaceria*	*Bacteria*	*Enveloped Virus*
Sterilization	✓	✓	✓	✓	✓	✓
HLD	*Some*	✓	✓	✓	✓	✓
LLD	×	*Some*	*Some*	×	✓	✓

✓: the microorganism is affected by the level of disinfection
×: the microorganism is not affected by the level of disinfection

The present study is focused on new green alternatives for surfaces and medical equipment disinfection in health care facilities, *i.e.,* for LLD and HLD purposes.

2.2.1 Traditional Healthcare Equipment and Surface Disinfection

The efficiency of surface disinfection is mainly dependent on two aspects: the disinfectant used, and the technique applied. The perfect product for health care disinfection has yet to be discovered, nonetheless there is a large spectrum of outstanding disinfectants that offer a variety of qualities. William *et al.*[14], examined the properties of an ideal disinfectant, which included: broad spectrum activity; fast acting; easy to use; economical; stability and cleaner.

Understanding the mechanisms of antibacterial activity of disinfectants has made considerable progress, however this topic has not been extensively studied. Despite that, the disinfectants' action is likely to be caused by a consistent sequence of events, regardless of the microbial entity. This may be foreseen as the disinfectant interacting with the cell surface, then penetrating the cell wall and acting at the target site [15]. There are a wide variety of biocides used for disinfection, which may be divided into various categories depending on the functional group they possess or the target on which they act. When it comes to functional groups, they are classified into at least 22 categories, as described in *Figure 1*, however they are broadly divided into four groups based on their target of action: proteins, membranes, nucleic acids and cell walls [16].

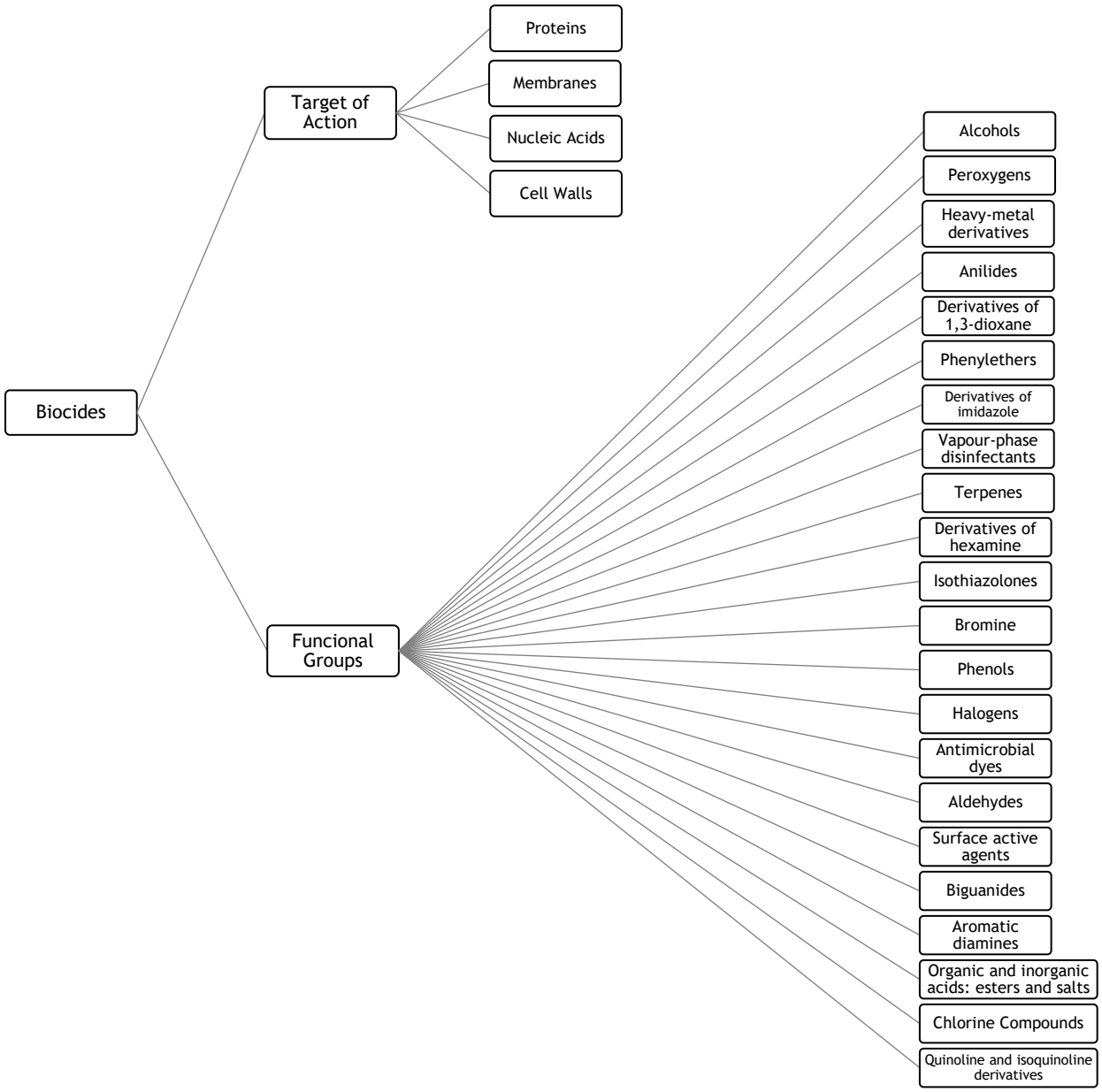


***Figure 1**: Classes of biocides based on the target of action and functional groups [16].*

On the other hand, bacterial tolerance to disinfectants may be developed as a result of frequent exposure to these substances. Despite the lack of research in comparison to antibiotic resistance, disinfectant tolerance is a significant clinical issue since these compounds play a critical role in preventing hospital infections [17,18].

2.2.2 Resistance

Russel [17] defined that a strain is resistant if it is insusceptible to a concentration of disinfectant that is used in practice or if it is not inactivated (or inhibited) by a concentration of disinfectant that inactivates (or inhibits) the majority of that organism's strains. Depending on how bacteria resist to antimicrobial agents, antimicrobial resistance may be divided into two broad groups: natural and acquired resistance [19,20]. The first one can either be **intrinsic** (*i.e.*, always present in the species) and is considered as a feature that is conserved across all species and is independent of past antimicrobial agents' exposure; or **induced** (the genes occur naturally in the bacteria but are only expressed to resistance levels after exposure to an antimicrobial substance) [21]. Acquired resistance can either be temporary or permanent and stands for the bacteria's acquisition of any genetic materials (through horizontal gene transfer) or mutations in its chromosomal DNA [22,23].

Bacterial resistance has been developed in microorganisms by different mechanisms of action, as described in *Figure 2*: (1) chemical modification of the drug; (2) making it inactive by physically removing it from the cell (efflux pumps); (3) by causing the modification of the target site such that the antimicrobial agent does not recognize it; (4) by decreasing the permeability of the membrane that surrounds the bacterial cell; (5) drug lysis/inactivation by hydrolytic enzymes; (6) production of alternative enzymes that can be used instead of the antibiotic-inhibited enzymes [24-26]. Another recognized defense against antimicrobial agents is the formation of biofilms, which aids in the growth and spread of microorganisms, leading to persistent infections.

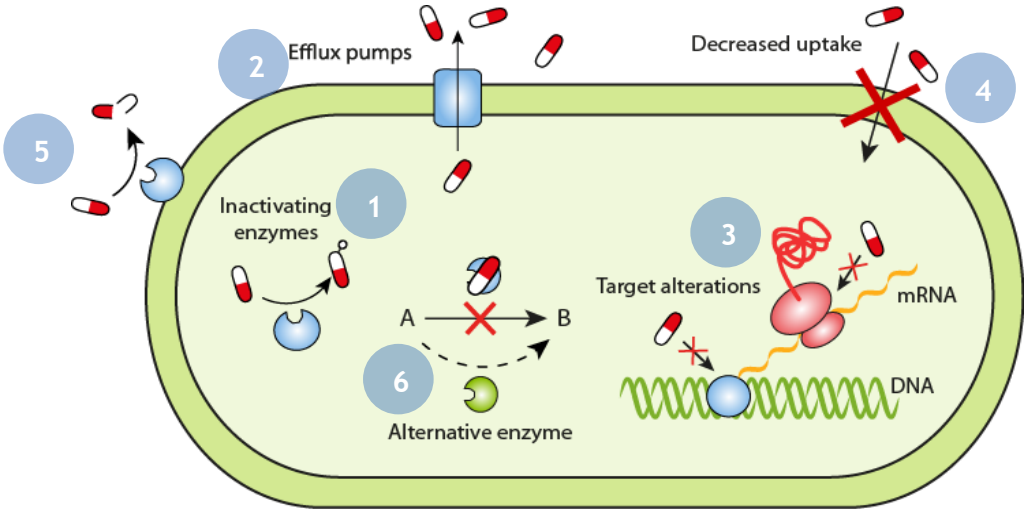


***Figure 2**: Scheme of the main strategies developed for bacteria resistance [26].*

2.2.3 Biofilms

Microorganisms are classified as planktonic primarily based on their growth characteristics, however, it is now well known that 99% of all microorganisms adhere to surfaces and grow as biofilms, as it is an overarching strategy for their survival [27].

Biofilms are surface-attached clusters of microbial cells, with increased tolerance to antimicrobial action that are embedded in a self-produced extracellular matrix, known as Extracellular Polymeric Substance (EPS). EPS benefits the microorganisms in biofilms by preserving the biofilms' structure and protecting microorganisms from aggressive agents [28]. Biofilms may adhere to a variety of surfaces, including metals, polymers, plant and body tissue, MD, implant materials, among others [29]. Bacterial motility, higher shear pressures, and hydrodynamic and electrostatic interactions between the microbe and the surface are significant factors that affect microbial adherence to (bio)materials [30].

The formation of biofilms by microorganisms represents a basic survival mechanism in which the organisms are protected from environmental conditions such as host immune responses and normal levels of conventional antimicrobial agents [31]. Biofilms have a wide range of properties, which play a significant role in their survival strategy. A few of them are the presence of one or more microbial species, adherence to each other and to surfaces and three-dimensional structure [32].

Biofilm development by bacterial pathogens on any substrate/layer follows a five-step process, as presented in *Figure 3*: (1) Attachment: free planktonic cells connect reversibly to biotic or abiotic surfaces by weak interactions such as acid-base, hydrophobic, Van der Waals, and electrostatic forces; (2) Colonization: Bacterial pathogens use stronger connections, including collagen-binding sticky proteins, lipopolysaccharides, flagella, and pili to permanently adhere to the surface; (3) Proliferation: multilayered bacterial cells accumulate significantly, and massive volumes of EPS are generated, creating the biofilm matrix; (4) Maturation: the multilayered bacterial cells connect to form the typical three-dimensional biofilm structure; (5) Dispersion: once fully developed, biofilms are destroyed using mechanical and active methods [33].

The production of EPS that forms the biofilm matrix is an essential step in biofilm growth because it provides vital architectural support and protection for the microbial populations it surrounds, accounting for over 90% of the biofilm mass in most cases. Although the physical and chemical properties of EPS vary by species, it is mostly composed of polysaccharides, proteins, lipids, and extracellular DNA [34]. In a variety of bacterial model systems, EPS production is regulated via Quorum-Sensing (QS) control, which consists of molecular communication between cells controlled by chemical signaling molecules called autoinducers. This allows

bacteria to recognize population density by measuring the accumulation of signaling molecules secreted by community members [35]. Therefore, QS allows bacteria to initiate EPS secretion in the biofilm stage, *i.e.* at high cell density, where it provides a competitive advantage, rather than in the planktonic state, when it could be a waste of resources [36].

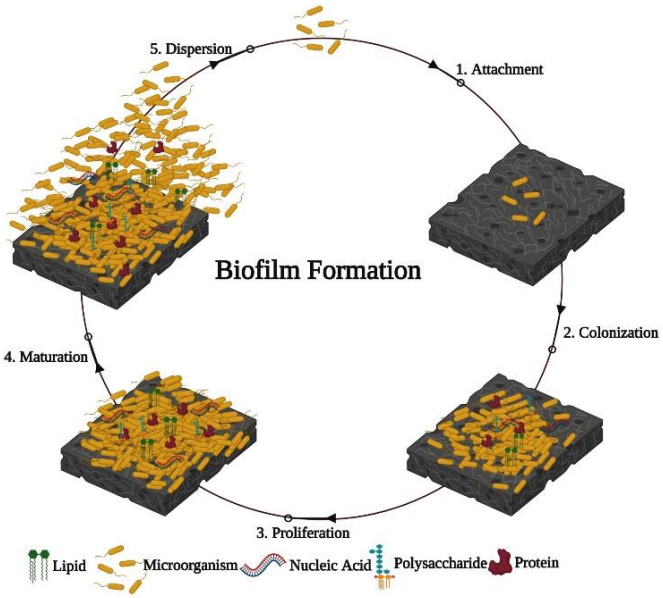


***Figure 3**: The stages of development involved in bacterial biofilm production [33].*

The mechanism through which biofilms develop resistance is not fully understood, however some theories suggest that EPS acts as a polymer that interacts with antimicrobials, inhibiting their activity before it reaches the cells embedded in the matrix. EPS can bind to antimicrobials, slowing their diffusion or chemically react with them, rendering them ineffective [37]. Due to all of these characteristics of biofilms, biofilm-forming bacteria are difficult to destroy with traditional antimicrobial agents, resulting in an urgent need for innovative and effective approaches.

2.3 Eco-friendly Alternatives

Aside from bacterial resistance to commonly used disinfectants, the growing concern for the preservation of the environment places green chemistry and green solvents in the spotlight. Among the several issues that green chemistry focuses on, the most important is the development of new green solvents, particularly to replace the common organic solvents that are toxic, carcinogenic and can cause air and water pollution as well as land contamination [38,39]. In this regard, ionic liquids (ILs) and deep eutectic solvents (DES) have received considerable attention over past few years.

ILs are organic salt mixtures with a low melting point, often below 100 ºC, composed of organic cations and organic or inorganic anions [40,41]. Great chemical and thermal stability, high selectivity, and a low vapor pressure are just a few of the qualities of these liquids [40]. The latter is the primary driver of increased interest in these liquids as green solvents [38]. However, the low biodegradability and biocompatibility of ILs limit their potential as ecologically acceptable solvents [42].

DES are an alternative to ILs, which consist of a combination of two or more solid organic compounds, with a melting point that is lower than the melting point of each individual component due to the formation of intermolecular hydrogen bonds [41]. DES are similar to ILs in terms of physical qualities, but they offer advantages such as being less poisonous, simpler to produce with high purity at a lower cost, and biodegradable [43].

2.3.1 Natural Deep Eutectic Solvents

Recently, Dai *et al*. [38] discovered a significant number of stable Natural Deep Eutectic Solvents (NADES) based on plant-abundant primary metabolites such as organic acids, sugars, and amino acids. In fact, Choi *et al*. [44] observed that the compounds capable of forming NADES are so abundant in living cells that it is illogical to consider them as only intermediates in metabolic pathways. Therefore, they hypothesized that these compounds formed a third liquid medium, in addition to water and lipids [45]. This finding was able to address fundamental biological issues about how organisms cope with non-water or lipid soluble compounds, as well as how they endure harsh environments like drought or cold [46].

NADES, parallel to DES, are mixtures of Hydrogen Bond Acceptors (HBAs, usually ammonium salts, such as choline chloride) and Hydrogen Bond Donors (HBDs, such as urea and lactic acid) with melting points lower than any of their separate individual components [47]. The interaction between HBDs and HBAs is the major contributor to the melting point depression, however the type and asymmetry of organic salts, as well as charge delocalization generated by hydrogen bonding, can also be significant factors [48]. NADES can be prepared using one of three methods: thermal mixing (the components are mixed at a high temperature (about 80 ºC) until a colorless clear liquid is formed), vacuum evaporation (the components are evaporated at 50ºC with a rotary evaporator, after being dissolved in water) or freeze drying (the components are dissolved in water before being freeze dried) [47].

Despite the fact that NADES were only recently discovered, they already have a wide range of biotechnology applications. They are used as an extraction medium, as a chromatographic media [49], for biocatalysis, for example, to enhance the activity and stability of enzymes [47], and can also be used as a replacement for organic solvents in biological assays [49]. Furthermore, antimicrobial properties of NADES against planktonic bacteria have been

reported by many authors, as described in *Table 2*. Nevertheless, research on this topic continues to grow, as there has been no extensive investigation into its efficacy against biofilms.

In light of this, the aim of this project is to evaluate the antimicrobial activity of Choline Chloride-based NADES against *E. coli* and *S. aureus* biofilms, as these two species are the most common causes of infections in the health care industry [50,51].

Table 2: *Antimicrobial activity of NADES reported in the literature.*

NADES	*Molar Ratio*	*Microorganism*	*Antimicrobial Activity*	*Ref.*
ChCl : p-toluenesulfonic acid	1:1	*E. coli*	MIC (mM) = 18	[52]
		S. enteritidis	MIC (mM) = 26	
		S. aureus	MIC (mM) = 18	
		L. monocytogenes	MIC (mM) = 30	
ChCl : Oxalic acid	1:1	*E. coli*	MIC (mM) = 12	[52,53]
			d (mm) = 47 ± 2	
		S. enteritidis	MIC (mM) = 12	
		S. aureus	MIC (mM) = 12	
			d (mm) = 73 ± 3	
		L. monocytogenes	MIC (mM) = 14	
		P. mirabilis	d (mm) = 49 ± 1	
		S. typhimurium	d (mm) = 45 ± 4	
		P. aeruginosa	d (mm) = 50 ± 2	
ChCl : Levulinic acid	1:2	*E. coli*	MIC (mM) = 12	[52]
		S. enteritidis	MIC (mM) = 12	
		S. aureus	MIC (mM) = 14	
		L. monocytogenes	MIC (mM) = 12	
ChCl : Malonic acid	1:1	*E. coli*	MIC (mM) = 18	
		S. enteritidis	MIC (mM) = 20	
		S. aureus	MIC (mM) = 16	
		L. monocytogenes	MIC (mM) = 24	

ChCl : Malic acid	**1:1**	*E. coli*	MIC (mM) = 14	52
		S. enteritidis	MIC (mM) = 18	
		S. aureus	MIC (mM) = 14	
		L. monocytogenes	MIC (mM) = 22	
ChCl : Citric acid	1:1	*E. coli*	MIC (mM) = 12	52,54
			MIC (%) = 0.78	
		S. enteritidis	MIC (mM) = 16	
		S. aureus	MIC (mM) = 12	
			MIC (%) = 0.39	
		L. monocytogenes	MIC (mM) = 20	
		P. aeruginosa	MIC (%) = 0.39	
		S. enterica	MIC (%) = 0.78	
ChCl : Tartaric acid	2:1	*E. coli*	MIC (mM) = 14	52
		S. enteritidis	MIC (mM) = 18	
		S. aureus	MIC (mM) = 12	
		L. monocytogenes	MIC (mM) = 16	
ChCl : Glycerol	1:2	*S. pyogenes*	MIC (mM) = 0.38	55
		E. coli	MIC (mM) = 0.05	
		S. aureus	MIC (mM) = NA	
		P. aeruginosa	MIC (mM) = 0.24	
		C. albicans	MIC (mM) = 0.24	
		B. cereus	MIC (mM) = 0.24	
Citric Acid : 1,2-Propanediol	1:4	*S. pyogenes*	MIC (mM) = 0.00019	
		E. coli	MIC (mM) = 0.00039	
		S. aureus	MIC (mM) = 0.0001	
		P. aeruginosa	MIC (mM) = NA	
		C. albicans	MIC (mM) = 0.00019	
		B. cereus	MIC (mM) = NA	

Betaine : Malic acid : Glucose	**1:1:1**	*E. coli*	d (mm) = 44 ± 2	[53]
		P. mirabilis	d (mm) = 62 ± 3	
		S. typhimurium	d (mm) = 24 ± 3	
		P. aeruginosa	d (mm) = 45 ± 3	
ChCl : Fructose	1:1	*E. coli*	MIC (%) = 25	[54]
		S. aureus	MIC (%) = 25	
		P. aeruginosa	MIC (%) = 25	
		S. enterica	MIC (%) = 25	
	1:2	*E. coli*	MIC (%) = 25	
		S. aureus	MIC (%) = 25	
		P. aeruginosa	MIC (%) = 25	
		S. enterica	MIC (%) = 25	
ChCl : Acetamide	1:1	*E. coli*	EC_{50} (mM) = 275.2	[56]
ChAc : Ethylene glycol	1:1	*E. coli*	EC_{50} (mM) = 281.1	

E. coli – Escherichia coli; S. enteritidis – Salmonella enteritidis; S. aureus – Staphylococcus aureus; L. monocytogenes – Listeria monocytogenes; P. mirabilis – Proteus mirabilis; S. typhimurium – Salmonella typhimuruim; P. aeruginosa – Pseudomonas aeruginosa; S. enterica – Salmonella enterica; S. pyogenes – Streptococcus pyogenes; C. albicans – Candida albicans; B. cereus – Bacillus cereus; MIC – Minimum Inhibitory Concentration; d – Growth inhibition zone diameter; EC_{50} – Half maximal effective concentration; ChCl – Choline Chloride; ChAc – Choline Acetate.

3 Materials and Methods

3.1 Chemicals

Choline Chloride ($C_5H_{14}ClNO$, CAS [67-48-1], ≥ 98%, white crystalline powder) and Lactic Acid ($C_3H_6O_3$, CAS [50-21-5], 90%, clear colorless to yellow liquid) were both purchased from VWR International, LLC. Citric Acid monohydrate ($C_6H_8O_7.H_2O$, CAS [5949-29-1], < 99%) and Xylitol ($C_5H_{12}O_5$, CAS [87-99-0], > 98%) were purchased from Scharlau and Tokyo Chemical Industry, respectively. Sucrose ($C_{12}H_{22}O_{11}$, CAS [57-50-1]), 1,2 Propanediol ($C_3H_8O_2$, CAS [57-55-6]) and Urea ($CO(NH_2)_2$, CAS [57-13-6]) were all purchased from Merck KGaA (all with purity > 99%). As choline chloride is a hygroscopic material, it was placed in a glass desiccator with silica gel prior to use, to remove any moisture content.

3.2 Preparation of NADES

NADES were synthesized using the thermal mixing method by mixing choline chloride and HBDs in glass vials at predetermined molar ratios. In a water bath, the mixtures were heated to 80 ºC and manually stirred until a clear, homogenous liquid was formed (for about 60 min to 90 min). All prepared NADES were allowed to cool to room temperature and are summarized in *Table 3*, present in *Section 4*. To achieve the concentrations used for biofilm treatment assays, all dilutions were made in Phosphate-Buffered Saline, PBS (8 $g{\cdot}L^{-1}$ of NaCl, 0.2 $g{\cdot}L^{-1}$ of KCl, 1.44 $g{\cdot}L^{-1}$ of Na_2HPO_4 and 0.24 $g{\cdot}L^{-1}$ of KH_2PO_4 at pH 7.4, all purchased from VWR International, LLC).

3.3 Computational Analysis of NADES Components

The COSMO-RS [57] (Conductor-like Screening Model for Real Solvents) developed by Nick Austin, was used to estimate the properties of the NADES' individual components. For each component, it was determined the molar mass, density, melting point, and enthalpy of fusion.

3.4 Bacterial Strains and Culture Conditions

The antimicrobial activity was evaluated using *Escherichia coli* CECT 434 and *Staphylococcus aureus* CECT 976 obtained from the Spanish Type Culture Collection. Both bacterial strains were cultured overnight at 37 ºC and 100 rpm (New Brunswick Scientific I26 shaker), in 250 mL flasks containing 100 mL of sterile Mueller-Hinton Broth (MHB, purchased from VWR International, LLC).

3.5 Biofilm Formation

The bacteria grown as described in *Section 3.4* were centrifuged (Eppendorf centrifuge 5810R) at 3700 g for 10 min, washed and resuspended twice in sterile MHB. Afterwards, the number of cells in suspension was adjusted to roughly 1×10^8 colony-forming units per milliliter (CFU·mL^{-1}). Then 200 µL of the prepared bacteria suspension was added to each well of a 96 wells microtiter plate, to form single-species biofilms. Filled microtiter plates were incubated at 37 ºC and 100 rpm for 24 h. Multi-species biofilms were produced in the same way as single-species biofilms, with the exception that 100 µL of each bacterial suspension was added to each well. Negative controls were obtained by incubating the wells only with nutrient medium, without the addition of any bacteria.

3.6 Biofilm Treatment

After the incubation period, all wells were washed with 200 µL of PBS to remove all non-adherent and weakly adhered bacteria. Biofilms were then exposed, for 30 min, to 200 µL of the different concentrations of the selected NADES (1, 10, 50, 100, 250 and 500 mg·mL^{-1}). After the exposure time, the antimicrobial agent was discarded and a neutralization test took place, by adding 200 µL of a Universal Neutralizer consisting of 1 g·L^{-1} α-histidine (Merck KGaA), 3 g·L^{-1} lecithin (Thermofisher), 5 g·L^{-1} sodium thiosulphate (Labkem), 30 g·L^{-1} saponin (VWR International, LLC), 30 g·L^{-1} tween 80 (VWR, International, LLC), in phosphate buffer 0.0025 M. After 15 min, the neutralizer was discarded, and all wells were washed with PBS to remove any non or weakly-attached bacteria. The positive control (untreated cells) was carried out using PBS instead of the antimicrobial agent.

3.6.1 Biofilm Culturability Reduction

The number of CFU per cm^2 was calculated to assess the culturability reduction of biofilm cells produced by each NADES. Following the processes outlined in *Section 3.6*, biofilms from each well were scrapped for 1 min with the addition of 250 µL of PBS to re-suspend the adhering sessile cells and transferred to microcentrifuge tubes. This procedure was repeated twice, to ensure that all biofilm cells were removed. Each microcentrifuge tube was then filled with 500 µL of PBS and serial dilutions of the biofilm suspension were prepared in sterile saline solution (8 g·L^{-1} NaCl, VWR International, LLC) until 10^{-5}. The number of CFUs was determined using the drop plate method. For both *E. coli* and *S. aureus*, the growth medium used was Plate Count Agar (PCA, VWR International, LLC), and the plates were incubated for 24 h at 37 ºC for cell count. The number of biofilm colonies was evaluated as log CFU·cm^{-2}. For multi-species biofilms, the number of colonies of *E. coli* and *S. aureus* was counted separately since the colonies from each species are distinct and are easily distinguished. Three independent assays were performed with duplicates.

3.6.2 Biofilm Removal Quantification

Crystal Violet (CV) staining was used to quantify the amount of biofilm removed. CV is a basic dye that binds to negatively charged surface molecules and polysaccharides in the extracellular matrix in both live and dead cells, thereby being well suited to quantify total biofilm mass. For this procedure, after the steps mentioned in *Section 3.6*, the remaining attached bacteria were fixed with 250 µL per well of absolute ethanol (VWR International, LLC) for 15 min. Then, the ethanol was discarded, and the plates were dried for 5 min at room temperature. Afterwards, the fixed bacteria were stained with 200 µL per well of 1 % (v/v) CV (Merck KGaA), and after 5 min, the excess stain was removed. Lastly, 200 µL per well of 33 % (v/v) of acetic acid (Fisher Scientific UK) was used to dissolve the dye bound to the adhering biomass. The absorbance was then measured at 570 nm, using a microplate reader (Spectrostar Nano, BMG Labtech). The percentage of biofilm removal was given by Equation 1, where %BR is the percentage of biofilm removal, A_C indicates the A_{570nm} value for the positive control wells and A_X indicates the A_{570nm} value for biofilm exposed to each antimicrobial agent. At least three independent assays were performed with triplicates.

$$\%BR = \frac{A_C - A_X}{A_C} \times 100 \quad \text{(Eq.1)}$$

3.7 Dose-dependence of Antimicrobial Activity

The Chick-Watson mathematical model of disinfection was assumed to evaluate the antimicrobial concentration dependency of biofilm killing [58]. This model is represented by Equation 2.

$$\frac{dX}{dt} = k_{dis} C^n t \quad \text{(Eq.2)}$$

The solution to this model is found by integration, assuming a constant biocide concentration, as shown in Equation 3.

$$\ln\left(\frac{X}{X_0}\right) = -k_{dis} C^n t \quad \text{(Eq.3)}$$

Where X_0 and X are the count of CFU·cm^{-2} before and after biocidal exposure; k_{dis} is a disinfection rate coefficient ((mL·mg^{-1})^n·min^{-1}); C is the biocide concentration (mg·mL^{-1}); n is a concentration exponent and t is the exposure time (min). The concentration dependence of killing (n) was determined using a toolbox for linear regression of GraphPad Prism 9.3 for Windows 10 (GraphPad Software, La Jolla California, USA) and the disinfectant rate coefficient (k_{dis}) was determined by Equation 4, for different NADES concentrations after 30 min of

exposure. The quality of the model was evaluated through the coefficient of determination (R^2).

$$\ln(r) = \ln(k_{dis}) + n \ln(C) \quad \text{(Eq. 4)}$$

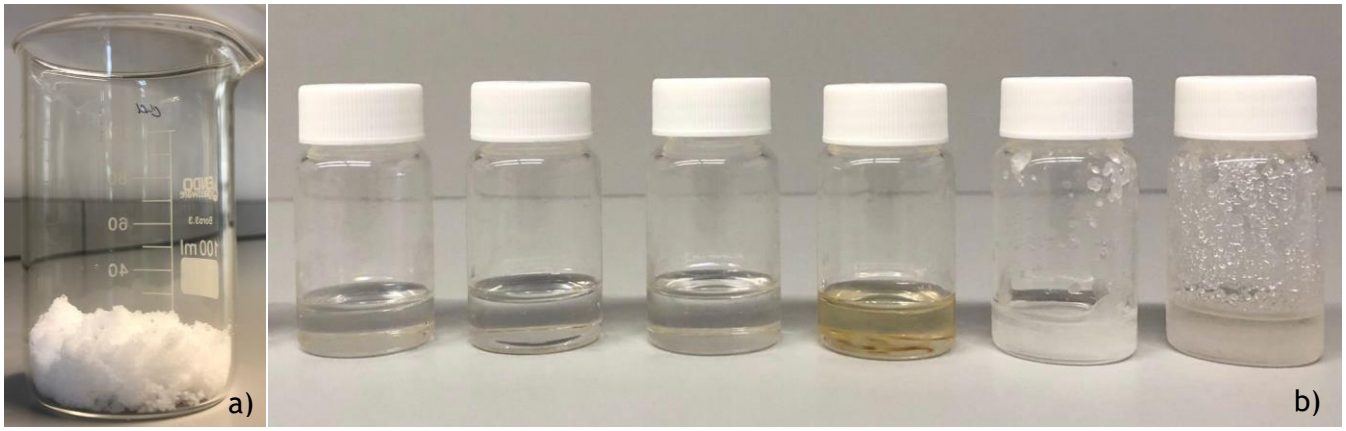
3.8 Statistical Analysis

The statistical software GraphPad Prism 9.3 for Windows 10 (GraphPad Software, La Jolla California, USA) was used to analyze the experimental data. At least three independent experiments in duplicate were conducted for each tested condition. For all cases, the mean and standard deviation (SDs) within samples were calculated. First, the Shapiro-Wilk test was used to determine if the data acquired for each test followed a normal distribution or not, and hence whether they were parametric or non-parametric. Then, for parametric data, the differences between measures were calculated using ANOVA with Tukey's and Dunnett's post hoc tests. For non-parametric data, the measurements were analyzed using the Kruskal-Wallis test with Dunn's post test. Statistical computations were based on a 95 % confidence level for all tests ($p < 0.05$ was deemed statistically significant).

4 Results and discussion

4.1 NADES production and stability

In this study, multiple NADES were prepared from readily available compounds. The produced NADES were selected for their potential antimicrobial effects described in the literature. NADES are recognized to have melting points lower than any of their separate individual components, due to the interaction between HBAs and HBDs. Therefore, NADES are liquids at room temperature or even at lower temperatures [49]. *Figure 4a)* presents the HBA (choline chloride) in its solid form at room temperature. Choline chloride was used as HBA in the production of all selected NADES. All the produced NADES are shown in *Figure 4b)*, and, as expected they all prove to be liquid at room temperature, with the exception of ChCl:XY2 and ChCl:SU that crystallized. The molecular structures of the individual components of the NADES produced are present in *Figure 4c)*. *Table* 3 presents properties of the produced NADES: their molar ratios and their appearance immediately after the heating process and after 24 h.



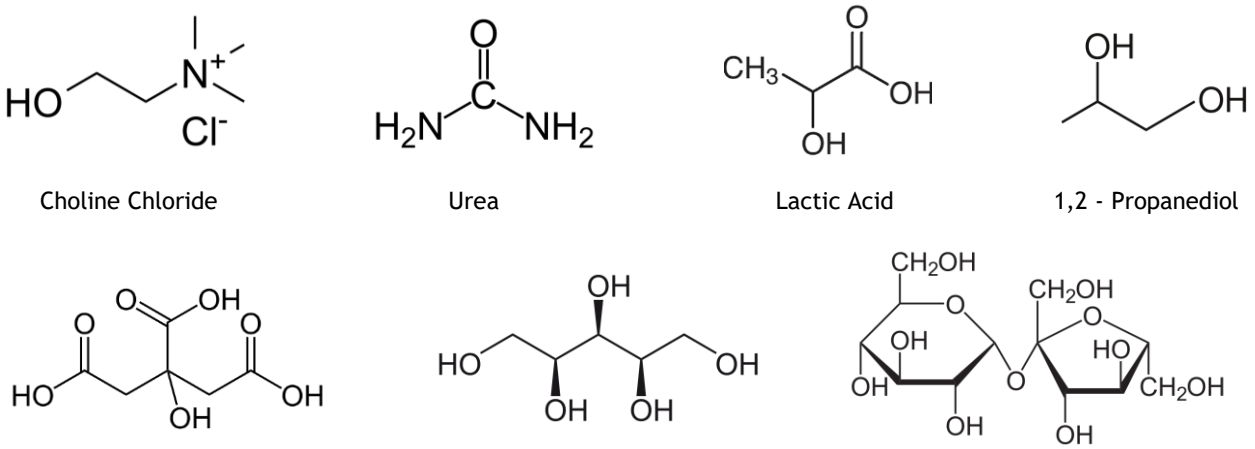


***Figure 4**: a) Aspect of choline chloride at room temperature; b) Aspect of produced NADES 24 h post-production. From left to right: ChCl:UR; ChCl:LA; ChCl:PP; ChCl:CA; ChCl:XY2; ChCl:SU; c) Molecular structures of the individual components of the NADES production* [59-65].

Table 3: NADES produced and their respective molar ratio and appearance immediately after synthesis and 24 h later.

NADES					Observation		
HBA	HBD	Abbreviation	Molar Ratio	Water Content (%)	After Heating	After 24h	Ref.
Choline Chloride	Lactic Acid	ChCl:LA	1:2	6.67	Clear Liquid	Clear Liquid	[66]
	Citric Acid	ChCl:CA	1:1	33.3	Ligh Yellow Liquid	Ligh Yellow Liquid	[67]
	1,2-Propanediol	ChCl:PP	1:2	-	Clear Liquid	Clear Liquid	[68]
	Urea	ChCl:UR	1:1	33.3	Clear Liquid	Clear Liquid	[69]
	Xylitol	ChCl:XY1	1:1	-	Clear Liquid	Crystallized	[52]
		ChCl:XY2	1:2	-	Clear Liquid	Crystallized	[70]
	Sucrose	ChCl:SU	1:1	-	Crystallized	Crystallized	[38]

The COSMO-RS [57] (Conductor-like Screening Model for Real Solvents) was employed to estimate certain properties of the NADES' individual components. This thermodynamic method is based on quantum chemistry and it is often used to predict several features of fluids, such as chemical potential, screening charges, activity coefficients, solubility and partition coefficients [57]. In the scope of this work, the molar mass, density, melting point, and enthalpy of fusion for each component were determined. The values estimated are present in *Table A.1*, present in the appendix section.

Following the production of the NADES, PBS dilutions were prepared for the experimental research. Test concentrations of 1, 10, 50, 100, 250 and 500 $mg \cdot mL^{-1}$ were selected since they cover a wide range of concentrations and could provide a better understanding of NADES antimicrobial effects.

Taking into consideration the quaternary ammonium compounds (QACs), *e.g.*, benzalkonium chloride, the usual concentration of these biocides used as disinfectants for hospital use is typically between 400 and 500 ppm and almost always below 1000 ppm [71]. This corresponds to a concentration between 0.4 and 0.5 $mg \cdot mL^{-1}$, which is much lower than the concentrations tested in the present study. In fact, QAC-bearing waste and wastewater originate from domestic, medical, and industrial use of QACs. The majority of trace pollutants, including QACs, pass through wastewater treatment facilities and are discharged into the environment because standard wastewater treatment plants are built to remove large, easily degradable organics [72]. Although choline chloride is a QAC, it has been proven to be biodegradable and non-toxic [73]. According to the literature, a good correlation between biodegradability and toxicity was found for choline-based NADES, suggesting that they have a

green profile potential [74]. Since all of these solvents are made from natural ingredients, it may not be a problem to use concentrations that are much higher than what is usually used for biocides.

The NADES stability is related to the structures of the precursors and their molar ratio [75] and it was assessed by the occurrence of crystallization of the synthesized solvents. As described in *Table 3*, when ChCl:LA, ChCl:CA, ChCl:PP and ChCl:UR were formulated, they presented as clear homogeneous liquids that remained clear after 24 h, suggesting they are stable. Despite that, ChCl:CA presented a light-yellow color. This observation was also reported by Abbot *et al.*[67], who synthesized NADES with different molar ratios of choline chloride and citric acid, and did not find residues or crystals during a Polarized Light Microscopy (POM) analysis.

When it comes to NADES that contain xylitol, on a first try, ChCl:XY1 was formulated. After 24 h, crystallization was observed so a second attempt was made, adjusting the molar ratio (ChCL:XY2), yet the same phenomena occurred. Moreover, during the synthesis of ChCl:SU, a clear liquid was not even obtained. These events can be explained according to Dai *et al.*[76], who tested the stability of mixtures of sugars-choline chloride with different molar ratios. The authors reported the appearance of a solid precipitate, which might be explained because one chloride ion from choline chloride may form two hydrogen bonds with two hydroxyl groups from sugars, acting similarly to a choline chloride and carboxylic acid interaction.

Attending the stability, the NADES selected to carry out further work in controlling bacterial biofilms were ChCl:LA, ChCl:CA, ChCl:PP and ChCl:UR.

4.2 Effect of NADES on the Control of Single-Species Biofilms

4.2.1 Biofilm Culturability Reduction

Pre-established biofilms were treated with different concentrations of selected NADES for 30 min. The antibiofilm properties of NADES were evaluated in terms of the culturability reduction of biofilm cells. For that, the number of CFU per cm^2 was calculated and the number of biofilm colonies was evaluated as log CFU·cm^{-2}. Both bacteria, *E. coli* and *S. aureus*, were capable of forming biofilms, however total biofilm removal was not achieved, independently of the NADES tested and its concentration.

When analyzing the culturability reduction of ChCl:LA and ChCl:CA towards *E. coli* (*Figure 5A* and *Figure 5B)*, it is observed that their antibacterial potential increased for increasing concentrations, achieving a maximum of 2.4 ± 0.6 and 2.7 ± 0.5 log CFU·cm^{-2}

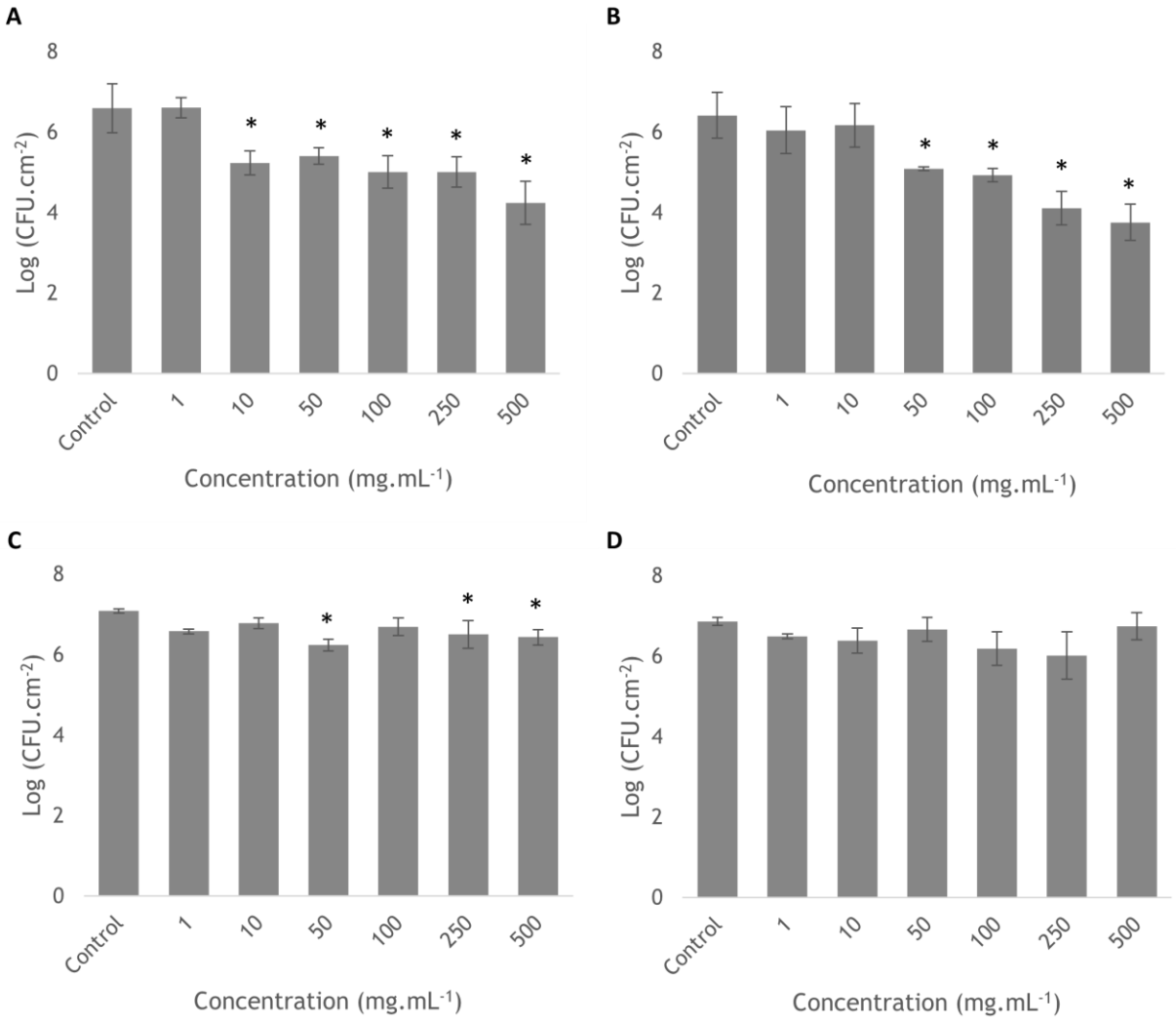


***Figure 5**: Effect of the selected NADES at different concentrations after 30 min of exposure towards E. coli biofilms in terms of biofilm culturability. A - ChCl:LA; B - ChCl:CA; C - ChCl:PP; D - ChCl:UR. * - samples were statistically different from unexposed bacteria (control) ($p < 0.05$, Dunnett's test).*

reduction, respectively. In both cases, no significant differences were found between the control and lower concentrations ($p < 0.05$).

Regarding the tolerance of *E. coli* to ChCl:PP, the obtained results, presented in *Figure 5C*, demonstrated a higher tolerance to this solvent, in comparison to ChCl:LA and ChCl:CA. In contrast to the preceding NADES, a maximum reduction of 0.9 ± 0.2 log CFU·cm^{-2} was obtained at a concentration of 5 mg·mL^{-1}, yet for the highest concentration there was only a reduction of 0.7 ± 0.2 log CFU·cm^{-2} (both were found to be significantly different from the control, *i.e.*, $p < 0.05$). Regarding ChCl:UR (*Figure 5D*), none of the concentrations tested were considered statistically different from the unexposed bacteria ($p > 0.05$).

In contrast to the scenario outlined above, when assessing the culturability reduction of ChCl:LA towards *S. aureus* (*Figure 6A*), a maximum reduction of 3.37 ± 0.31 log CFU·cm^{-2} is detected at 100 mg·mL^{-1} ($p < 0.05$). However, there is no statistically significant differences

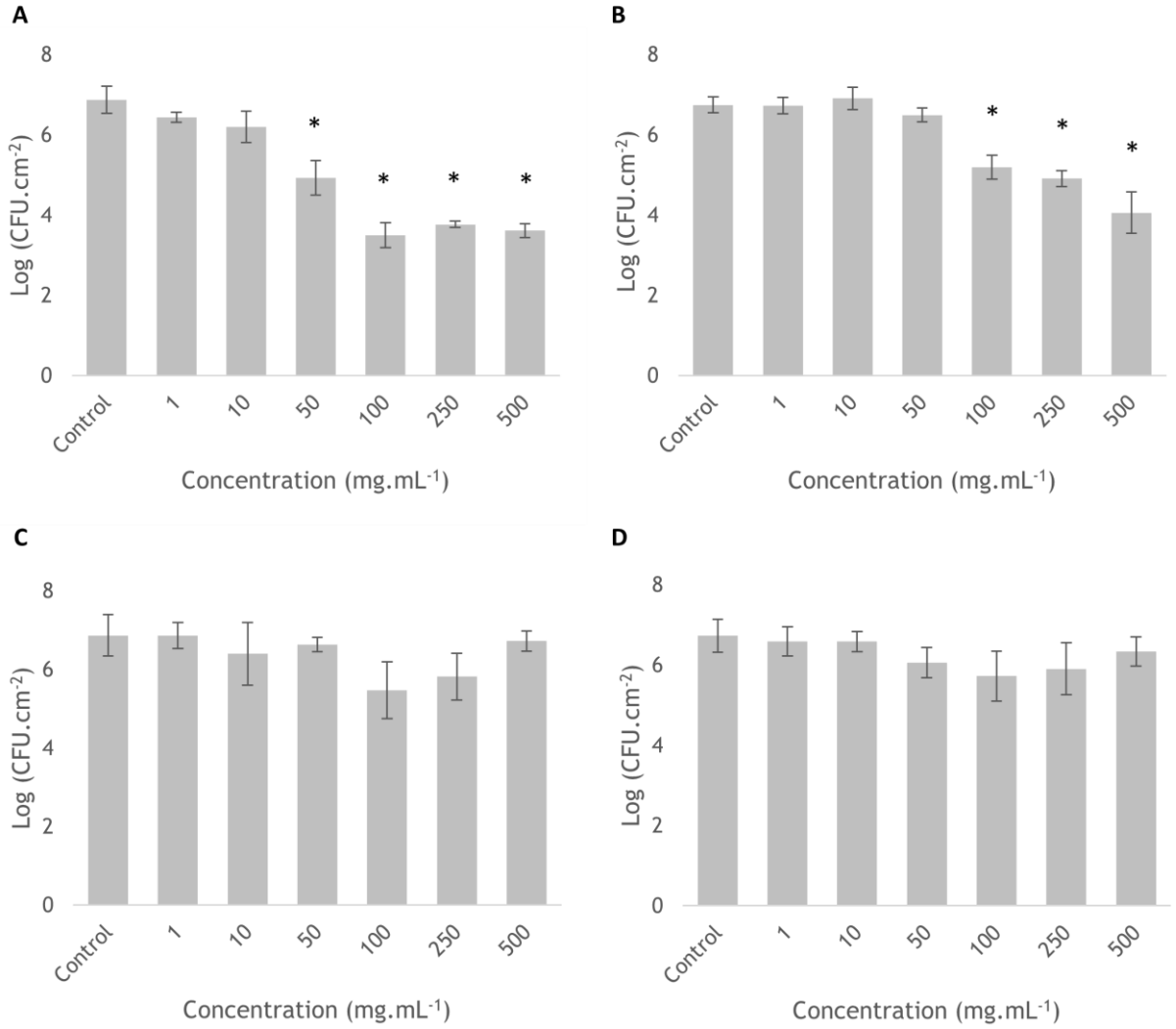


***Figure 6**: Effect of the selected NADES at different concentrations after 30 min of exposure towards S. aureus biofilms in terms of biofilm culturability. A - ChCl:LA; B - ChCl:CA; C - ChCl:PP; D - ChCl:UR. * - samples were statistically different from unexposed bacteria (control) (p < 0.05, Dunnett's test).*

between the higher concentrations: 100, 250 and 500 mg·mL^{-1} ($p > 0.05$, Tukey's test). Therefore, it is possible to observe that *S. aureus* is more susceptible to ChCl:LA than *E. coli*. However, the maximum biofilm inactivation was obtained at 100 mg·mL^{-1} and the increase of ChCl:LA concentration until 500 mg·mL^{-1} is not advantageous in terms of biofilm control.

The culturability reduction of *S. aureus* caused by ChCl:CA *(Figure 6B)* is quite similar to its effect towards *E. coli*, since there is a maximum reduction of 2.58 ± 0.51 log CFU/cm^2 at 500 mg·mL^{-1} ($p < 0.05$).

Tolerance of *S. aureus* to ChCl:PP and ChCl:UR (*Figure 6C* and *Figure 6D*, respectively) is also similar to that observed for *E. coli*. Yet, in this situation, there are no significant differences between the concentrations tested and the control nor between the concentrations themselves, in both ChCl:PP and ChCl:UR ($p > 0.05$).

The data obtained was adjusted to the Chick-Watson model. The values estimated for the biocide concentration dependence parameter are expressed in *Table 4*, as well as the values obtained for the disinfection rate coefficient and the R^2, that translates the adjustability to the model. The figures obtained from the adjustability to the model are presented in *Figure 7*.

***Table 4**: Estimated values of NADES concentration dependence parameter (n), adapted to Chick-Watson Model and the disinfection rate coefficient (k_{dis}) for single-species biofilms. The values are the adjustment to the mean values of three independent assays. Model adjustability was assessed by R^2.*

	E. coli			***S. aureus***		
NADES	$\boldsymbol{n}$	$\boldsymbol{k_{dis}}$ $((mL \cdot mg^{-1})^n \cdot min^{-1})$	$\boldsymbol{R^2}$	$\boldsymbol{n}$	$\boldsymbol{k_{dis}}$ $((mL \cdot mg^{-1})^n \cdot min^{-1})$	$\boldsymbol{R^2}$
ChCl:LA	0.2439	0.0166	0.8880	0.2758	0.0222	0.9035
ChCl:CA	0.3570	0.0099	0.9774	0.4911	0.0041	0.8196
ChCl:PP	0.0512	0.0153	0.1038	0.024	0.0198	0.0063
ChCl:UR	0.0843	0.0128	0.1672	0.1609	0.0093	0.3211

If $n = 1$, the decrease in log CFU·cm^{-2} increased linearly with the NADES concentration; if $n = 2$, the reduction in log CFU·cm^{-2} rose in direct proportion to the NADES square concentration. Accordingly, mathematically, NADES with a high n value have an antimicrobial activity that is greatly influenced by changes in concentration, whereas those with a low n have less of an impact. For both biofilms, the calculated concentration exponent (n) from Chick-Watson model values were < 1 for all NADES tested (*i.e.*, less dose-dependent). Therefore, the antibacterial activity of the NADES tested was not considered to be dose-dependent for both bacteria. However, the values of R^2 obtained for ChCl:PP and ChCl:UR are very low, when

compared to the values obtained for the acidic NADES, indicating that the model does not adjust properly and supporting the negligible antibiofilm activity of these solvents.

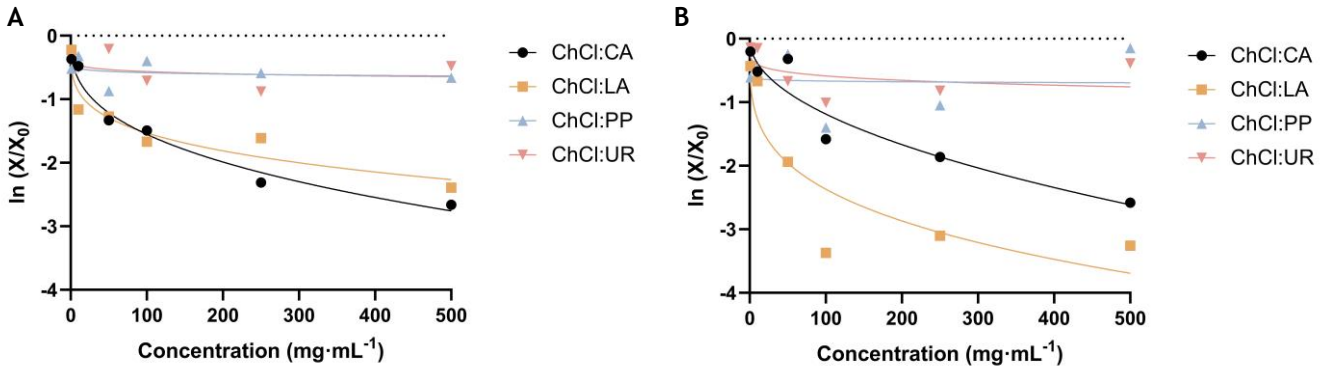


***Figure 7**: Logarithmic reduction of E. coli (A) and S. aureus (B) biofilms, as a function of biocide concentration (mg·mL^{-1}) after 30 min of NADES exposure. The lines represent the Chick-Watson model of experimental data, and the points represent the means for at least three independent assays.*

These findings reveal amide- and alcohol-based NADES did not reduce bacterial growth, while organic acid-based NADES had a considerable inhibitory impact, indicating their potential use as antibacterial agents. This was already reported by Zhao *et al.* [52], when testing the inhibitory effect on planktonic *E. coli* of choline-based NADES with urea and citric acid. In this study, there was no inhibition for the solvent with urea as a component, however for the solvent with citric acid it was obtained a growth inhibition zone diameter of $1.93 \pm 0.13\ cm$. Studies that evaluate the antibiofilm activity of NADES are still scarce in the literature, nevertheless, knowledge of the antibiofilm activity of these solvents is a step forward to obtain consistent results and a more realistic idea of the potential of NADES as disinfectants.

De Morais *et al.* [77], claimed that choline-based NADES will pass through cell membranes and exert their harmful effects in the cytoplasm, which are often attributed to their acid counterparts, changing the pH and anion pool of the cytoplasm and so harming cell viability. The detrimental effect of organic acids used as HBDs was supported by Yang *et al.* [78], when examining the biocompatibility of different choline-based NADES with different bacteria, and only the ones prepared with organic acids exhibited a strong inhibitory effect on bacterial growth, corroborating other studies that proved that acidic HBDs have the ability to inactivate cells by denaturing proteins present on the cell wall, which causes cell death and collapse [79]. Even if choline chloride is nontoxic and approved by the EFSA (European Food Safety Authority), the influence of the hydrogen bonding, between the HBD and the anion of the salt, affects both the chemical structure and physical qualities. Since compounds with delocalized charges are more toxic than those with localized charges, it has been proven that charge delocalization caused by a hydrogen bond makes a combination more poisonous than its separate components.

Delocalized charges have a stronger influence because the hydrogen bond network in acid-based NADES is dense and compact [80,81].

The fundamental distinction between *E. coli* and *S. aureus* is their cell envelope, and they are gram-negative and gram-positive, respectively. Gram-negative bacteria are characterized by a lipopolysaccharide-containing outer membrane that surrounds the peptidoglycan cell wall. Although gram-positive bacteria lack an outer membrane, they are encased in layers of peptidoglycan that are much thicker than those found in gram-negative bacteria [82]. Even though *S. aureus* has a thicker outer layer of peptidoglycan, it was more susceptible to ChCl:LA than *E. coli*, which may be somewhat unexpected, but can be explained by the polysaccharide backbone of peptidoglycan that is formed of equal amounts of alternate N-acetylglucosamine and N-acetylmuramic acid residues, which are cross linked by short peptides. The cholinium cation present in NADES can interact with the polysaccharide or peptide chains through H-bonding or electrostatic contact when is partly dissociated in aqueous solution, which might result in cell wall deformation or rupture [56]. Additionally, the peptidoglycan-based bacterial cell wall is relatively porous and is not thought to represent a permeability barrier for small substrates.

4.2.2 Biofilm Removal Quantification

Biofilm removal quantification was assessed by CV staining assay, which is based on staining biomass (cells and EPS) that compose the biofilm attached to cell culture plates. During the assay, detached cells are washed away, and the remaining attached cells are stained with CV. The results of biofilm removal obtained for *E. coli* and *S. aureus* biofilms are presented in *Table 5* and *Table 6*, respectively.

When analyzing the biofilm removal of ChCl:LA towards *E. coli*, there is a clear increase in biofilm removal with the increase of the NADES concentration, with the exception of 250 $mg{\cdot}mL^{-1}$. The biofilm removal in this concentration suffers a sudden decrease to $16.96 \pm 0.02\%$. There was no explanation found for this phenomenon, since the highest concentration tested translated into the highest percentage of biofilm removal ($74.38 \pm 0.14\%$). However, this fact was observed in each independent assay performed, which demands further investigation into the effects of this compound at different concentrations on biofilm structure and matrix.

Regarding the effect of ChCl:CA towards *E. coli*, a maximum percentage of biofilm removal ($34.36 \pm 0.14\%$) was obtained for 50 $mg{\cdot}mL^{-1}$. The further increase of ChCl:CA concentration did not improve biofilm removal. This indicates that a higher concentration is not advantageous in terms of biofilm removal. When comparing these results to the ones obtained in the culturability reduction assay, it can be noted that the bacterial reduction increased with the increase of the concentration. This corroborates the fact that biofilm

removal and killing are distinct phenomena, which means that the log CFU·cm^{-2} reduction could be due to the presence of dead cells that remained attached to the surface.

***Table 5**: Effect of the selected NADES at different concentrations after 30 min of exposure towards E. coli Biofilms in terms of biofilm removal by CV assay.*

Concentration (mg·mL^{-1})	*Biofilm Removal (%)*			
	ChCl:LA	*ChCl:CA*	*ChCl:PP*	*ChCl:UR*
1	9.34 ± 0.01	31.02 ± 0.10	6.12 ± 0.01	2.43 ± 0.001
10	28.45 ± 0.10	33.44 ± 0.01	6.41 ± 0.06	7.28 ± 0.01
50	41.67 ± 0.08	39.36 ± 0.01	9.84 ± 0.02	11.97 ± 0.01
100	43.33 ± 0.13	35.74 ± 0.12	12.88 ± 0.03	25.48 ± 0.05
250	16.96 ± 0.02	17.35 ± 0.04	22.39 ± 0.05	22.70 ± 0.03
500	74.38 ± 0.14	17.56 ± 0.07	34.51 ± 0.11	20.34 ± 0.03

When it comes to ChCl:PP towards *E. coli*, the biofilm removal increases with the concentration of the NADES tested. A maximum removal of $34.51 \pm 0.11\%$ was obtained after treatment with ChCl:PP at 500 mg·mL^{-1}. In this case, the culturability reduction is negligible, suggesting that this solvent is more effective in removing biofilm than cell inactivation. In fact, ChCl:PP at higher concentrations had a better performance on *E. coli* biofilm removal than ChCl:CA. The case of ChCl:UR is very similar to the case of ChCl:CA, since there is a maximum removal of $25.48 \pm 0.05\%$ at 100 mg·mL^{-1}.

***Table 6**: Effect of the selected NADES at different concentrations after 30 min of exposure towards S. aureus Biofilms in terms of biofilm removal by CV assay.*

Concentration (mg·mL^{-1})	*Biofilm Removal (%)*			
	ChCl:LA	*ChCl:CA*	*ChCl:PP*	*ChCl:UR*
1	8.62 ± 0.03	14.70 ± 0.04	6.40 ± 0.02	5.75 ± 0.03
10	17.08 ± 0.02	19.60 ± 0.01	3.98 ± 0.02	6.59 ± 0.03
50	28.85 ± 0.02	20.21 ± 0.12	9.12 ± 0.03	3.79 ± 0.03
100	38.68 ± 0.03	25.23 ± 0.002	23.02 ± 0.04	17.19 ± 0.04
250	37.70 ± 0.02	24.68 ± 0.01	12.12 ± 0.01	6.58 ± 0.01
500	22.08 ± 0.02	5.81 ± 0.02	18.05 ± 0.02	22.46 ± 0.09

When analyzing the biofilm removal of the NADES towards *S. aureus*, the acidic-based NADES along with ChCl:PP demonstrate a similar behavior, with a maximum removal at 100 mg·mL^{-1}. ChCl:LA caused the highest value for biomass removal: $38.68 \pm 0.03\%$. Regarding ChCl:UR towards *S. aureus*, the results obtained are too low, however, there is a maximum biofilm removal at 500 mg·mL^{-1} of $22.46 \pm 0.09\%$.

Comparing the values obtained for the different strains, it is noted that there was a higher biomass removal towards *E. coli* biofilms than *S. aureus*. However, independently of the biocide concentration tested in this study, complete biofilm removal was not accomplished. Nava-Ocampo *et al.* [69], studied NADES as potential cleaning agents for single-biofilm removal and suggested that the NADES are capable of solubilizing the proteins that are part of the EPS. However, the low values of biomass removal obtained in the present work do not seem to express this fact. However, the assays performed by Nava-Ocampo *et al.* [69] lasted for 24 h of NADES contact with biofilm, whereas the biofilm treatment in this study was performed in 30 min. Moreover, the biofilm models used in both works are very distinct which probably means that biofilms with very different compositions were used. Nava-Ocampo *et al.* [69] used aerobic granules from a lab- or pilot- scale reactors and in the present work, a younger biofilm structure attached to a polystyrene surface is used. The use of granules in a reactor instead of a biofilm attached to a surface from a microtiter plate may also benefit from mass transfer phenomena that may contribute to a better penetration of NADES in granules than in the biofilm. This means that the biofilm tolerance reported in the present work may be due to slow or incomplete penetration of the biocide into the biofilm due to diffusion limitations in the EPS [83]. Nevertheless, this topic would require the characterization of biofilm composition and structure.

4.3 Effect of NADES on the Control of Multi-Species Biofilms

4.3.1 Biofilm Culturability Reduction

Undoubtedly, biofilms in nature are complex and formed by different microorganisms inserted into the same community. Therefore, in this research, the effect of NADES on multi-species biofilms, composed by *E. coli* and *S. aureus*, was also investigated. The results obtained are presented in *Figure 8*.

The culturability reduction of ChCl:LA in multi-species biofilms reaches a maximum of 2.74 ± 0.14 log CFU·cm^{-2} and 1.16 ± 0.73 log CFU·cm^{-2}, at 500 mg·mL^{-1}, for *E. coli* and *S. aureus*, respectively (total reduction of 2.45 ± 0.41 log CFU·cm^{-2}). The fact that *E. coli* has decreased more than *S. aureus*, which is exactly the opposite of what happens in single-species biofilms, shows that there may exist a protective effect towards *S. aureus*. When harmful substances are present, sensitive strains benefit from the protection provided by more resistant populations, *e.g.*, when the antibacterial agent is entirely detoxified by the resistant species [84].

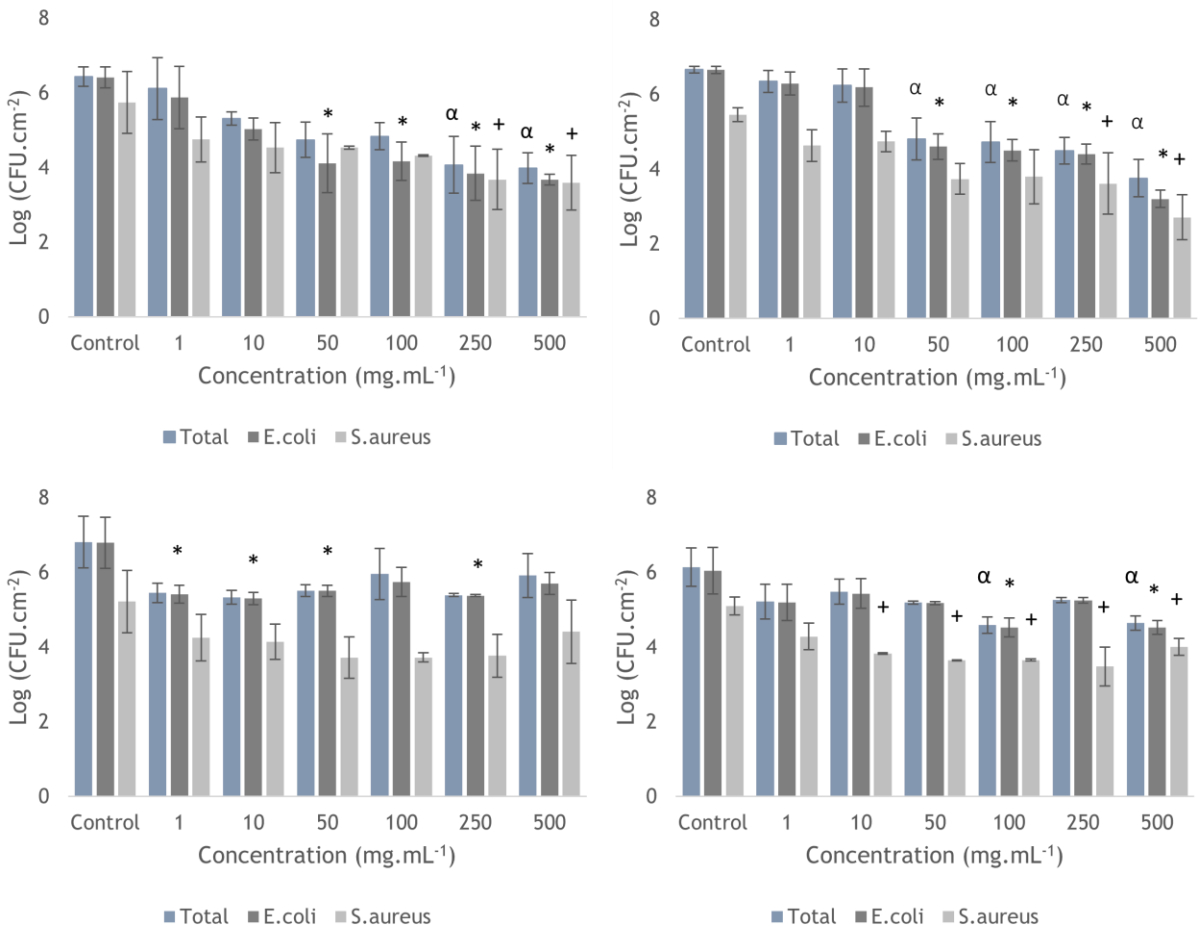


***Figure 8**: : Effect of the selected NADES at different concentrations after 30 min of exposure towards Multi-Species biofilms in terms of biofilm culturability. A - ChCl:LA; B - ChCl:CA; C - ChCl:PP; D - ChCl:UR. * - samples of E. coli were statistically different from unexposed bacteria (control) (p < 0.05, Dunnett's test). + - samples of S. aureus were statistically different from unexposed bacteria (control) (p < 0.05, Dunnett's test). a - total biofilm count was statistically different from unexposed bacteria (control) (p < 0.05, Dunnett's test).*

When it comes to ChCl:CA, a maximum reduction was also detected at 500 mg·mL^{-1} of 3.46 ± 0.23 log CFU·cm^{-2} for *E. coli* and 1.92 ± 0.60 log CFU·cm^{-2} for *S. aureus* (total reduction of 2.9 ± 0.5 log CFU·cm^{-2}). Once again, for lower concentrations, there are no statistically significant differences between the concentrations and the unexposed biofilm. It is plausible to conclude that the protective effect also applies in this instance, given that the decrease in *S. aureus* is significantly less than in biofilms composed of single-species.

Regarding ChCl:PP and ChCl:UR, similarly to the previous situations, there is a much weaker antimicrobial potential than the acidic NADES. In the case of ChCl:PP, there is a total maximum reduction of 1.47 ± 0.19 log CFU·cm^{-2} at 10 mg·mL^{-1}. Unlike the previous assays for ChCl:UR, in this situation there are differences between the concentrations tested and the control. A total maximum reduction of 1.5 ± 0.4 log CFU·cm^{-2} is reached both at 10 and 50 mg·mL^{-1}.

As with the previous assays, the data was adjusted to the Chick-Watson model. The values of the estimated parameters are presented in *Table 7*. The figures obtained from the adjustability to the model are presented in *Figure 9*. Similarly to single-species biofilms, the model is only well adjusted for acidic-based NADES. The values of R^2 for ChCl:PP and ChCl:UR suggest that their antibiofilm potential is negligible, which also explains the negative value of n for ChCl:PP. The antibacterial activity of the NADES tested was not considered to be dose-dependent.

***Table 7**: Estimated values of NADES concentration dependence parameter (n), adapted to Chick-Watson Model and the disinfection rate coefficient (k_{dis}) for multi-species biofilms. The values are the adjustment to the mean values of three independent assays. Model adjustability was assessed by R^2.*

	E. coli			***S. aureus***		
NADES	$\boldsymbol{n}$	$\boldsymbol{k_{dis}}$ $((mL \cdot mg^{-1})^n \cdot min^{-1})$	$\boldsymbol{R^2}$	$\boldsymbol{n}$	$\boldsymbol{k_{dis}}$ $((mL \cdot mg^{-1})^n \cdot min^{-1})$	$\boldsymbol{R^2}$
ChCl:LA	0.1913	0.0299	0.9036	0.5492	0.0014	0.8671
ChCl:CA	0.3348	0.0139	0.9051	0.3551	0.0062	0.7246
ChCl:PP	-0.0318	0.0482	0.2899	0.0230	0.0372	0.0495
ChCl:UR	0.1081	0.0222	0.3351	0.0296	0.0383	0.086

Evaluating the total biofilm count, it would be expected to have a lower reduction of colonies in multi-species biofilms than in single-species biofilms, since it has been suggested that cell-to-cell signaling in multi-species biofilms raises the capacity of organisms to withstand disinfection by increasing transcription of genes responsible for the survival of oxidative stress, changing the composition and viscosity of the EPS, or affecting biofilm architecture and the

spatial distribution of various strains. These QS-induced systems, provide bacteria the ability to respond critically to environmental hazards including oxidative stress [85]. However, the total reduction of multi-species biofilms was higher than in single-species biofilms. This may be due to competition between bacteria, already reported in other studies with multi-species biofilms of *E. coli* and *S. aureus* [86], forming an unstable biofilm that ends up being more susceptible to antibacterial agents than single-species biofilms. Nonetheless, this topic would have to be further explored.

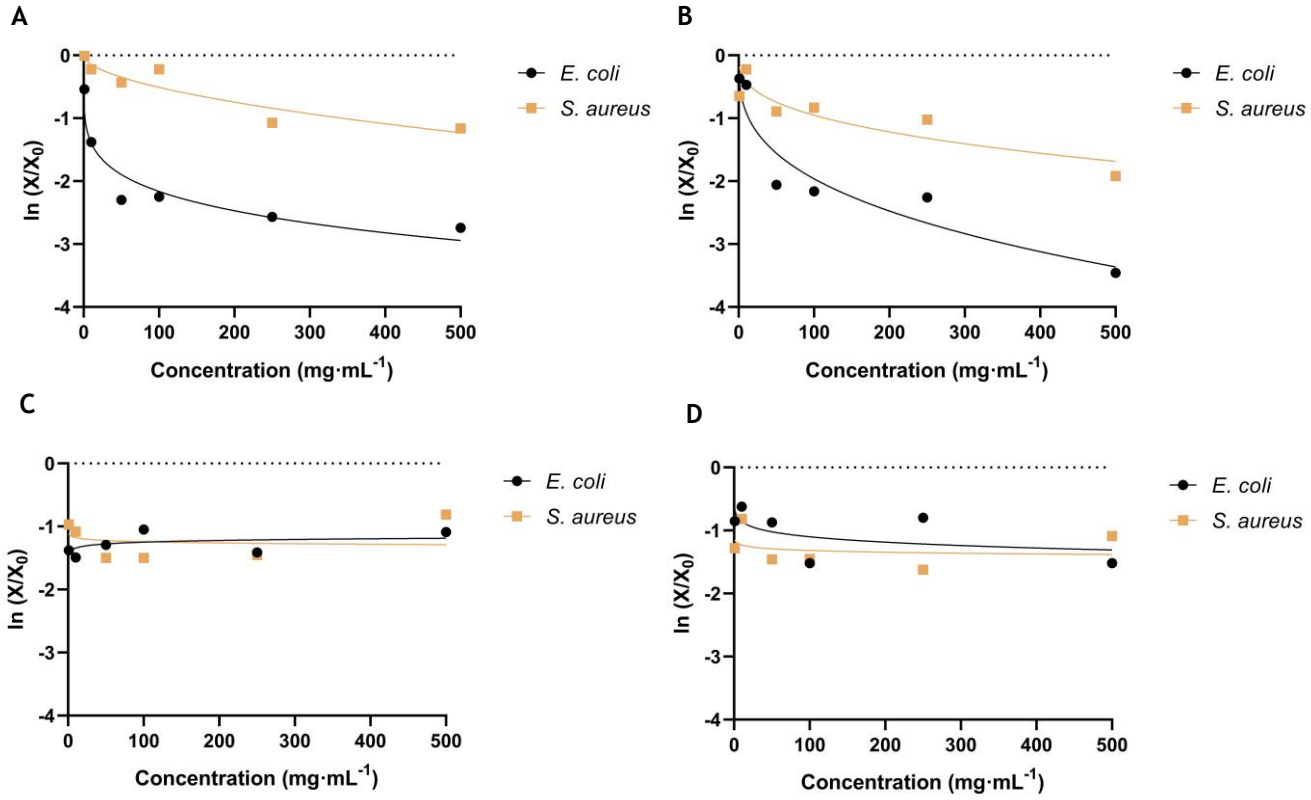


***Figure 9**: Logarithmic reduction of multi-species biofilms, as a function of biocide concentration (mg·mL^{-1}) after 30 min of NADES exposure: (A) ChCl:CA; (B) ChCl:LA; (C) ChCl:PP; (D) ChCl:UR. The lines represent the Chick-Watson model of experimental data, and the points represent the means for at least three independent assays.*

Broadly speaking, there was a higher reduction of *E. coli* than *S. aureus* in multi-species biofilms. As mentioned before, this may be due to the protective effect of binary biofilms. Several species can coexist in biofilms, with their behavior resulting from the combination of synergetic and antagonistic interactions, since they can create metabolites that can interfere negatively or favorably with growth and biofilm development [87].

4.3.2 Biofilm Removal Quantification

The impact of selected NADES as possible biofilm disruptors against multi-species biofilms was assessed through the quantification of biomass removal by CV staining, and the results obtained are presented in *Table 8*.

It is evident that the multi-species biofilm removal caused by NADES is generally lower than that observed for single-species biofilms. Regarding ChCl:LA, there is a maximum reduction of $29.38 \pm 0.02\%$ at 50 mg·mL^{-1}. At both 10 and 250 mg·mL^{-1}, the maximum biofilm removal of ChCl:CA is obtained. For ChCl:PP there is a maximum biomass removal of $30.17 \pm 0.12\%$ at 1 mg·mL^{-1}. For ChCl:UR there is a maximum removal of $18.89 \pm 0.03\%$ at 250 mg·mL^{-1}.

Contrary to single-species biofilms, there is no apparent correlation between the concentration utilized and the percentage of biofilm removal in multi-species biofilms.

***Table 8**: Effect of the selected NADES at different concentrations after 30 min of exposure towards Multi-Species Biofilms in terms of biofilm removal by CV assay.*

Concentration (mg·mL^{-1})	*Biofilm Removal (%)*			
	ChCl:LA	*ChCl:CA*	*ChCl:PP*	*ChCl:UR*
1	22.00 ± 0.07	14.22 ± 0.01	30.17 ± 0.12	4.58 ± 0.01
10	2.56 ± 0.002	21.39 ± 0.02	1.84 ± 0.01	8.39 ± 0.004
50	29.38 ± 0.02	10.50 ± 0.04	3.59 ± 0.001	2.50 ± 0.02
100	22.01 ± 0.02	6.24 ± 0.03	5.47 ± 0.02	15.97 ± 0.09
250	8.63 ± 0.004	21.39 ± 0.03	9.33 ± 0.10	18.89 ± 0.03
500	13.91 ± 0.04	21.27 ± 0.06	0.81 ± 0.002	3.30 ± 0.04

The low removal values and fluctuating behavior of the values appear to demonstrate the limited efficacy of selected NADES as biofilm disruptors. Even if the bacteria are neutralized, the biofilm matrix may not be eliminated, serving as the foundation for future biofilm formation. Positively charged components like polysaccharides, proteins, or nucleic acids attach to negatively charged ones like CV. Any antimicrobial drug that increases the amount of negatively charged residues will also promote the development of the CV complex, leading to an incorrect perception of an increase in biofilm mass. It may be caused by an increase in negatively charged components released from dead cells retained in the matrix, an overproduction of negatively charged polysaccharides (*i.e.,* as a reaction to antimicrobial agents), or by the influence of EPS enzymatic degradation [88]. In fact, multi-species biofilms seemed to be more susceptible to organic acid-based NADES than single-species biofilms in terms of cellular culturability, however an opposite behavior was found in terms of biomass removal, which may be due to significant composition and structural differences between single and multi-species biofilms. Once again, further studies on the characterization of biofilm composition and structure as well as on the interaction of NADES with biofilm components would provide significant details to better understand NADES action.

5 Conclusions

The main objectives of this work were the production of choline-based solvents and the assessment of the antibiofilm potential of each selected NADES against single and multi-species biofilms. For this purpose, the NADES were synthesized using the thermal mixing method, by mixing choline chloride and a HBD of choice. The mixture was heated in a water bath at 80 ºC until a clear, homogenous liquid was formed. The antimicrobial activity was evaluated using *E. coli* and *S. aureus* as bacterial models to form biofilms.

Acidic-based NADES were the most promising NADES to control single and multi-species biofilms formed by *E. coli* and *S. aureus*. This fact may be due to the ability of acidic HBDs to inactivate cells by denaturing proteins present on the cell wall, which causes cell death and collapse. In most cases, the reduction increased as the concentration tested increase. A maximum reduction of 2.7 ± 0.5 log CFU·cm^{-2} was obtained with ChCl:CA at 500 mg·mL^{-1} for *E. coli* biofilms, 3.37 ± 0.31 log CFU·cm^{-2} obtained with ChCl:LA at 100 mg·mL^{-1} for *S. aureus* biofilms and 2.9 ± 0.5 log CFU·cm^{-2} obtained with ChCl:CA at 500 mg·mL^{-1} for the total biofilm count in multi-species biofilms.

Independent of the type of biofilm studies, the amide- and alcohol-based selected NADES were shown to have negligible antibiofilm potential. In single-species biofilms, ChCl:PP showed a maximum reduction of 0.9 ± 0.2 log CFU·cm^{-2} at 5 mg·mL^{-1} for *E. coli* and no inhibition for *S. aureus*. ChCl:UR, on the other hand, showed no inhibition for both bacteria. In multi-species biofilms, there was a total maximum reduction of 1.47 ± 0.19 log CFU·cm^{-2} at 10 mg·mL^{-1} for ChCl:PP, and 1.5 ± 0.4 log CFU·cm^{-2} at 10 and 50 mg·mL^{-1} for ChCl:UR.

The data adjustment to the Chick-Watson mathematical model corroborates the better performance of acidic-based NADES. However, the model did not fit the data from ChCl:PP and ChCl:UR resulting in very low values of R^2, emphasizing its negligible antibiofilm properties.

For the biofilm removal quantification assay, total biofilm removal was not achieved for either type of biofilm. In fact, in the present study NADES did not seem to have a significant role as biofilm disruptors. In single-species biofilms, there was a higher biomass removal for *E. coli* biofilms than for *S. aureus*. In multi-species biofilms, the percentages of removal were lower than in single-species and the values obtained demonstrated no apparent correlation between the concentration used and the percentage of biofilm removal, which translates into the limited efficacy of NADES as biofilm disruptors.

In summary, acidic-based NADES were more effective than ChCl:PP and ChCl:UR on bacteria neutralization. However, all selected NADES had poor performance in removing the biofilm matrix, which means that even if the bacteria are neutralized, the biofilm matrix may not be

eliminated, serving as the foundation for future biofilm formation. Nonetheless, the results obtained demonstrated that NADES have indeed antibacterial potential even against biofilms. This potential should be thoroughly explored since acidic-based NADES are eco-friendly molecules with promising actions on biofilm control, specifically on the reduction of bacterial culturability.

6 Assessment of the work done

6.1 Objectives Achieved

One of the goals of this work was to produce NADES, as described in the literature, to combine two or more solid chemicals into a liquid combination that would remain liquid at normal temperature after the heating production process. This objective was achieved for the majority of NADES selected.

The main goal of this work was to evaluate the potential of selected NADES on biofilm control through the assessment of culturability reduction in log CFU·cm^{-2} and the percentage of biomass removal. This objective was fully achieved; the results obtained were consistent, and strong conclusions could be drawn about the most promising NADES for disinfection process and biofilm control. In fact, acidic-based NADES have a stronger effect on single and multi-species biofilms than amide- and alcohol-based ones, which is due to acidic HBDs' ability to inactivate cells by denaturing proteins present on the cell wall, resulting in cell death and collapse.

To summarize, all the objectives proposed were accomplished. This work is innovative not only by exploring NADES as antimicrobial agents but mostly by exploring their action against biofilms of planktonic cells. The use of biofilm models provides a more realistic scenario, providing more reliable information about the potential of NADES as disinfectants. In fact, this work aims to serve as a reference key for future research on the antibacterial properties of NADES and their potential use as a replacement for common disinfectants.

6.2 Perspectives for Future Works

Due to time limitations, certain areas were not explored in depth. However, it would be intriguing to better understand the behavior of sugar-based NADES crystallized and to test different molar ratios, and to evaluate the remaining NADES through POM analysis, to ensure there was no formation of residues. Additionally, it would be interesting to compare the results obtained with the antimicrobial properties of the individual components of each NADES, in order to see the difference between the two and to understand how the formation of NADES enhances certain properties of the substances.

This work is based on the assumption that NADES are biodegradable and non-toxic, and do not cause a problem when used in large quantities. Therefore, it would be interesting to evaluate its biodegradability, to ensure it does not become a problem for the environment.

In order to confirm the action of NADES in biofilms, it would be useful to analyze biofilm structure before and after treatment with the antimicrobial agent through confocal laser scanning microscopy. It would also be interesting to evaluate the membrane damage of the bacteria through contact angles or to evaluate the intake of propidium iodide through the live/dead method. It would be intriguing as well to investigate the effect of the sequential exposure time of these solvents on bacteria. Essentially, the antibiofilm potential of NADES needs to be further explored.

6.3 Final Assessment

This work presented itself as a difficult task due to scarcely available literature on NADES antibiofilm properties, requiring very intense research.

On a final note, I enjoyed working on this topic, and the laboratory work helped me develop technical and personal skills. I think this work was well-crafted, and it fits the present concern of the environment and the problems that involve the development of bacterial tolerance to disinfectants.

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Appendix A – Computational Analysis with COSMO-RS

Table A.1 presents the values obtained for each individual component of the NADES produced, estimated with COSMO-RS.

***Table A.1**: Properties for each individual components of the NADES produced, obtained with COSMO-RS software*

Compound	*CAS*	*Molar Mass (g· mol^{-1})*	*Density (kg·L^{-1})*	*T_m (K)*	*$\Delta_m h$ (kJ·mol^{-1})*
Choline Chloride	67-48-1	139.08	1.039	271.53	18.60
Lactic Acid	50-21-5	90.03	1.080	323.12	15.59
Citric Acid	77-92-9	192.03	1.374	562.25	31.50
1,2-Propanediol	57-55-6	76.05	0.949	243.50	15.35
Urea	57-13-6	60.03	1.100	433.50	0.00
Xylitol	87-99-0	152.07	1.283	359.40	37.18
Sucrose	57-50-1	342.12	1.364	568.01	63.17

T_m: melting (fusion) point; $\Delta_m h$: Enthalpy of fusion