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A closer look in the antimicrobial properties of deep eutectic solvents based on fatty acids

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ABSTRACT

Microbial infection is a serious and challenging clinical complication that has attracted widespread interest in last decades. In this context, we present a strategy based on deep eutectic solvents (DES) to explore these unmet medical needs. DES systems based on saturated fatty acids, namely, capric acid (CA), myristic acid (MA), lauric acid (LA) and stearic acid (SA) were produced and fully characterized at a physicochemical level. The thermal characterization results indicate a depression of the melting point in DES form when compared with the starting compounds to near-physiological levels, whereas via polarized optic microscopy insights on the homogeneity/separation of the counterparts were obtained. Regarding, physicochemical properties, temperature also has a great effect on the viscosity of the eutectic systems, the higher the temperature the lower the viscosity observed. The antimicrobial potential of DES systems was evaluated against a broad spectrum of microorganisms. The obtained results show that DES retain the antimicrobial of the counterparts possibly present synergistic effects between components, mainly in the CA:MA formulation. The systems revealed significant antimicrobial activity against the tested Gram-positive bacteria and *C. albicans*, with the CA:LA system showing the greatest overall inhibitory/bactericidal activity. This system was then used for a biofilm removal/detachment assay where relevant activity is evident against the prementioned organisms and *E. coli* without need of additional physical force. The obtained results illustrate the potential of saturated fatty acid-based DES when compared with isolated fatty acids as preventive/therapeutic options against microbial infections and/or components of novel thermoresponsive biomedical devices for antibacterial purposes.

1. Introduction

The development of new designer green solvents that exhibit far enhanced properties compared with those of organic solvents is a key feature in green chemistry, as it allows to circumvent the harsh conditions currently employed in several chemical processes (e.g., separation and extraction processes) (Hayyan et al., 2015; Liu et al., 2015; Mbous et al., 2017; Mouden et al., 2017). Introduced by Abbot and coworkers in 2004 (Abbott et al., 2003, 2004), deep eutectic solvents (DES) have emerged as a powerful alternative to the conventional organic solvents and also to their analogues ionic liquids (ILs) (Mouden et al., 2017; Alonso et al., 2016; Tang and Row, 2013; Xu et al., 2017). DES are commonly defined as a mixture of two or more compounds, which at certain molar ratio present a significant reduction on the melting point of the pure starting compounds (Liu et al., 2015; Mbous et al., 2017; Mouden et al., 2017; Smith et al., 2014; Tang et al., 2015). The depression on the melting point to values lower than the ones predicted from the known enthalpies of fusion of the individual compounds, is usually ascribed to the establishment of hydrogen bonding interactions between the compounds (Gutiérrez et al., 2009, 2010). Besides hydrogen bonding interactions, electrostatic interactions and Van der Waals forces may also play an important role (Alonso et al., 2016; Tang and Row, 2013; Smith et al., 2014; Abbott et al., 2006; del Monte et al., 2014; Paiva et al., 2014).

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The variety of compounds able to form a DES are virtually unlimited accounting for possibly to 10^6 different combinations, turning these solvents highly versatile and flexible for several applications (Alonso et al., 2016; Tang and Row, 2013; Smith et al., 2014; Abbott et al., 2006; del Monte et al., 2014). The biological and physicochemical properties of DES can also be finely tuned by the nature of the chemicals used to form a DES, coupled with a proper selection of the molar ratio, water content and temperature (Paiva et al., 2014; Dai et al., 2015; Jeong et al., 2015). DES share some of the promising characteristics of ILs but contrarily to ILs, DES fully obey the green chemistry metrics (Paiva et al., 2014; de María and Maugeri, 2011; Pena-Pereira and Namieśnik, 2014; Zhao and Baker, 2013). The major advantages over their analogue solvents are the easy preparation, lower cost of production, broader selection for design purposes and biodegradability (Alonso et al., 2016; Mainberger et al., 2017; Wagle et al., 2014; Zainal-Abidin et al., 2017). All these remarkable properties accelerate the pace to a strong entrance of DES in several fields, including the CO_2 capture, biocatalysis, electrochemistry, organic synthesis, cosmetics and biomedical applications (Alonso et al., 2016; Tang and Row, 2013; Smith et al., 2014; Abbott et al., 2006; del Monte et al., 2014). One of the major field of application for DES is the extraction of diverse bioactive compounds from various biomass types (e.g., phenolics compounds from plants, microalgae fractionation, lignocellulosic materials) due to the ability of DES to be used either as an extraction solvent or as a delivery medium of the extract (Kumar et al., 2018; Ruesgas-Ramón et al., 2017; Sed et al., 2018). In the biomedical field, DES have been extensively used to dissolve and extract biopolymers and also to preserve biological materials (e.g., DNA, G-quadruplexes, cells) (Paiva et al., 2014; Castro et al., 2018). DES have also shown ability to improve the solubility, permeation and absorption of a wide spectrum of solutes, including the active pharmaceutical ingredients (API's), being hence called, therapeutic deep eutectic solvents (THEDES) (StottWilliams and Barry, 1998; Aroso et al., 2016; Duarte et al., 2017; Silva et al., 2018; Morrison et al., 2009). Few recent publications have also made significant contribution on the development of DES with antibacterial properties which suggest the potential of DES as a broad-spectrum antiseptic agent for therapeutic and also preventive applications (Radošević et al., 2018; Wikene et al., 2015; García-Argüelles et al., 2013; Zakrewsky et al., 2016; Zhao et al., 2015; Hayyan et al., 2013).

Based on that, herein, the main goal was to unveil the antimicrobial potential of fatty acids while in DES form due to the possible synergistic effects among the counterparts. The antibacterial and antifungal properties of fatty acids are well-known and synergistic effects among fatty acids have also been reported (Batovska et al., 2009; Kitahara et al., 2004; Lee and Jo, 2016; Nakatsuji et al., 2009). Furthermore, fatty acid's antimicrobial properties are mainly attributed to nonspecific action mechanisms, i.e. membrane dissolution/destabilization, which is useful not only due to its widespread effect on multiple microorganisms but also due to the lower chance of resistance dissemination/acquisition (Desbois and Smith, 2010; Kabara et al., 1972; Ouattara et al., 1997; Zheng et al., 2005). However, to the best of our knowledge up to now the antimicrobial properties of DES based on fatty acids were not yet reported. The antimicrobial properties of fatty acids are dependent on several factors including chain size, hydrophobicity, number and position double bonds and functional groups (Desbois and Smith, 2010; Kabara et al., 1972; Zheng et al., 2005; Campbell and Cronan, 2001; Galbraith and Miller, 1973). Thereby, our main interest in this study was to get insights on the possible therapeutic use of DES based on saturated fatty acids, combining capric acid (CA) 10C with other saturated fatty acids with different chain size length (i.e., lauric acid (LA) 12C, myristic acid (MA) 14C, stearic acid (SA) 18C, as reported elsewhere (Wang and Meng, 2010). The selected blends present a melting temperature near the physiological temperature and are composed by fatty acids with well-known antibacterial properties. Our comprehensive strategy includes the physicochemical characterization followed by a screening of their antimicrobial properties against a wide spectrum of microorganisms, according to their prevalence in most of the health problems and also their common use for testing of different substances (Fig. 1). Additionally, the potential of these DES systems to promote a spontaneous detachment of biofilms were also evaluated without the use of additional antibiotics and physical forces.

2. Materials and methods

2.1. DES preparation

During the preparation of the DES, MA (ref.70082, Sigma Aldrich), SA (ref. 175366, Sigma Aldrich) and CA (ref. C1875, Sigma Aldrich) were used as raw materials. The systems were prepared according to a previous work reported elsewhere (Wang and Meng, 2010). Briefly, the systems were prepared by simply mixing the components at 70 °C, under constant stirring. After 30 min, a clear liquid solution was formed, and the mixture was slowly cooled down to room temperature (RT) until further use.

2.2. Polarized optical microscopy (POM)

Optical characterization of different formulations of DES (i.e., CA:LA; CA:SA; CA:MA) was carried out at RT by POM using and Olympus BH2 transmission microscope (Olympus, UK) coupled with a Leica digital camera DFC 280 (Leica, UK). The formulations were deposited on a microscopic glass slide and micrographs were obtained at RT.

2.3. Thermal analysis

Differential Scanning Calorimetry (DSC). The DSC experiments were performed in a TA instrument DSC Q100 model (Thermal analysis & analysers, USA). The temperature ramp included a heating step from 0C to 80 °C (heating rate 5 °C min^{-1}), followed by an isothermal step of 2 min prior to cooling down the system to 20 °C.

2.4. Viscosity measurements

The viscosity of the different DES was measured using a Kinexus Prot Rheometer (Kinuxus Prot, MaL 1097376, Malvern) fitted with parallel plate geometry with diameter of 20 mm (PU20 SR1740 SS) and gap of 1 mm. The viscosity of the different formulations of DES was assessed under controlled stress conditions and with a shear rate of 10 s^{-1}. After equilibrating the sample temperature at 20 °C during 5 min, a temperature scan was performed from 50 °C to 15 °C at 2 °C min^{-1}.

2.5. Disk diffusion assay

The antimicrobial activity of DES was determined using *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 700698 (Methicillin-resistant strain, MRSA), *S. epidermis* ATCC 35984 (Methicillin-resistant strain, MRSE), *Pseudomonas aeruginosa* ATCC 27853, *Escherichia coli* ATCC 25922 and *Candida albicans* ATCC 90029, according to the guidelines of Clinical and Laboratory Standards Institute (CLSI). Initially, the antimicrobial activity was screened out using the disc diffusion approach, adapting a methodology elsewhere described by Ferreira et al. (AbbottBoothby et al., 2004). Gentamicin sulphate (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 fatty acid was also evaluated. The discs (CT0998B, Oxford) were prepared by incubating them in DES formulations and controls, as elsewhere reported (Silva et al., 2019). The disks impregnated with the different formulations were placed on the inoculated agar. The plates were incubated during 24 h at 37 °C (bacterial strains) and 30 °C (*C. albicans*). After incubation, the diameter of the clear zone (inhibition halo diameter) was determined to assess the antimicrobial properties of each formulation.

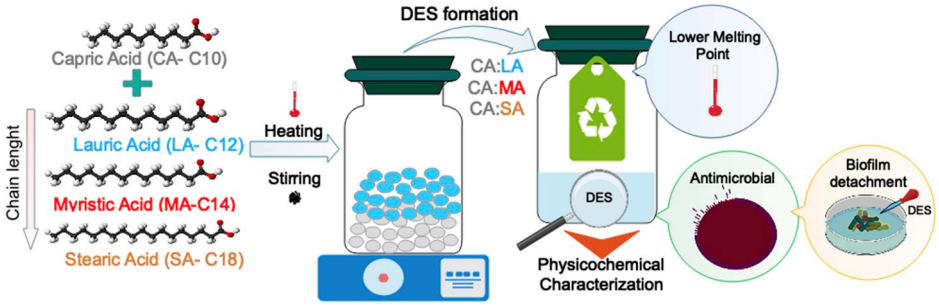


Fig. 1. Schematic illustration of the production steps of DES and their physicochemical and antimicrobial assessment.

2.6. Assessment of minimal inhibitory concentrations (MICs), minimal fungicidal concentration (MFCs) and minimal bactericidal concentration (MBCs)

MICs/MFCs/MBCs were determined by microbroth dilution technique. Briefly, standard solutions of fatty acids in powder and DES form were prepared under sterile conditions by first dissolving the weighted compounds in DMSO (ref. 276855, Sigma), followed by serial dilution in Müller-Hinton Broth medium (MHB, ref 70192-500 g, Sigma Aldrich) maintaining a final 10%(v/v) percentage of DMSO. During the dilution process, the microtubes were warmed up to 70 °C and homogenized using an ultrasonic bath. Afterwards, the microtubes containing the different formulations (500 μL/tube) were inoculated with 500 μL of bacterial suspension at 1–2x10^6 CFUs/mL in MHB. As control, MHB (i.e., bacteria-free), MHB (with no added compounds), a solution of 10% (v/v) DMSO in MHB and the pure components were used. The microtubes were incubated at 37 °C (bacterial strains) or 30 °C (*C. albicans*) under shaking for 24 h and the bacterial growth was monitored. The MICs/MFCs of each compound were considered as the concentrations at which the tested formulations prevent the formation of turbidity in the microtube. However, to confirm such results and to avoid underestimated values (i.e., the compounds itself may cause turbidity), subcultures of each concentration, were performed onto trypticase soy agar (TSA, Ref. 610052, Frilabo) for 24 h. This assay allows to confirm the MIC/MFC values, as well as, the determination of the MBC of each compound. The MBC were considered as the minimal concentration of each formulation required to lead to complete bacterial death.

2.7. Assessment of biofilm removal efficiency

S. aureus ATCC 700698, *C. albicans* ATCC 90029 and *E. coli* ATCC 25922 were spread into TSA plates and left growing for 24 h at optimal temperature for each organism, 37 °C and 30 °C for bacteria and yeast, respectively. Posteriorly, suspension cultures were prepared by inoculation of single colonies into TSB and grown for additional 24 h. Cultures were then harvested, resuspended in TSB and adjusted to approximately 1–2x10^4 CFUs/mL. Subsequently, 200 μL of the previously prepared dilution was transferred into wells, in a 96-well polystyrene flat-bottom plate and incubated during 20 h at optimal temperature, for biofilm formation. Following biofilm formation, microbial suspensions were carefully removed, and sample solutions of CA:LA were added at different concentrations (625, 1250 and 2500 μg/mL) in ddH_2O with 10%(v/v) DMSO. After different exposure times (5, 10, 20 and 30 min) sample solutions were removed and 200 μL of MHB media containing 10%(v/v) alamar blue dye (ref. BUF012B, Bio-Rad) added into the wells, followed by plate incubation for 1 h, at either 37 °C or 30 °C. Absorbance at 570 and 600 nm was then measured using a microplate reader (SYNERGY HT™) to calculate the removal efficiency of biofilms. SEM images were also obtained using a JEOL JSM-6010 LV (JEOL, Japan). Biofilms were established in cover slips (ref. 83.1840.002, Sarstedt) for 20 h and treated with CA:LA (at 1250 μg/mL) for the above-mentioned time points. Posteriorly, sample solutions were gently removed, and microorganisms fixed using a phosphate-saline buffer with 10% (v/v) formalin for 1 h and washed with deionized water. Fixed biofilm was then dehydrated using by immersion in solutions with increasing concentrations of alcohol (20%, 50%, 70%, 90% and 100%). Finally, samples were left dry in a safety cabinet overnight and then gold-sputtered for SEM image acquisition.

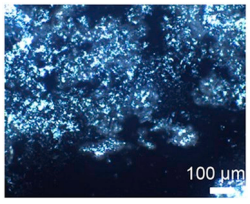
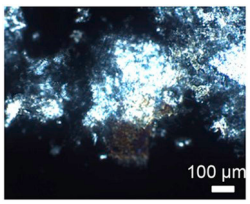
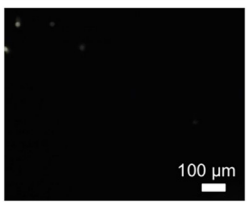
3. Results and discussion

3.1. DES preparation and characterization

In this work, DES based on saturated fatty acids were prepared as elsewhere reported by Meng and coworkers (Wang and Meng, 2010) and the formation of a liquid at RT was assessed (Table 1). DES based on CA and LA are liquid at RT (i.e., no insoluble particles are detected), whereas the other formulations are pasty-like solid at RT. The formation of a liquid is a strong indication of loss of lattice arrangement.

These results were further confirmed by POM, which is a common tool used to assess the existence of crystal-like structures in the eutectic mixture even if the crystals are mixed with a liquid phase (Aroso et al., 2016). The POM micrographs at RT corroborated the visual observation as in CA:LA no crystal-like formations are distinguished. Thereby, this formulation presents a micrograph with a full black background that is

Table 1
Summary of the different DES prepared in this study with their respective visual aspect and POM micrographs. The scale bar is 100 μm.

DES	Molar Ratio	Observation/visual aspect	POM at room temperature
CA:SA	4:1	White solid at RT	
CA: MA			
CA:LA	3:1	White solid at RT	
	2:1	Transparent liquid at RT	

an indicative of a homogeneous liquid phase at RT.

The POM assessment was combined with DSC analysis to get insights on the thermal events of these eutectic mixtures. According to DES definition, the melting point of the eutectic mixture should be far below than that of each counterpart due to the establishment of intermolecular hydrogen bonds and occasionally Van der Waals interactions (Gutiérrez et al., 2009, 2010). In order to evaluate this, the thermograms of the powder and DES were performed (Fig. 2) and the results corroborated previous data on the literature (Wang and Meng, 2010; Yuan et al., 2014). The thermograms of each fatty acid present a well-defined and unique sharp endothermic peak at ≈ 32.3 °C, ≈46.6 °C, ≈ 58.6 °C, ≈ 73 °C, for CA, LA, MA, SA, respectively. Additionally, the melting peak of CA:LA, CA:MA and CA:SA is ≈ 22.7 °C, ≈24.6 °C, ≈ 28.8 °C, respectively. The peaks obtained in DES systems are different from those of the parent species and a clear depression in the melting point occurs which further suggests the supramolecular rearrangement while the compounds are in DES form. Usually the depression on the melting point is dependent on the nature of the counterparts, molar ratio, lattice energy of DES and also entropy changes arising from the formation of DES (Smith et al., 2014; Abbott et al., 2006; Mainberger et al., 2017; Khandelwal et al., 2016). In all the DES a depression of the melting point occurs and it lowers up to near-physiological levels when compared with the starting compounds, which turns meaningful to explore the properties of these eutectic systems in different fields, namely in biomedical field.

The viscosity of the systems was also evaluated as a function of temperature for the different formulations of DES (Fig. 3), as it is a parameter that restricts the possible pharmaceutical application of DES since it influences the diffusion of a liquid out of a solid support. The results obtained indicate that the viscosity is inversely related with temperature, i.e., the viscosity decreases as the temperature increases. Similar results have been reported for other formulations of DES, being known as Arrhenius-like behavior (Aroso et al., 2016, 2017; Craveiro et al., 2016). The DES with higher viscosity is CA:SA, followed by CA:MA and lastly, CA:LA. This data also suggests that the viscosity is dependent on the chain size of the saturated fatty acids, corroborating the DSC data (Fig. 2) (Dai et al., 2015; Radošević et al., 2016; Florindo et al., 2018; Hayyan et al., 2012; Stefanovic et al., 2017). Beyond the effect of chain length size, it should be pointed out that the viscosity is also related with a vast hydrogen bonding network that is stablished between the counterparts of DES (Zainal-Abidin et al., 2017).

3.2. Antimicrobial assessment

To evaluate the antimicrobial potential of the various DES formulations, firstly a disk diffusion assay was carried out using the selected microbial strains. Inhibition halo measurements are presented in Table 2. The representative images of the obtained plates can be found as supplementary information (Tables S1–S6).

As can be easily observed by analyzing Table 2, the DES formulations only show antibacterial activity against the tested Gram-positive bacteria (resistant and non-resistant) and *C. albicans*, displaying no activity against the *P. aeruginosa* and *E. coli* (i.e., inhibition halo ≈ 0 mm) strains used. These results are in accordance with other accounts in the literature, as Gram-negative bacteria are usually resistant to the antibacterial activity of fatty acids due to a more complex membrane structure, with several studies reporting that the presence of lipopolysaccharides on the cell wall prevents the fatty acids from reaching cell membrane and exerting its effect (Kitahara et al., 2004; Desbois and Smith, 2010; Ouattara et al., 1997; McGaw et al., 2002). On the other hand, the cell wall of Gram-positive bacteria readily absorbs fatty acids allowing their passage into the inner membrane (Kitahara et al., 2004; Ouattara et al., 1997). C. *albicans* is reported as being able to modulate its membrane surface hydrophobicity during growth and morphogenesis, which may explain its susceptibility to these compounds as it displays a hydrophobic membrane surface during certain stages of its life cycle to promote, among other things, virulence by enhanced adhesion to cells (Glee et al., 1995; Hobden et al., 1995). Furthermore, it should be noted that the overall lower susceptibility observed in *C. albicans* is possibly due to the lower incubation temperature for this microorganism (30 °C) when compared with that used for the bacteria strains (37 °C), which affect the diffusion of the compounds/DES formulations through the solid media, together with the lower viscosity at a higher temperature (Fig. 3).

In fact, both LA and CA have documented inhibitory activity against *C. albicans* fact that for LA is not reflected in these results (Huang et al., 2011). Likewise, MA's lower activity and SA's complete inactivity may result from their inability to diffuse through the media as these fatty acids have substantially higher melting points than both LA and CA (Fig. 2).It should be noted that for all DES formulations present an inhibition halo for *C.albicans* (i.e., inhibition halo ≈ 12.7–15 mm), which further suggest the higher diffusion of DES formulations at lower temperature when compared with the individual counterpart. Taking into account the results of this initial screening, *S. aureus*, MRSA, MRSE and *C. albicans* were selected for MIC/MBC/MFC assessment and the determined concentration values are presented in Table 3. Firstly, it should be noted that the determination of antibacterial activity of fatty acids, in suspension culture, is not straightforward due to the turbidity of lipidic solutions and their overall lack of solubility in water, as elsewhere reported (Khuwijitjaru et al., 2002; Oliveira et al., 2009). To solve this issue, DMSO was added to the medium in such a percentage that did not show any effect on MICs and MBCs determination. It should be noted that the dilutions performed did not weaken and/or disrupt the vast network of intermolecular interactions, as reported elsewhere for other DES formulations in this concentration range (Hayyan et al., 2015, 2016; Silva et al., 2019; Juneidi et al., 2015; Radošević et al., 2015). The main advantage of DES concerns in the formation of a supramolecular

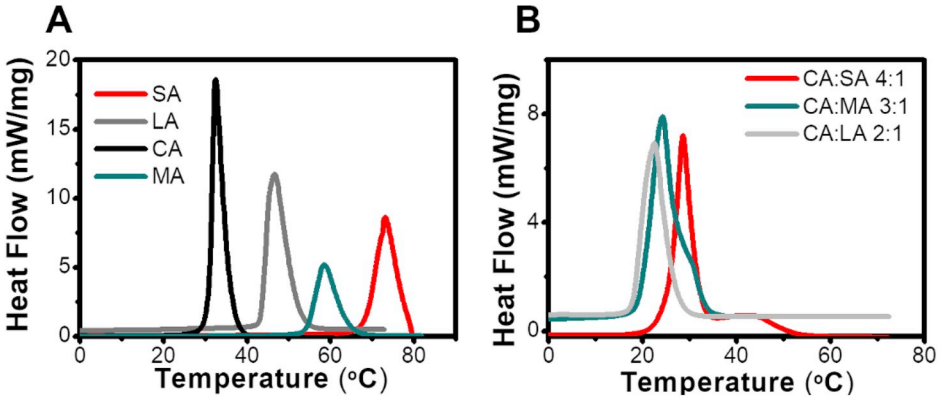


Fig. 2. DSC thermograms obtained for powders (A) and DES (B). Peaks arising above the baseline represent endothermic peaks.

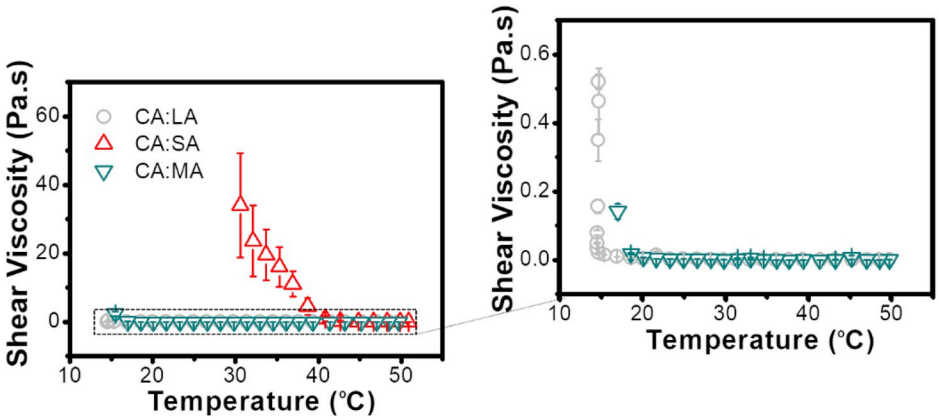


Fig. 3. Variation of the viscosity of the different formulations of DES as a function of the temperature.

Table 2
Inhibition halo measurements (diameter (mm) ± SD) for the various DES formulations and individual counterparts. Results are presented by formulation/compound for each microbial strain tested. NI – no inhibition, NT – Not tested.

DES/Compound	*E. coli*	*P. aeruginosa*	*S. aureus*	*MRSA*	*MRSE*	*C. Albicans*
CA	NI	NI	15.00 ± 0.00	13.33 ± 0.47	20.33 ± 0.94	17.33 ± 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
CA:LA	NI	NI	15.67 ± 0.58	16.50 ± 0.41	20.00 ± 0.82	13.5 ± 0.41
CA:MA	NI	NI	14.00 ± 1.00	14.33 ± 0.47	15.67 ± 0.47	12.7 ± 0.47
CA:SA	NI	NI	14.67 ± 0.58	15.67 ± 0.47	15.00 ± 0.82	15.0 ± 0.50
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	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Table 3
MIC/MBC/MFC values of individual counterparts and DES. Results are presented by formulation for each microbial strain tested. ND- Not dissolved.

DES/Raw materials	MIC (μg/mL)				MBC/MFC (μg/mL)			
	Bacterial strain			Yeast strain	Bacterial strain			Yeast strain
	S. aureus	MRSA	MRSE	C. albicans	S. aureus	MRSA	MRSE	C. albicans
CA	625	625	625	625	1250	1250	1250	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
CA:LA	625	625	625	625	1250	1250	1250	1250
CA:MA	625	625	625	1250	1250	1250	1250	2500
CA:SA	1250	1250	1250	1250	2500	2500	2500	2500

arrangement established due to intermolecular interactions such as hydrogen bonding interactions and van der waals. Thereby, when using a physical mixture of compounds different behaviors are obtained as in that situation a simple dissolution of each compound in a certain medium occurred without any supramolecular arrangement, as previously reported (Silva et al., 2019; Pereira et al., 2019).

The MIC/MBC/MFC results confirm the hypothesis that the inhibition halos were most likely influenced by the diffusion ability of the compounds and incubation temperature. This is particularly true for *C. albicans*, where both LA and MA that displayed no activity during the disk diffusion assay, possess MIC/MBC/MFC concentration values equal to those observed in the bacterial strains tested. As such, it can be stated that a negative result in the disk diffusion assay does not, by itself, suffice as an exclusion criterion for further downstream analysis in this particular context. Regarding the MIC/MBC/MFC values obtained for the DES formulations and individual counterparts, results are as expected with LA, CA and their correspondent eutectic blend CA:LA displaying the highest overall antimicrobial activity, followed closely by CA:MA who displayed only lesser inhibitory activity regarding *C. albicans* (i.e., ≈1250–2500 μg/mL), and finally CA:SA with the worst overall performance. The antimicrobial capabilities of individual LA and CA are well-reported in the literature with LA being the medium chain aliphatic fatty acid with the greatest overall antimicrobial activity, followed by CA. In fact, several authors have reported that, in general, medium and long-chain fatty acids tend to display a higher antibacterial effect towards gram-positive bacteria. Even when considering overall activity, the major consensus is that unsaturated fatty acids with 10 and 12 carbon chain length display the highest antimicrobial potential, which probably means that these fatty acids present the ideal hydrophobic balance for appreciable bioavailability and microbial membrane/wall permeation (Batovska et al., 2009; Kitahara et al., 2004; Nakatsuji et al., 2009; Huang et al., 2014). The less positive results observed for MA are most likely due to the slightly higher carbon chain (14C) in comparison with both CA (10C) and LA (12C), which greatly increases its hydrophobicity reducing its solubility and therefore bioavailability (Desbois and Smith, 2010; Ouattara et al., 1997; McGaw et al., 2002; Churchward et al., 2018; Yoon et al., 2018). In fact, SA dissolution was not achieved most likely due to its high carbon chain

length (18C) (Kabara et al., 1972). However, it should be noted that inclusion of MA in DES form appears to somewhat potentiate its dissolution, since CA:MA displays the same MIC/MBC/MFC values as CA, even though isolated MA is less potent. On the contrary, CA:SA shows lower inhibitory activity than isolated CA, as the established interactions in DES form in conjunction with the highly insoluble SA most likely leads to co-precipitation of both fatty acids. Thus, the data further suggest the supramolecular arrangement between both counterparts while in DES form. It is well-stablished in the literature that the supramolecular arrangements of DES may led to synergistic or additive effects, which in some cases led to more or less toxic systems in comparison with their individual counterpart (Hayyan et al., 2015, 2016; Radošević et al., 2015, 2018; Juneidi et al., 2015). Regarding DES efficacy for each of the tested microbial strains, the results are also as expected, with lower values for *S. aureus* when compared with the MRSA and MRSE strains which are, by nature, more competitive and resilient organisms (Bes et al., 2018; Igbinosa et al., 2016). Furthermore, the MIC/MFC values determined for *C. albicans* are similar to the ones obtained for both MRSA and MRSE, illustrating the potential of these DES based on saturated fatty acid as an effective treatment and preventive measure for not only bacterial, but also yeast-related infections (Huang et al., 2011; Bergsson et al., 2001). CA:LA, the most promising formulation, showed MIC/MBC/MFC concentration values of 625 μg/mL (395.21 μg of CA + 229.21 μg of LA) and 1250 μg/mL (790.42 μg of CA + 459.58 μg of LA), respectively which are in accordance with the determined values for the isolated components and existing literature (Kitahara et al., 2004; Kabara et al., 1972). The absolute mass composition of the various DES formulations is presented in Fig. 4. This widespread antimicrobial potential observed is derived, most likely, from the non-specific antimicrobial action mechanism of fatty acids, as these primarily actuate on the membrane leading to its destabilization/dissolution causing a wide range of direct and indirect inhibitory effects (Desbois and Smith, 2010; Yoon et al., 2018). Thereby, using DES based on saturated fatty acids no drug resistance is induced due to the non-specific mechanism of action. Nonetheless, depending on the counterpart used in combination with CA, the results are different, being the overall antimicrobial activity increased with saturated fatty acids with lower chain size length.

3.3. Biofilm removal-efficiency

CA:LA formulation was selected for additional antimicrobial studies, particularly for the evaluation of biofilm permeation and dissolution capability. The results obtained via the alamar blue assay after biofilm exposure to CA:LA for different time frames are presented in Fig. 5.

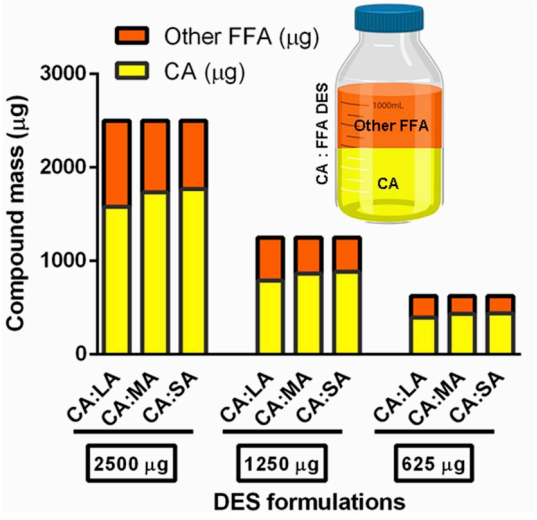


Fig. 4. Absolute mass composition of DES based on CA and other saturated fatty acids.

The results indicate that for all tested organisms CA:LA was effective in dispersing or disrupting established biofilm. Moreover, it appears that even the lowest concentration (625 μg/mL) applied over time can exert an effect comparable to the two higher concentrations used (2500 and 1250 μg/mL) with removal rates over 50% being achieved, for most cases, in 10 min. These results are comparable with previous studies performed using DES based on betaine and erythrol for the detachment of *Streptococcus mutants* biofilm (Lim et al., 2017). To the best of our knowledge, this recently published study by Lee et al., in 2017 is the only reported instance of the use of DES for microbial biofilm disruption. A noteworthy point is that even though these systems showed no bactericidal/inhibitory activity against the tested gram-negative strains, it appears that CA:LA possesses the ability to effectively disrupt the biofilm of the *E. coli* strain used in this assay. This is probably due to the system direct impact on the biofilm matrix which is majorly composed of hydrated extracellular polymeric substance that depending on the bacterial species may vary from cellulose to alginate (Archer et al., 2011; Chandra et al., 2001; Serra et al., 2013). Furthermore, it should be highlighted that the results obtained show that CA:LA can successfully disrupt biofilm without the need of additional physical force or antibiotics (Archer et al., 2011; Chandra et al., 2001; Høiby et al., 2010). Based on the obtained results, an action mechanism of DES in established biofilms where, upon exposure to CA:LA at tested concentrations there is simultaneously both biofilm detachment, due to destabilization of the polymeric matrix, and also a bactericidal effect due to the compounds bactericidal effect on 2 of the 3 tested microorganisms at the applied concentrations is proposed. This dual action mechanism explains why, even though saturated fatty acid-based DES displayed no significant antibacterial activity against *E. coli*, an effect on this gram-negative bacteria's biofilm was observed. To further validate the proposed model and the obtained results in the metabolism-based alamar blue assay, samples of cultivated biofilm exposed to 1250 μg/mL for the different established time periods were prepared for SEM microscopy (Fig. 6).

The obtained results support the above proposed model as one can observe a progressive dispersion of the biofilm over time in all microorganisms subjected to DES exposure. Furthermore, the biofilm dispersion does not appear to directly correlate 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 bactericidal effects are observed (Fig. 7).

This is most likely due to a perturbation of the biofilm's polymer matrix leading both to detachment and enhanced permeation of CA:LA. Moreover, considering the observed results from both the alamar blue assay and SEM imaging, it is reliable to state that the effect which is primarily enhanced over time is the removal/dispersion of biofilm, as a gradual reduction of microbial cells is observed in all cases. Overall, these results are very appealing since both a gradual dispersion of biofilm as well as bactericidal effects are achieved.

4. Conclusions

Inspired by the well-known antimicrobial properties of fatty acids and by an unmet medical challenge, our comprehensive strategy resulted in the production of DES based on saturated fatty acids taking advantage of possible synergistic effects among the counterparts and also from the depression on the melting point of the DES when compared with the one of individual starting compounds. The antimicrobial activity of the different formulations was evaluated against a wide panel of microorganisms. The most promising system was the CA:LA formulation with relevant antimicrobial activity towards the gram-positive bacterial strains used and *C. albicans*. Furthermore, the results obtained show that this system can promote biofilm detachment/removal in all microorganisms tested, even for gram-negative bacteria, although most likely

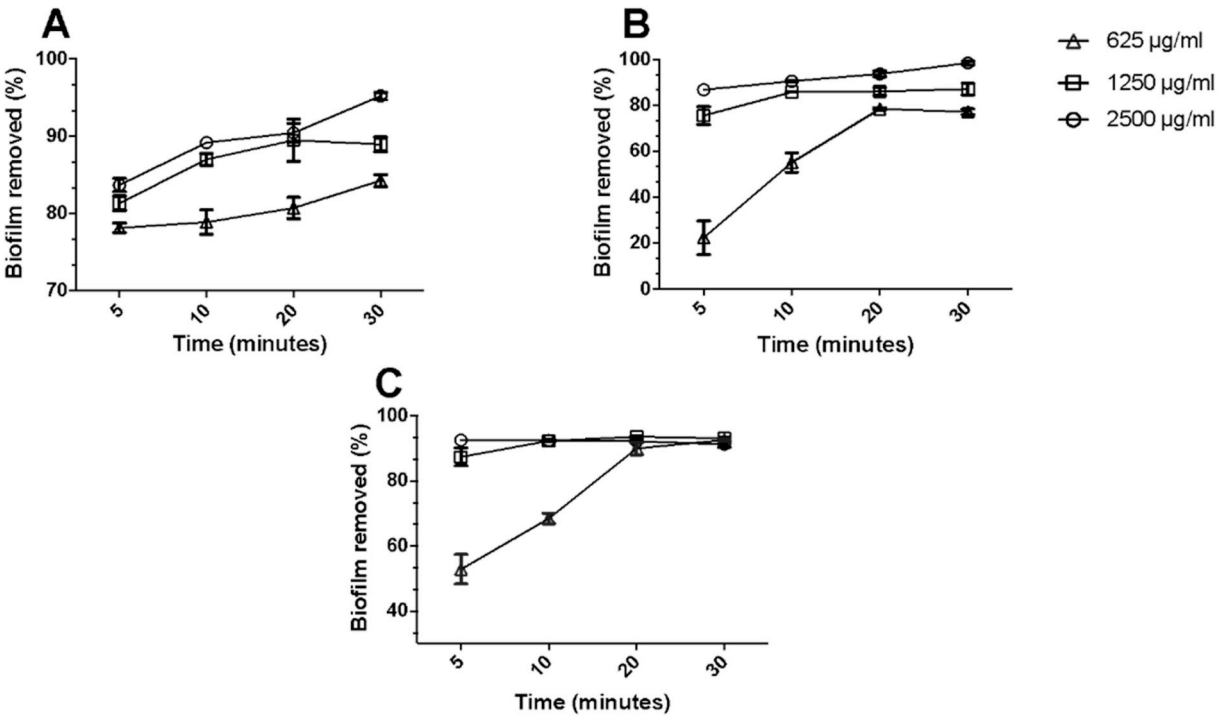


Fig. 5. Percentage of biofilm removed upon exposure to different concentrations of CA:LA (625, 1250 and 2500 µg/ml) for a total period of 30 min for (A) *E.coli*, (B) *MRSA* and (C) *C.albicans*.

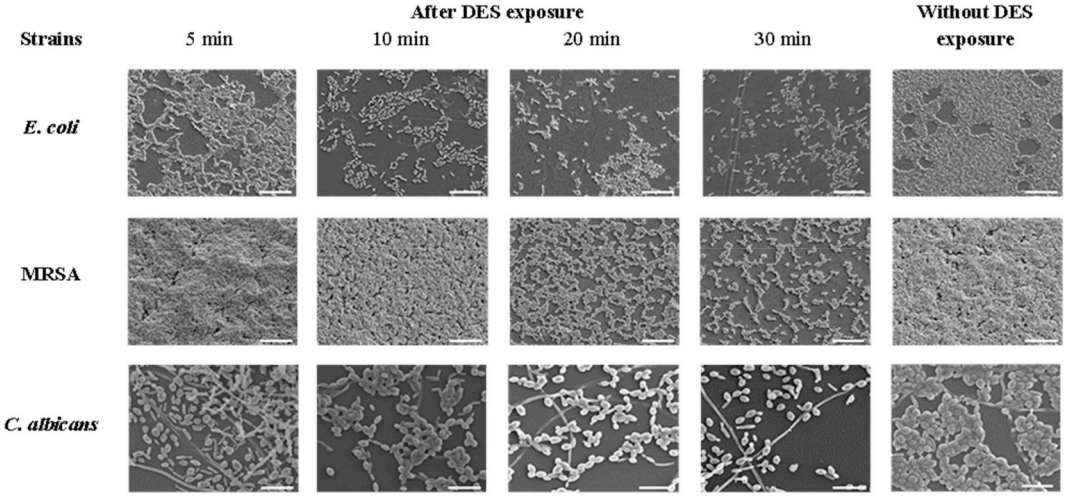


Fig. 6. SEM imaging of biofilm from three distinct microorganisms seeded onto cover slips with different exposure times to CA:LA eutectic blend. Results are presented by microorganism for the various exposure times applied. Scale bar is 10 µm-.

via different mechanisms. This study provides insight on the potential of DES based on saturated fatty acids as a possible broad-spectrum antimicrobial agent that if properly harnessed can be translated into clinical practice as an effective preventive and/or therapeutic option against microbial infections in various settings. Taking advantage of the observed thermo-responsiveness at near physiological temperature, these systems are strong candidates as components to produce novel thermosensitive biomaterials with enhanced properties. Furthermore, saturated fatty acid-based DES also show promise as potential alternatives to classical antibiotics, such as gentamicin, whose specific mechanism of action somewhat facilitates the dissemination of antibiotic resistance, in an age where the emergence of multi-resistant pathogens is a matter of great concern in the Public health agenda.

Author contributions

J.M.S. and A.R.C.D conceived of the study. J.M.S prepared and characterized the NADES. E.S. conducted the experiments with Microorganisms. J.M.S., E.S., R.L.R., A.R.C.D R. analysed the data and participated in the interpretation of results. All authors have read and approved the final manuscript.

Declaration of competing interest

None.

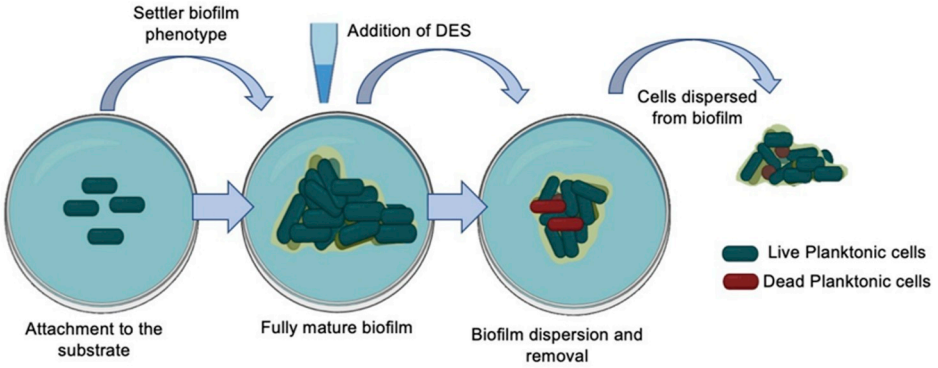


Fig. 7. Proposed mechanism by which CA:LA eutectic blend leads to biofilm dispersion and removal from substrates.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scp.2019.100192.

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