



## Menthol-based deep eutectic systems as antimicrobial and anti-inflammatory agents for wound healing

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### ABSTRACT

Effective antimicrobial treatment has been identified as a serious and unmet medical need. Herein, we present a strategy based on deep eutectic systems (DES) to overcome current limitations, answering the need not only to effectively kill bacterial agents but also to avoid their adhesion and proliferation, which is associated with biofilm formation and have a crucial impact on bacterial virulence. To achieve such a goal, natural deep eutectic systems (NADES) based on menthol (Me) and saturated free fatty acids (FFA) were produced, fully physico-chemical characterized, and its bioactive properties were described. The antimicrobial potential of menthol-based NADES with FFA, namely, myristic acid (MA), lauric acid (LA), and stearic acid (SA) were investigated towards a broad panel of microorganisms. The obtained data indicates that NADES possess effective antimicrobial properties towards the Gram-positive bacterial and fungal strains tested. Among the tested formulations, Me:LA at a molar ratio of 4:1 molar was used to carry out a biofilm detachment/removal assay due to its superior microbiological properties. This formulation was able to effectively lead to biofilm removal/dispersion of not only methicillin-resistant *Staphylococcus aureus* (MRSA) and *Candida albicans*, but also *Escherichia coli*, without the need of any additional physical force or antibiotic. Furthermore, since microbial invasion and biofilm formation is highly undesired in wound healing, namely in chronic wound healing, the wound healing properties of these eutectic formulations was also investigated. The results suggest that these NADES can cope with microbial invasion and biofilm detachment while not compromising normal keratinocyte proliferation and migration verified in wound healing and epidermis repair, while also contributing to the reduction of cell stress and inflammation via the control of ROS production. In conclusion, these results provide the indication that NADES based on Me and FFA holds great interest as antimicrobial agents for preventive and therapeutic applications in various clinical settings, including wound healing.

### 1. Introduction

Over the past 30 years, it has been widely recognized that the attachment of bacteria on biological surfaces is a prerequisite to invasion and a critical step during the infection process (Hori and Matsumoto, 2010; Donelli, 2015). Microbial adhesion is strongly dependent on the chemical character of the surface, namely its hydrophilicity and charge of its materials. Once adhered to the surface, microorganisms proliferate and colonize surfaces, developing organized structures

known as biofilms (Hori and Matsumoto, 2010; Donelli, 2015; Khameneh et al., 2016). Considering healthcare costs, these are significantly increased for patients with infections associated with biofilms since these are difficult to treat and usually require higher antibiotic doses and prolonged treatment times (Donelli, 2015; Hetrick and Schoenfisch, 2006). The routinely clinical approach is based on the delivery of antibiotics either by the conventional systemic approach or by local administration. The conventional systemic approach of antibiotic administration often leads to poor tissue penetration, whereas its local

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administration may enable the maintenance of a high local antibiotic concentration while avoiding unnecessary selective pressure among the patient's natural microbiota (Donelli, 2015; Hetrick and Schoenfisch, 2006; Ribeiro et al., 2016). Several antibiotics including vancomycin, tobramycin, cefamandole, cephalothin, carbenicillin, amoxicillin and gentamicin, have already been incorporated into controlled-release devices (Donelli, 2015; Khameneh et al., 2016; Hetrick and Schoenfisch, 2006). However, the increasing resistance of certain microorganisms to conventional therapies has prompted focus on the research for new strategies to reduce surface colonization and consequently reduce microbial virulence in earlier stages of the invasion process. For example, it has been reported attempts to solve the unfavorable antimicrobial properties of medical devices, including strategies such as modification of surfaces with polyethylene glycol, polyethylene oxide brushes, silver, betaine esters, and quaternary ammonium compounds (QAC's) (Khameneh et al., 2016; Hetrick and Schoenfisch, 2006; Zilberman and Elsner, 2008). Most recently, the antimicrobial properties of deep eutectic systems (DES) have been reported (Zakrewsky et al., 2014; Aroso et al., 2016; Mano et al., 2015; Zakrewsky et al., 2016; García-Argüelles et al., 2013). DES properties have been pushing forward these systems as versatile alternative agents in a wide range of applications (Pena-Perreira and Namieśnik, 2014). Such systems can be simply defined as a mixture of two or more components, combining a hydrogen bond donor (HBD) with a hydrogen bond acceptor (HBA), which at a specific molar ratio present a lower melting point than either of its individual components (Smith et al., 2014; del Monte et al., 2014; Zhang et al., 2012). In other words, such combinations result in a liquid system with tailor-made characteristics corresponding to the initial chosen individual components. The simplicity of eutectic systems preparation, its tailor-made potential, alongside with their remarkable properties which follow the green chemistry metrics, have driven their research for example, in synthesis, metal-catalyzed organic reactions, electrochemistry, nanomaterials, biochemistry, separation, material chemistry and also biomedical applications (Smith et al., 2014; del Monte et al., 2014; Zhang et al., 2012; Paiva et al., 2014). In biomedical applications, DES are receiving increasing interest not only as efficient carriers of active pharmaceutical ingredients (APIs), where they may enhance the APIs bioavailability, but also for the synthesis of a wide panel of polymer-related materials, as anticancer therapeutics, antituberculosis, among several others (Smith et al., 2014; Paiva et al., 2014; Abo-Hamad et al., 2015; Alonso et al., 2016; Dai et al., 2013; Mbous et al., 2017; Silva et al., 2019).

DES can, therefore, be designed with antimicrobial and antibiofilm potential by the combination of individual components with those properties (Zakrewsky et al., 2014; Aroso et al., 2016; Mano et al., 2015; Zakrewsky et al., 2016; García-Argüelles et al., 2013; Hayyan et al., 2015; Hayyan et al., 2016). Interestingly, the bioactivity of such systems can also be associated with wound healing potential as described by Silva et al. (Silva et al., 2019). Therefore, our main goal in this work was to survey different DES, combining menthol and saturated free fatty acids, that could work as antibacterial and biofilm dispersal agents, thus controlling microbial infection in the advent of an epidermis disruption. Me is a monocyclic monoterpene described for its: i) antimicrobial properties; ii) anti-inflammatory activity since it is able to inhibit inflammatory mediators and cytokines; iii) analgesic properties, where its antinociceptive has been associated with its ability to activate the central kappa opioid system; and iv) anesthetic effect (Juergens et al., 1998; Tsuk, 1990; Abbaszadeh et al., 2014; Daneshkazemi et al., 2016; Galeotti et al., 2002; Guimarães et al., 2013). On the other hand, FFA presents potent antimicrobial properties towards a wide range of clinically relevant pathogenic bacteria and fungi (Desbois and Smith, 2010; Kabara et al., 1972; Kitahara et al., 2004; Silva et al., 2019). In this work, the prepared DES were evaluated towards a wide panel of microorganisms, such as *S. aureus*, *P. aeruginosa*, *E. coli*, MRSA, MRSE and *C. albicans*, and their potential as biofilm dispersal/inhibiting agents was assessed. Furthermore, the wound healing properties of these eutectic

systems were evaluated by assessing their influence on the proliferation and migration of keratinocyte and their impact on controlling an inflammatory state on this epidermis cell line. This provides clues for the design of enhanced therapies towards modern medicine relevant challenges such as control of microbial invasion and proliferation, which could thereafter foresee applications in for example, chronic wounds from diabetic patients (Fig. 1).

## 2. Materials and methods

### 2.1. NADES preparation

For the preparation of eutectic systems, menthol (Me; ref. M2772, Sigma Aldrich), lauric acid (LA; Ref. W261408-SAMPLE-K, Sigma Aldrich), myristic acid (MA; ref.70082, Sigma Aldrich), and stearic acid (SA; ref. 175,366, Sigma Aldrich), were used as initial individual compounds. Briefly, Me was mixed with LA, MA, and SA at different molar ratios and heated to 70 °C, under constant stirring for 5–10 min.

### 2.2. Thermal properties

Differential scanning calorimetry (DSC) experiments were carried out in a TA instrument DSC Q100 model (Thermal analysis & analyzers), using aluminum pans. The formulations were equilibrated at 40 °C for 5 min followed by cooling to –40 °C, an isothermal period for 5 min, and heating to 110 °C at 5 °C /min.

### 2.3. Disk diffusion assay

The antimicrobial activity of NADES was determined using *S. aureus* ATCC 25,923, *S. aureus* ATCC 700,698 (Methicillin-resistant strain, MRSA), *S. epidermis* ATCC 35,984 (Methicillin-resistant strain, MRSE), *P. aeruginosa* ATCC 27,853, *E. coli* ATCC 25,922, and *C. albicans* ATCC 90,029. Initially, the antimicrobial activity was assessed by a disk diffusion approach. Gentamicin sulfate (ref. G1914, Sigma Aldrich) at 50 mg/mL, Fluconazole (ref. Y0000557, Sigma Aldrich) at 5 mg/mL, and sterile water were used as positive and negative controls, respectively. Additionally, the effect of each FFA and Me was evaluated. The assay was performed as described elsewhere (Silva et al., 2019; Silva et al., 2019).

### 2.4. Assessment of minimal inhibitory concentrations (MIC), minimal bactericidal concentration (MBC), and minimal fungicidal concentrations (MFC)

The MIC and MBC/MFC were determined by the microbroth dilution technique as previously described (Silva et al., 2019; Silva et al., 2019). Briefly, standard solutions of the isolated components and the NADES were prepared under sterile conditions by dissolving the compounds in DMSO (ref. 276,855, Sigma), followed by serial dilution in Mueller-Hinton Broth medium (MHB, ref 70,192–500 g, Sigma Aldrich). Afterward, the microtubes containing the sample solutions (500 µL/tube) were inoculated with 500 µL of microbial suspension at  $1-2 \times 10^6$  CFUs/mL in MHB. As a control, MHB (i.e., bacteria-free), MHB (with bacteria), a solution of 10% (v/v) DMSO in MHB. The tubes were incubated at 37 °C (bacterial strains) or 30 °C (*C. albicans*) under shaking for 24 h and microbial growth monitored. Posteriorly, it was carried out sub-culturing in trypticase soy agar (TSA, Ref. 610,052, Frilabo, Lda) followed by 24 h incubation to determine MBC/MFC and confirm MIC values. Experiments were carried out in triplicate and repeated on three independent occasions.

### 2.5. Biofilm formation

*S. aureus* ATCC 700,698 (Methicillin-resistant strain, MRSA), *C. albicans* ATCC 90,029 and *E. coli* ATCC 25,922 were spread onto TSA

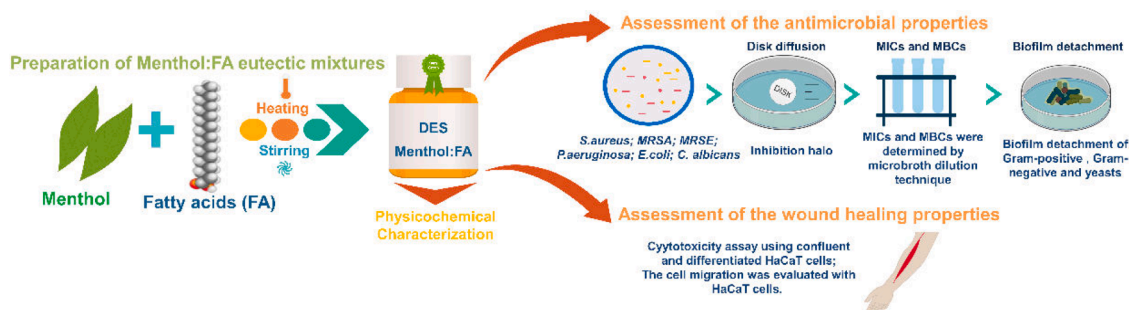


Fig. 1. Schematic illustration of DES production steps, considering their physicochemical and bioactivity assessment.

plates and grown overnight at optimal temperature for each organism (37 °C and 30 °C for bacteria and yeast, respectively). Thereafter, suspension cultures were prepared by inoculating single colonies in TSB, and posterior incubation for 24 h at optimal growth temperatures. Cultures were then harvested, resuspended in TSB, and adjusted to approximately  $1-2 \times 10^6$  CFUs/mL. Posteriorly, 200  $\mu$ L of the previously prepared bacterial suspension was transferred to a 96-well polystyrene flat-bottom plate and incubated for an additional 20 h, at optimal growth temperature, for biofilm formation.

## 2.6. Assessment of biofilm removal efficiency

Following biofilm formation, microbial suspensions were carefully removed, and sample solutions with the individual components and eutectic systems were added. After different exposure times (5, 10, 20 and 30 min) sample solutions were removed and 200  $\mu$ L of MHB media containing 10%(v/v) alamar blue (ref. BUF012B, Bio-Rad) was added to the wells, followed by plate incubation for 1 h, at either 37 °C or 30 °C, protected from light. Posteriorly, absorbances at 570 and 600 nm, using a microplate reader (SYNERGY HT™) were used to calculate biofilm removal efficiency, based on values obtained for biofilm treated with deionized water. Scanning electron microscope (SEM) images were also obtained using a JEOL JSM-6010 LV (JEOL, Japan). For this purpose, biofilms were established in coverslips (ref. 83.1840.002, Sarstedt) for 20 h and treated with the formulations for the prementioned time points. Posteriorly, sample solutions were gently removed, and microorganisms fixed using a 10% (v/v) formalin in PBS solution for 1 h. Fixed biofilms were then dehydrated by immersion in solutions with increasing ethanol concentrations (50%, 70%, 90% and 100%). Finally, samples were air-dried to remove residual ethanol, followed by gold-sputtering for SEM image acquisition.

## 2.7. Cytotoxicity assessment

An immortalized human keratinocyte cell line (HaCaT) was obtained from Deutsches Krebsforschungszentrum (DFKZ, Germany). Cells were subcultured as a confluent monolayer in DMEM medium, supplemented with 10% (v/v) of heat-inactivated fetal bovine serum (FBS) and 1% (v/v) of penicillin-streptomycin (PS), under standard growth conditions (5% CO<sub>2</sub>, 37 °C, humidified atmosphere). The cell culture medium and supplements were acquired from Corning Life Science (Corning®, USA). The cytotoxicity of the different systems was assessed using confluent and differentiated HaCaT cells. The cells were seeded in a 96-well plate at a density of  $4.5 \times 10^4$  cells/well and allowed to grow for 3 days. Upon reaching confluency, the cells were incubated with the different systems diluted in culture medium and allowed to grow for 24 h. Cells only incubated with culture medium were considered as negative control. Thereupon, cells were washed twice with PBS (Fischer Scientific, USA) and cell viability was estimated using CellTiter 96® Aqueous One Solution Cell Proliferation Assay (Promega, USA), comprising MTS viability reagent, according to manufacturer's instructions. The MTS viability reagent was diluted in DMEM medium supplemented with 5%

(v/v) FBS. Absorbances were measured at 490 nm using UV-vis spectrophotometry (VICTOR Nivo™, PerkinElmer, USA), and cell viability was expressed in percentage normalized with the control. At least three independent experiments were performed in triplicate.

## 2.8. Wound healing assay

The wound healing potential of DES was assessed using HaCaT cells. The cells were seeded at a density of  $1 \times 10^5$  cell/cm<sup>2</sup> in a 12-well plate and allowed to grow until 100% confluence (48 h). Afterwards, a disruption on cells confluent monolayer – here considered as a model for superficial epithelial wound – was formed with a 200  $\mu$ L micropipette tip, and each well was washed twice with PBS in order to remove the nonadherent cells. Thereafter, the DES formulations and the individual components were incubated for 24 h. Micrographs were taken by inverted optic microscopy (Zeiss Axio Vert A1, Germany) at two different time points: 0 and 24 h. The wound area was measured and calculated using the Zeiss Zen 3.1 software. The wound area recovered was expressed in terms of percentage using the following equation and considering three isolated experiments performed in triplicate:

$$\text{Wound area recovered (\%)} = \frac{\text{Initial area} - \text{Final area}}{\text{Initial area}} * 100$$

## 2.9. Reactive oxygen species assessment

NADES potential for reducing reactive oxygen species in human keratinocytes (HaCaT) was assessed using a dichlorofluorescein-diacetate (DCFH-DA) probe-based assay, which is widely used to detect oxygen species in different in vitro models (Kalyanaraman et al., 2012; Wang and Joseph, 1999). Briefly, HaCaT cells were seeded in a 24-well plate at a density of  $1.52 \times 10^5$  cells/mL and allowed to grow overnight. Thereafter, the cell culture was exposed to NADES and their corresponding individual counterparts diluted in culture medium, and on culture medium (control), for 1 h. After, the culture media was removed, and the cell culture washed twice with PBS. To quantify the presence of ROS, 600  $\mu$ L of DCFH-DA at 25  $\mu$ M was added to each plate well for 1 h. Fluorescence was measured in a microplate reader (HH35L2019044, Victor Nivo 3S, Perkin Elmer), applying an excitation wavelength of 480 nm and an emission wavelength of 530 nm. At least three independent experiments were performed in triplicate.

## 3. Results and discussion

### 3.1. Natural deep eutectic systems physicochemical characterization

NADES based on menthol combined with lauric acid, myristic acid or stearic acid, with optimized molar ratios were produced as previously described elsewhere (Silva et al., 2019). Namely, Me:LA with 4:1 molar ratio as well as Me:MA and Me:SA, both 8:1 molar ratio, were produced. The individual components and eutectic systems melting temperatures were determined (Table 1) and corroborated previously described data

**Table 1**

Summary of the different NADES prepared and their melting point. Melting points of the individual components are included for comparison.

NADES	Molar Ratio	Melting Point ( °C)
Me:LA	4:1	≈10.0
Me:MA	8:1	≈16.1
Me:SA	8:1	≈34.7
Me	–	≈28.3
LA	–	≈46.6
MA	–	≈58.6
SA	–	≈73.0

(Silva et al., 2019). The large depression of the formulation's melting point when compared with those of the isolated compounds, suggests the establishment of intermolecular interactions between Me and the various saturated free fatty acids, forming a deep eutectic system supramolecular structure mostly attributed to hydrogen bonding (El Achkar et al., 2020).

Furthermore, NMR analysis of the systems, reported by Silva et al. also supports the formation of a deep eutectic system supramolecular structure between menthol and the three different fatty acids (Silva et al., 2019).

### 3.2. Antimicrobial potential evaluation

As an initial screening to assess NADES antimicrobial activity a disk diffusion assay was carried out with microbial strains ranging from MRSA to clinically relevant Gram-negative bacteria (i.e. *E. coli* and *P. aeruginosa*), and also including *C. albicans*, the main cause of cutaneous and vulvovaginal candidiasis (Gonçalves et al., 2016). Inhibition halo measurements are presented in Table 2. Representative images of obtained plates can also be found in the supplementary information (Table S1-S6).

The results from Table 2 reveal that all the DES formulations only display inhibitory activity towards the tested Gram-positive bacteria (*S. aureus*, MRSA and MRSE) and *C. albicans*, not displaying activity towards the tested Gram-negative bacteria species (*E. coli* and *P. aeruginosa*). These results are in accordance with other reports in the literature, as Gram-negative bacteria are usually resistant to the antibacterial activity of FFA and essential oils derivatives due to a more complex cell membrane structure (Desbois and Smith, 2010; Silva et al., 2019; Al-Bayati, 2009; Ouattara et al., 1997; Karapinar and Aktuğ, 1987; Pattnaik et al., 1997). Since these compounds work primarily via destabilization of the lipid membrane, their effects are therefore heavily dependent on both lipid composition and membrane net surface charge (Kabara et al., 1972; Yoon et al., 2018). As the exterior layer of Gram-negative bacteria outer membrane is mostly composed of lipopolysaccharides, a hydrophilic barrier is formed, protecting these bacteria from the effects of hydrophobic compounds such as Me and FFA (Vaara and Nurminen, 1999; Wiener and Horanyi, 2011). On the other

hand, the cell wall of Gram-positive bacteria is more permeable to hydrophobic compounds allowing both compounds to exert their inhibitory effects upon the cells (Kitahara et al., 2004; Al-Bayati, 2009; Ouattara et al., 1997; Schelz et al., 2006). Likewise, *C. albicans* is reported in the literature as being able to modulate its membrane surface hydrophobicity during growth and morphogenesis, which in turn may explain its susceptibility to these types of compounds, as it displays a hydrophobic membrane surface during certain stages of its life cycle, among other things, to promote virulence by enhanced adhesion to cells (Glee et al., 1995; Hobden et al., 1995). Based on the results of this preliminary screening *S. aureus*, MRSA, MRSE and *C. albicans* were selected for further MIC and MBC/MFC determination for the tested DES formulations. The obtained results are presented in Table 3. Firstly, it should be highlighted the challenges of determining the antimicrobial activity of the DES formulations and individual compounds towards the selected microbial species in suspension culture, due to the turbidity of the solutions and overall low solubility of the compounds (Chandra et al., 2001; Bes et al., 2018). To solve this issue, DMSO was added to the medium to a final concentration of 1.5%(V/V) which did not show any effect on MICs and MBCs/MFCs determination.

The MIC and MBC/MFC values for the raw components corroborated values mentioned in literature, namely for Me between 125 and 4000 µg/mL, for LA between 30 and 1600 µg/mL, for MA between 498 and 1600 µg/mL (Silva et al., 2019; Silva et al., 2019; Karapinar and Aktuğ, 1987; Pattnaik et al., 1997; Yoon et al., 2018; Schelz et al., 2006; Lee and Jo, 2016; McGaw et al., 2002; Singh et al., 2015). The concentration values determined for all tested strains are concordant with the values obtained for the major counterpart of the deep eutectic systems formulations, namely menthol. The widespread antimicrobial effects observed, with these systems displaying relevant impact over both the tested Gram-positive bacteria strains and the yeast *C. albicans*, support the proposed action mechanism for saturated free fatty acids and terpenic essential oils constituents (Lee and Jo, 2016; McGaw et al., 2002; Soković et al., 2010; Sokovic et al., 2008). In this model, both compounds primarily exert their effects via membrane dissolution, a non-specific action mechanism, which explains the observed effect on the different microorganisms selected for MIC/MBC determination. Furthermore, it should be noted that working with these individual compounds in DES form, a supramolecular structure, did not hamper the antimicrobial capabilities of the individual compounds. While at first one may expect that the Me:LA 4:1 system would be more efficient, when analyzing the absolute mass composition for each system at different concentrations (2500, 1250 and 625 µg/mL), as illustrated in Fig. 2, we can verify that the absolute quantities of LA are either lower than 312.5 µg, the determined MBC of LA, or overlap with the necessary amounts of Me to achieve inhibitory/bactericidal effect (1250 and 2500 µg/mL).

In contrast, regarding SA specifically, its inclusion in a DES formulation appears to improve its antimicrobial effects. This is reflected by the fact that the Me:SA (81.46% Me + 18.54% SA) NADES formulation

**Table 2**

Inhibition halo (diameters (mm)±SD) for the various NADES formulations and individual components. Results are presented by formulation/compound for each microbial strain tested. NI – no inhibition, NT – Not tested, D- Deposit (Eutectic blend precipitated on the agar media impossibilities halo measurement).

DES/ Compound	Bacterial strain					Yeast strain
	<i>E. coli</i>	<i>P. aeruginosa</i>	<i>S. aureus</i>	MRSA	MRSE	<i>C. albicans</i>
Me	NI	NI	13.00±0.81	14.33±0.47	13.33±0.47	18.33±0.47
LA	NI	NI	15.67±1.53	13.33±0.47	14.67±0.47	NI
MA	NI	NI	11.33±0.58	11.67±0.47	11.67±0.47	NI
SA	NI	NI	NI	NI	NI	NI
Me:LA 4:1	NI	NI	14.17±0.62	16.70±0.47	17.16±0.62	17.00±1.63
Me:MA 8:1	NI	NI	13.00±0.82	12.16±1.18	12.16±0.85	19.33±0.47
Me:SA 8:1	NI	NI	D	D	D	19.33±1.25
Gentamicin	29.17±0.85	35.17±0.62	36.33±0.58	19.00±0.00	27.83±0.62	NT
Fluconazole	NT	NT	NT	NT	NT	32.67±0.47
Sterile water	NI	NI	NI	NI	NI	NI

**Table 3**

MIC and MBC/MFC values of individual counterparts and NADES. Results are presented by formulation for each microbial species and strains tested. ND- Not dissolved.

DES/Raw materials	MIC (µg/mL)				MBC/MFC (µg/mL)			
	Bacterial strain			Yeast strain	Bacterial strain			Yeast strain
	<i>S. aureus</i>	MRSA	MRSE	<i>C. albicans</i>	<i>S. aureus</i>	MRSA	MRSE	<i>C. albicans</i>
Me	625	1250	1250	625	1250	2500	2500	1250
LA	312.5	312.5	312.5	312.5	625	625	625	625
MA	625	1250	1250	1250	1250	2500	2500	2500
SA	ND	ND	ND	ND	ND	ND	ND	ND
Me:LA 4:1	625	1250	1250	625	1250	2500	2500	1250
Me:MA 8:1	625	1250	1250	625	1250	2500	2500	1250
Me:SA 8:1	625	1250	1250	625	1250	2500	2500	1250

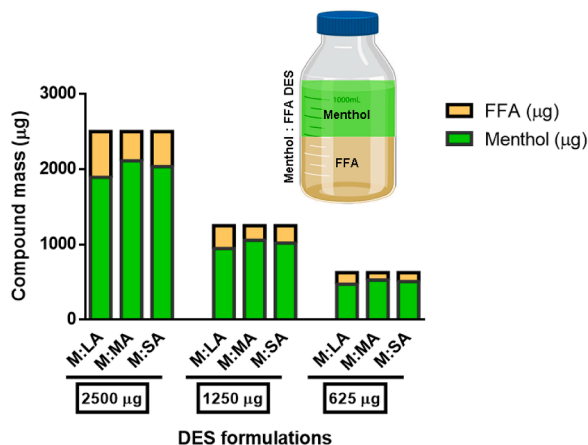


Fig. 2. Absolute mass composition of DES based on menthol and FFA.

displayed similar MIC/MBC values to that of isolated Me, even though isolated SA showed no antibacterial effect. The most likely explanation for this phenomenon is an enhancement of the compound’s bioavailability resultant from an increase in solubility, due to its incorporation in a DES supramolecular structure.

Considering the overall obtained results, the Me:LA 4:1 NADES formulation was selected to conduct an additional assay regarding biofilm detachment/removal, due to the relevant antimicrobial capabilities of both the DES formulation and its individual components. For that assessment, an alamar blue assay was used as shown in Fig. 3A. The results obtained for different time points are depicted in Fig. 3B.

From the obtained results, it is evident that Me:LA 4:1 could, over time, act on the tested microorganism’s biofilm leading to its disruption. This effect is more pronounced on *C. albicans* (Hobden et al., 1995). On the other hand, *E. coli* being a Gram-negative bacteria, requires a higher concentration (2500 µg/mL) and greater actuation time (10 min) for any relevant dispersion effect. Furthermore, MRSA show itself as a mid-point in biofilm removal effectiveness when compared with *E. coli* and *C. albicans*. This is probably due to the organism’s classification as a strong biofilm producer when compared with the two other species, which may, in part, explain the difference in the results when compared

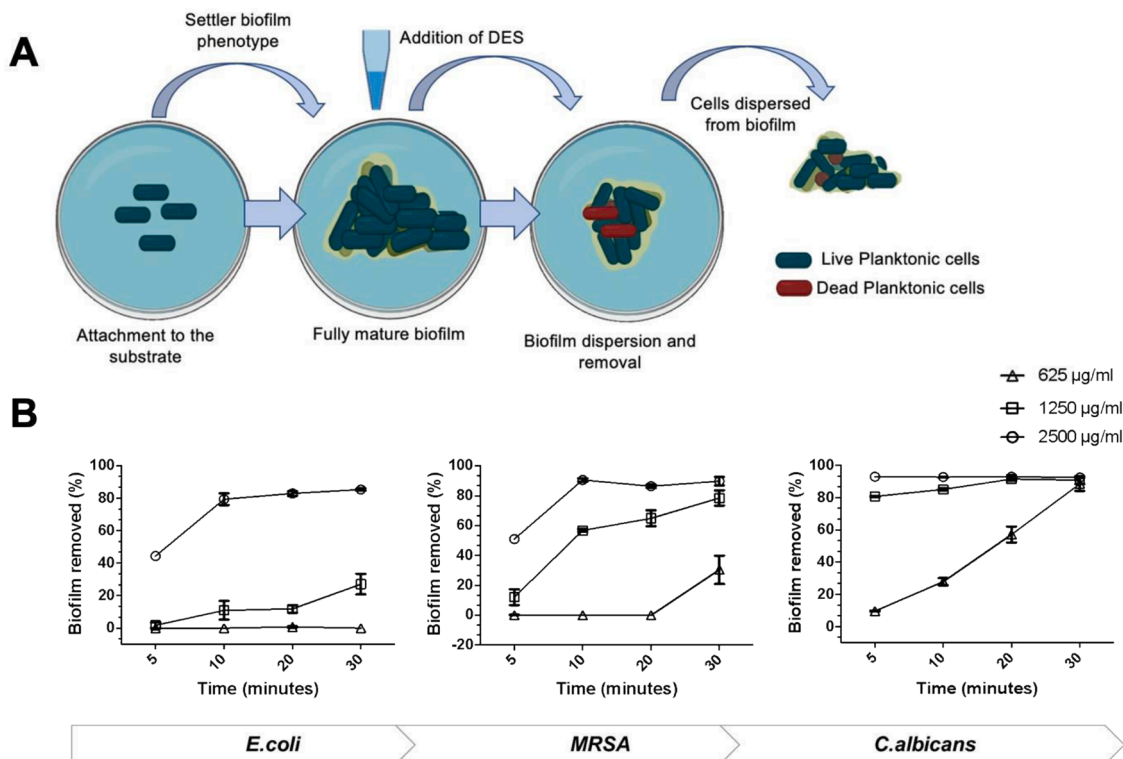


Fig. 3. (A) Proposed mechanism by which the Me:LA 4:1 eutectic formulation leads to biofilm dispersion and removal. (B) Percentage of biofilm removed upon exposure to different concentrations of Me:LA 4:1 (625, 1250 and 2500 µg/mL) for a total period of 30 min.

with *C. albicans* (Archer et al., 2011; Chandra et al., 2001; Bes et al., 2018; Igbinoso et al., 2016). Additionally, it should be highlighted that Me:LA 4:1 showed efficacy towards the used *E. coli* strain in the biofilm removal assay even though no antibacterial activity was verified in the susceptibility assay. This most likely reflects the system's direct impact on the biofilm matrix, which is majorly composed of hydrated extracellular polymeric substances that depending on the microbial species, may vary from cellulose to alginate (Archer et al., 2011; Chandra et al., 2001; Serra et al., 2013). Therefore, these results lead us to propose a dual-action mechanism for Me:LA 4:1-mediated biofilm removal/dispersal, where the system acts by both compromising the biofilm matrix and exerting cytotoxic effect towards vulnerable microorganisms. To further validate this proposal, a cultivated biofilm was exposed to 1250 µg/mL for the different established time periods and then observed under SEM microscopy (Fig. 4).

The obtained results validate the previous assumption since a progressive biofilm dispersion over time for all the microorganisms subjected to DES can be observed. Furthermore, the biofilm dispersion does not appear to be directly correlated with the biofilm reduction percentage observed in the alamar blue assay, which directly supports the theory that a dual-action mechanism of both biofilm dispersion and antimicrobial effects are in place. As previously noted, this must likely result from a disturbance of the biofilm's polymer matrix, leading not only to its detachment but also to enhancement of Me:LA 4:1 permeation. Moreover, considering the observed results from both the metabolic-based alamar blue assay and SEM imaging, it is safe to assume that the effect, which is primarily enhanced over time, is in fact the removal/dispersal of biofilm, since a gradual reduction of microbial cells is observed. Overall, these results are very appealing, since, not only a gradual dispersion of biofilm is achieved but also antimicrobial effects come into play, most likely consequence of the applied concentrations being within the MIC/MBC range. Thus, these DES formulations may represent a promising antimicrobial agent regarding all situations where microbial virulence and survival is associated with biofilm formation and maintenance, which often represents a great therapeutic challenge. From the best of our knowledge, this is the first time that the inhibition/dispersal ability of these systems were systematically studied and reported for Gram-positive, Gram-negative and fungi.

### 3.3. DES bioactivity

The bioactivity evaluation of the Me-based eutectic formulations was subdivided in three main aspects: i) cytotoxicity, ii) wound healing potential, ii) and anti-inflammatory activity indirectly assessed by DES

ability to control ROS production. The DES cytotoxic effect towards an immortalized keratinocyte cell line was assessed in order to select the concentration range which would be used in further assays (Table 4). Keratinocytes are the main type of cells found in the epidermis, the outermost layer of the skin. They constitute the vast majority of epidermal skin cells and are distributed in all epidermis layers from *stratum basale* to *stratum corneum* (McGrath et al., 2004; Barbieri et al., 2014). So, by assessing DES cytotoxic effect, one can have a preliminary safety indication of the toxicity/safety associated with using such DES in epidermal applications.

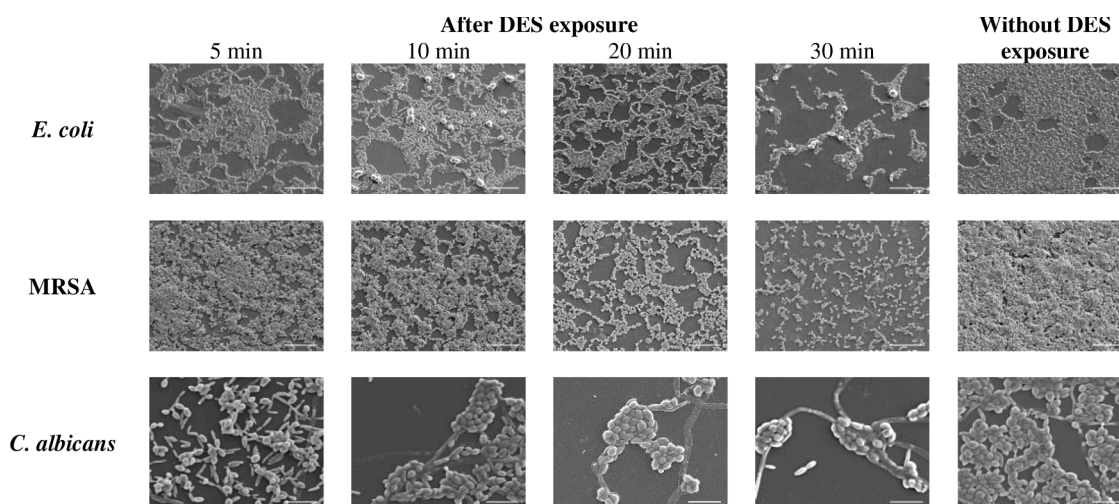
As previously reported by Silva and coworkers none of the our saturated free fatty acids isolated present cytotoxicity towards HaCaT cells. On the contrary, menthol alone presents a similar effect on cell viability compared to Me:LA (4:1) (Silva et al., 2019). Thus, from the EC<sub>50</sub> values described in table 4 it could be hypothesized that within the range of concentrations tested, the observed cytotoxic effect of these NADES formulations may be attributed to the presence of menthol. Considering the opposite cytotoxic profile of these FFA compared to Me, within the range of concentrations tested, these results highlight the synergetic/addictive effect of the deep eutectic system supramolecular structure on keratinocyte cell viability. Previous reports already provide an indication on the wound healing potential of DES comprising Me:SA (Silva et al., 2019).

Human skin and specially its outermost layer, epidermis, plays an essential role as a physical barrier against a wide range of external aggressions. Most importantly, its organized epithelium serves as a preventive barrier from invading pathogens but also plays an essential role as a sensitive organ concerning external stimuli allowing thereafter the body to respond accordingly (Campbell and Lichtensteiger, 2003). Thus, in other words, skin preserves the internal organism environment while providing protection against external negative stimulus (McGrath et al., 2004; Barbieri et al., 2014). With such a challenging task to perform,

**Table 4**

EC<sub>50</sub> values of DES obtained in the cytotoxicity assay. Results were obtained from three independent experiments performed in triplicate.

System	EC <sub>50</sub> (mM)
Me:LA (2:1)	13.99 ± 1,41
Me:LA (4:1)	4.38 ± 0,75
Me:LA (8:1)	34.17 ± 1,62
Me:MA (4:1)	24.44 ± 1,90
Me:MA (8:1)	9.13 ± 1,72



**Fig. 4.** Biofilm SEM imaging from three distinct microorganisms seeded in cover slips with different exposure times to Me:LA 4:1. Results are presented by microorganism for the various exposure times applied. Scale bar is 10 µm and magnification is 1000x.

one of skin's most challenging situations is upon skin disruption, which often represents a severe perturbation of the organism's homeostasis. Thus, upon a skin disruption, or simply called a wound, the skin immediately starts the regeneration process. The wound healing process is divided in 4 main overlapping steps i) hemostasis; ii) inflammation; iii) proliferation and iv) remodeling (Enoch and Leaper, 2008). Among them, keratinocytes have a protagonist role in the proliferation and remodeling stages in restoring the epidermis normal structure (Mansbridge and Knapp, 1987).

In this study, considering that Me:LA (4:1) presented the most promising antimicrobial and antibiofilm performance, this system was selected for further wound healing assays.

Fig. 5 discloses the micrographs of the scratch assay performed in a 2D cell monolayer of keratinocytes and it is possible to conclude that, although it was not observed a wound closure enhancement over the

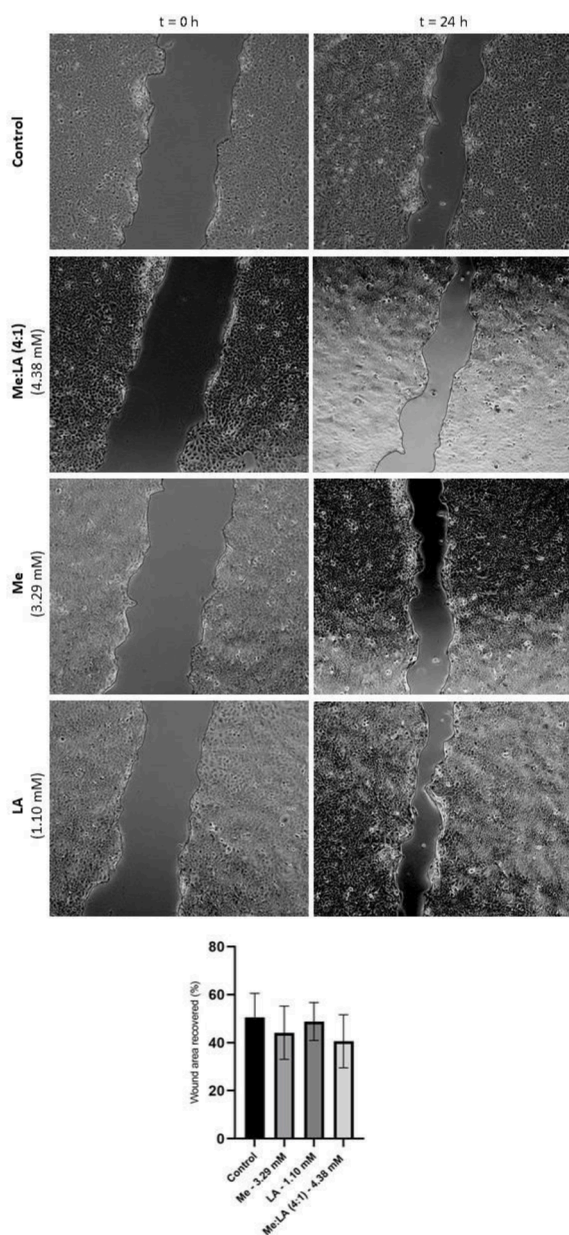


Fig. 5. Wound healing assay. (A) Optical microscopy micrographs of the scratch assay performed in a 2D cell monolayer of HaCaT (magnification 50x). (B) Results were expressed in terms of percentage of wound closure relative to the control using the mean  $\pm$  SD of six independent experiments performed in duplicate.

control in the performed assays, the DES formulations did not compromise normal keratinocytes cell viability and retained similar wound healing properties as the control (Fig. 5).

In this work we have studied further these DES, namely in which concerns the role of reactive oxygen species as this has been widely described in cell homeostasis and survival (Dunnill et al., 2017). Moreover, the production and increase of ROS is also associated with the inflammatory cascade (Trenam et al., 1992). To evaluate the production of intracellular ROS and the effect of the NADES in controlling ROS production, cells were exposed to the DES formulations in order to evaluate if the intra ROS production was affected. To assess that, a DCFH-DA probe was used since it is the most widely used probe to detect oxygen species. When in the presence of ROS, DCFH is oxidized to fluorescent dichlorofluorescein (DCF) and so the fluorescence emitted by the DCF is directly proportional to the concentration of ROS (Yu et al., 2021). The obtained results for Me-based DES, and their respective individual compounds (Fig. 6), shown that all the tested DES formulations promoted a significant decrease in the intracellular ROS production, when compared with the control, indicating a possible role of the Me-based NADES as anti-inflammatory agents.

#### 4. Conclusion

As efforts are ongoing to identify alternatives to antimicrobial treatments, in this work we present a strategy based on DES to explore these limitations, responding to the need of avoiding microorganism's adhesion and/or proliferation. Our comprehensive strategy resulted in the identification of DES based on Me and FFA that are effective in restraining microbial adhesion and proliferation, while possessing desirable physicochemical properties. In specific, the observed thermo-responsiveness of Me:LA 4:1, at near physiological temperature, has great potential regarding the functionalization of surfaces for wound dressing or other on-contact applications. The results show the antimicrobial capabilities of the developed DES against clinically relevant Gram-positive bacterial and fungal strains. Furthermore, while no direct bactericidal/bacteriostatic effect was verified for the Gram-negative bacteria tested, our results show that Me:LA 4:1 formulation was able to promote biofilm detachment/removal in all microorganisms tested, although most likely via different mechanisms. Considering the current

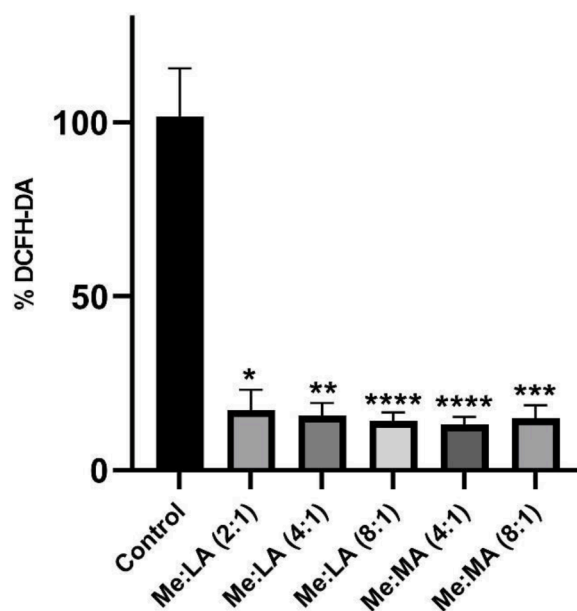


Fig. 6. Intracellular ROS results obtained for the Me-based DES. Results were obtained from three independent experiments performed in triplicate. Data indicated as mean  $\pm$  SD.

paradigm of microbial resistance towards conventional antibiotics and having in mind the importance of biofilm formation in microbial virulence in skin, the capacity to promote biofilm detachment/removal of the presented eutectic formulations could represent a strategy to deal with non-desired microbial invasion without promoting microbial drug resistance. Moreover, these Me-based DES formulations revealed a non-cytotoxic profile towards epidermal cells and did not compromise the normal cell proliferation and migration expect for these cells upon a tissue disruption. Additionally, the presented DES positively contribute to decrease cell stress by diminishing the production of ROS, which could thereafter be correlated with a positive role in control of the inflammatory cascade associated with wounds.

This study provided insights on the potential of Me and FFA-based DES, as alternative antimicrobial, and anti-inflammatory agents with the potential to be properly translated into clinical practice.

### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. Ana Rita C. Duarte has patent WOUND HEALING ENHANCERS, METHODS OF OBTAINING AND USES THEREOF pending to Universidade Nova de Lisboa.

### Data Availability

The data that has been used is confidential.

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