Research in Microbiology 172 (2021) 103880

Contents lists available at ScienceDirect

Research in Microbiology

journal homepage: www.elsevier.com/locate/resmic

Original Article

Pentadecanoic acid against *Candida albicans-Klebsiella pneumoniae* biofilm: towards the development of an anti-biofilm coating to prevent polymicrobial infections

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ARTICLE INFO

Article history:
Received 12 July 2021
Accepted 9 September 2021
Available online 24 September 2021

Keywords:
Polymicrobial biofilm
Pentadecanoic acid
Anti-biofilm coating
Candida albicans
Klebsiella pneumoniae

ABSTRACT

The ability to form biofilms is a common feature of microorganisms, which can colonize a variety of surfaces, such as host tissues and medical devices, resulting in infections highly resistant to conventional drugs. This aspect is particularly critical in polymicrobial biofilms involving both fungi and bacteria, therefore, to eradicate such severe infections, new and effective anti-biofilm strategies are needed. The efficacy of pentadecanal and pentadecanoic acid as anti-biofilm agents has been recently reported against different bacterial strains. Their chemical similarity with diffusible signal factors (DSFs), plus the already known ability of fatty acids to act as anti-biofilm agents, suggested to explore their use against *Candida albicans* and *Klebsiella pneumoniae* mixed biofilm. In this work, we demonstrated the ability of both molecules to prevent the formation and destabilize the structure of the dual-species biofilm. Moreover, the pentadecanoic acid anti-biofilm coating, previously developed through the adsorption of the fatty acid on polydimethylsiloxane (PDMS), was proved to prevent the polymicrobial biofilm formation in dynamic conditions by confocal laser scanning microscopy analysis. Finally, the evaluation of the expression levels of some biofilm-related genes of *C. albicans* and *K. pneumoniae* treated with pentadecanoic acid provided some insights into the molecular mechanisms underpinning its anti-biofilm effect.

1. Introduction

A biofilm is a microbial community in which cells are surrounded by a self-produced matrix of extracellular polymeric substances. The biofilm matrix promotes microbial adhesion to biotic and abiotic surfaces, protects cells, giving resistance to physical and chemical stresses, increases nutrient availability, and stimulates complex community interactions, keeping cells in close proximity [1]. Moreover, in biofilm, cells have a different phenotype and gene expression patterns compared to planktonic cells, showing a significant increase in resistance to drugs, which represents their most clinically relevant feature. Indeed, microbial biofilms are usually pathogenic and responsible for several diseases or biofilm-related infections, especially those associated with medical devices, including heart-circulatory devices, urinary catheters, contact lenses, and orthopaedic implants [2–4].

Given the high heterogeneity of human microflora, bacteria, fungi, and viruses are often isolated together from *in vivo* polymicrobial biofilms. In these microbial consortia, various types of interactions can take place among microorganisms, such as mutualism, antagonism, and commensalism, which makes polymicrobial biofilms even more complex to manage than monomicrobial ones [5].

In the clinical setting, in fact, polymicrobial biofilm-related infections complicate the diagnosis and the choice of suitable therapeutic treatment and cause significantly higher mortality compared to single-species biofilm infections; in particular, infections caused by mixed fungal-bacterial biofilms have become one of the main public health problems in clinics [6].

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https://doi.org/10.1016/j.resmic.2021.103880

Candida albicans is the most prevalent fungus isolated from fungal–bacterial biofilms; it interacts with both Gram-positive and Gram-negative bacterial partners [6,7]. *C. albicans* is a commensal fungus and, being part of the normal microflora of most humans, generally colonizes the mucocutaneous tissues of oral cavity, vagina, and gastro-intestinal tract [5,8]. It is, however, also an opportunistic pathogen, able to penetrate the deeper tissues and to enter the bloodstream, causing invasive candidiasis, a severe systemic infection that can affect blood, heart, brain, eyes, bones, and other parts of the body [9]. Moreover, *C. albicans* can attach and form biofilms on indwelling medical devices, and the increase in *Candida* infections in the last decades has almost paralleled the widespread and rising use of medical devices themselves, mainly in immunocompromised individuals [10].

Klebsiella pneumoniae is the second leading cause of bloodstream infections caused by Gram-negative bacteria that occur in hospitalized or immunocompromised patients [11]. *K. pneumoniae* is a ubiquitous Gram-negative opportunistic bacterium that generally resides in the mucosal tissues, but which typically switch to pathogenic in hospitalized or otherwise immune-deficient patients, causing broad spectra of diseases and showing increasingly frequent acquisition of resistance to antibiotics [12]. *K. pneumoniae* has several intrinsic virulence factors, among those its ability to produce biofilms on medical devices surfaces such as urinary catheters or tracheal tubes.

In the case of *Candida*-mixed-species infections, *C. albicans* and *K. pneumoniae* are often isolated together, especially from patients with bloodstream and oral infections or respiratory diseases [13–17].

Although biofilm-associated implant infections involving *C. albicans* and *K. pneumoniae* have gained a certain clinical relevance [18], so far, the description of the interactions between *C. albicans* and *K. pneumoniae* in mixed biofilms is limited to few observations [19–21].

Few current therapies can effectively act on the different species that cohabit a polymicrobial biofilm, not only for the biological differences between bacteria and fungi but also because polymicrobial biofilms exhibit higher microbial load and recalcitrance to classic antimicrobial treatments [22]. Our previous results demonstrated that Polar bacteria, belonging to different genera, showed different biological activities [23], among which a clear anti-biofilm activity against *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* [23,24]. In particular, it has been demonstrated that the Antarctic bacterium *Pseudoalteromonas haloplanktis* TAC125 produces a long-chain fatty aldehyde, the pentadecanal [25], endowed with strong biofilm-inhibiting activity against different *S. epidermidis* strains [25–27]. Then, some pentadecanal derivatives were synthesized, which resulted to be active against *S. epidermidis*, moreover, they resulted biocompatible on fibroblast and keratinocytes [28].

To create a novel effective strategy against device-associated infections, we recently proposed an anti-biofilm coating, developed through the physical adsorption of pentadecanoic acid on a polydimethylsiloxane (PDMS) surface [29]. PDMS is a material frequently used for the manufacture of medical devices, such as phonatory and mammary prostheses, but also catheters or drug-delivery systems [30], due to its physiological inertness, high blood compatibility, low toxicity, good thermal and oxidative stability [31]. Though the physico-chemical properties of PDMS make it a good candidate for medical applications, in some cases surface and bulk modifications are necessary. For instance, bulk modification methods like blending, copolymerization, or functionalization processes are employed in controlled drug delivery systems for drug transport throughout the polymer [32]. The proposed anti-biofilm coating was obtained by drop-casting method and its surface properties were evaluated in terms of hydrophobicity and roughness by atomic force microscopy (AFM) and water contact angle (WCA) analysis. Then, the anti-adhesive and biofilm-inhibiting effects of the proposed coatings were studied in vitro using a parallel plate flow chamber with *in situ* observation and image analysis systems [29]. Reported results demonstrated that the PDMS coated with pentadecanoic acid was able to strongly reduce the biofilm formation of *S. epidermidis*, furthermore the long-term efficacy of the proposed anti-biofilm coatings was also demonstrated, which showed a clear biofilm-inhibiting effect even after 21 days under buffer flow conditions [29].

Since the chemical structure of pentadecanal and pentadecanoic acid is similar to that of diffusible signal factors (DSFs), *quorum sensing* signals involved in the regulation of biofilm formation, and also, other long-chain fatty acids were reported to effectively reduce the biofilm of bacteria and yeasts like *C. albicans* [33,34]. The cis-11-methyl-2-dodecenoic acid produced by *Xanthomonas campestris* was the first member of the DSF family of signals found to induce dispersion of its own biofilm. Subsequently, a variety of DSFs have been identified, for example, the cis-2-Decenoic acid induces the dispersal of preformed biofilms and inhibited biofilm formed by several microbes (e.g., *K. pneumoniae*, *Bacillus subtilis*, *S. aureus*, *Escherichia coli*, *Proteus mirabilis*, *Streptococcus pyogenes* and *C. Albicans*) [33,34].

In this paper, we investigated the effect of pentadecanal and pentadecanoic acid in preventing and eradicating *C. albicans/K. pneumoniae* dual-species biofilm. Moreover, the polymicrobial biofilm formation on both untreated and modified PDMS was examined, using convertible flow cells. Finally, the influence of pentadecanoic acid on the expression levels of some biofilm-related genes (*HWP1*, *ALS3* for *C. albicans*, *luxS*, and *mrkA* for *K. pneumoniae*) was investigated in the dual-species biofilm.

2. Materials and methods

2.1. Strains and culture conditions

In this study, the reference fungal and bacterial strains *C. albicans* ATCC 90028 and *K. pneumoniae* ATCC 10031, obtained from American Type Culture Collection, were used. Planktonic growths and biofilm formation assays were performed at 37 °C in Tryptic Soy Broth (TSB) for *K. pneumoniae* and in TSB supplemented with glucose 1% w/v for *C. albicans* or mixed cultures. For strain maintenance, Sabouraud dextrose agar and Trypticase Soy Agar (TSA) media were respectively used for the fungus and the bacterium.

2.2. Pentadecanal synthesis

Pentadecanoic acid was purchased from Sigma (Sigma–Aldrich, Inc., St. Louis, 2018 Merck KGaA, Darmstadt, Germany). Pentadecanal was synthesized starting from the corresponding alcohol (Sigma–Aldrich) as already reported [25]. 1-Pentadecanol (15 mg) was charged into a 10 mL round-bottom flask equipped with a magnetic stir bar. The solid was then dissolved in toluene (1 mL) at 20 °C, and an aqueous solution of sodium bicarbonate (0.1 g in 1 mL of deionized water) was prepared and charged into the toluene slurry. Solid iodine (30 mg) was then charged to the alcohol followed by solid TEMPO (0.9 mg) [35]. The reaction mixture was then left under stirring for 16 h at 20 °C. The batch was cooled to 5 °C, diluted with ethyl acetate (1 mL), and quenched at 5 °C by adding an aqueous solution of sodium sulfite 10% (155 mg sodium sulfite in 1 mL of deionized water). The quenched reaction mixture was transferred into a separatory funnel, rinsed with additional ethyl acetate, and deionized water (1:1 v/v), and the aqueous layer was

cut away. The organic layer was then washed with 5 mL of saturated aqueous sodium bicarbonate, followed by 5 mL of brine. The organic layer was then dried over sodium sulfate, filtrated, and concentrated in vacuum, completely dried under a stream of argon (yield 95%). The purity of the synthesized compound was checked by ^{1}H -NMR spectroscopy (Bruker, 600 MHz, $CDCl_3$) and gas chromatography mass spectrometry (GC–MS), by using an Agilent 7820A GC System-5977B MSD spectrometer equipped with the automatic injector 7693A and a Zebron ZB-5 capillary column (Phenomenex, Toornace, CA, USA; flow rate 1 mL min^{-1}; He as carrier gas).

2.3. Minimal inhibitory concentration

Minimal inhibitory concentration, MIC 90, of pentadecanal and pentadecanoic acid were determined according to the broth microdilution protocol of the Clinical and Laboratory Standards Institute (CLSI), with few modifications [19]. 100 μl of fungal or bacterial culture were diluted to a final concentration of 1×10^6 colony forming units (CFU) mL^{-1} in TSB with or without glucose, respectively, and added into each well of a 96-well-microplate, in the presence of pentadecanal or pentadecanoic acid at concentrations up to 200 μg mL^{-1}. Both pentadecanal and pentadecanoic acid had been previously solubilized in DMSO and then added to the culture medium (0.5% v/v DMSO final concentration). Proper controls in DMSO were included in the experiments. Microbial cultures were incubated at 37 °C for 24 h and microbial growth was determined at 590 nm wavelength with a microplate reader (SYNERGY H4 BioTek).

2.4. Development and quantification of mono- and polymicrobial biofilms

C. albicans or *K. pneumoniae* culture was adjusted to 1×10^6 CFU mL^{-1} with the respective culture medium (TSB with or without glucose). For the development of monomicrobial biofilms in static conditions, 100 μL of each culture was pipetted into wells of a polystyrene 96-well microplate. Similarly, in the case of polymicrobial biofilms, a suspension of both microorganisms (1:1) in TSB 0,1% glucose was added to wells of a 96-well microplate. Biofilms were allowed to develop for 24 h or 48 h at 37 °C. The medium was replaced every 24 h. Quantification of total biofilm biomass was performed with crystal violet staining (CV) as previously described [20].

The quantification of viable cells of each strain in dual-species biofilms was determined using the plate counting technique on selective media. In detail, biofilms were scraped from the polystyrene surface of the wells, and microbial cells recovered in PBS. Next, they were serially diluted in PBS and plated on TSA and Rose Bengal agar plates, respectively supplemented with 1 μg mL^{-1} amphotericin B with 20 μg mL^{-1} chloramphenicol, to selectively count the CFU of *K. pneumoniae* and *C. albicans*. Plates were incubated at 37 °C for 48 h.

2.5. Pentadecanal and pentadecanoic acid anti-biofilm activity

To assess the inhibition activity of pentadecanal and pentadecanoic acid towards the formation of both mono- and polymicrobial biofilms, the latter were allowed to develop for 24 h as previously described, but in the presence of the anti-biofilm molecule, at concentrations ranging from 25 to 200 μg mL^{-1}. After 24 h, residual biofilm biomass was quantified by CV staining.

To test the effect of the same molecules against established biofilm, a fresh growth medium containing pentadecanal or pentadecanoic acid ranging from 25 up to 200 μg mL^{-1} was added to the 24 h old-biofilm and incubated for other 24 h (48 h in total). Then, residual biofilm total biomass was quantified as previously described.

Both pentadecanal and pentadecanoic acid had been solubilized in DMSO and then added to the culture medium (0.5% v/v DMSO final concentration). Proper controls in DMSO were included in the experiments.

2.6. RNA extraction and expression profiling by qPCR

After 24 h exposure to pentadecanoic acid at concentration of 100 μg mL^{-1}, fungal and bacterial cell suspensions were collected in a 2 mL tube, kept on ice immediately and were further homogenized in TRIzol (Invitrogen, Paisley, UK) using a TissueLyser II (Qiagen, Valencia, CA, USA) and steal beads of 7 mm diameter (Qiagen, Valencia, CA, USA). Total RNA was extracted and purified using Direct-zolTM RNA Miniprep Plus Kit (ZYMO RESEARCH). The amount of total RNA extracted was estimated by the absorbance at 260 nm and the purity by 260/280 and 260/230 nm ratios, using a NanoDrop spectrophotometer 2000 (Thermo Scientific Inc., Waltham, MA USA), to exclude the presence of proteins, phenol and other contaminants. For each sample, 1000 ng of total RNA was retrotranscribed with an iScript™ cDNA Synthesis kit (Bio-Rad, Milan, Italy), following the manufacturer's instructions. Afterwards, the variations in gene expression of *mrkA, luxS,* and normalizer *16S rRNA* for *K. pneumoniae*; *HWP1, ALS3* and normalizer *ACT1* for *C. albicans* [19] were evaluated. In Table 1 the list of primers is reported. Undiluted cDNA was used as a template in a reaction containing a final concentration of 0.3 mM for each primer and 1 × SensiFASTTM SYBR Green master mix (total volume of 10 μL) (Meridiana Bioline). PCR amplifications were performed in a AriaMx Real-Time PCR instrument (Agilent Technologies, Inc.), according to the manufacturer's instructions System thermal cycler using the following thermal profile: 95 °C for 10 min, one cycle for cDNA denaturation; 95 °C for 15 s and 60 °C for 1 min, 40 cycles for amplification; 95 °C for 15 s, one cycle for final elongation; one cycle for melting curve analysis (from 60 °C to 95 °C) to verify the presence of a single product. Each assay included a no-template control for each primer pair. To capture intra-assay variability, all real-time qPCR reactions were carried out in triplicate. Fluorescence was measured using Agilent Aria 1.7 software (Agilent Technologies, Inc.). The expression of each gene was analyzed and normalized against *16S rRNA* and *ACT1* gene for *K. pneumoniae* and *C. albicans*, respectively, using REST software (Relative Expression Software Tool, Weihenstephan, Germany, version 1.9.12) based on the Pfaffl method [36,37]. Relative expression ratios greater than ±1.5 were considered significant.

2.7. CLSM analysis

The anti-biofilm activity of the selected samples was also evaluated by Confocal laser scanning microscopy (CLSM). Biofilms were formed on NuncTM Lab-Tek® 8-well Chamber Slides (n◦ 177445; Thermo Scientific, Ottawa, ON, Canada). Briefly, the wells of the chamber slide were filled with overnight cultures diluted at 1×10^6 colony forming units (CFU) mL^{-1} (the mixed suspension of *C. albicans* and *K. pneumoniae*). The culture was incubated at 37 °C for 48 h to allow the biofilm formation. Then, the mature biofilms were incubated for 24 h in the absence and in the presence of pentadecanoic acid (100 μg mL^{-1}) in order to assess its anti-biofilm activity and its influence on cell viability. The biofilm cell viability was determined by the FilmTracerTM LIVE/DEAD® Biofilm Viability Kit (Molecular Probes, Invitrogen, Carlsbad, California), containing the SYTO® 9 green fluorescent nucleic acid stain (10 μM) and

Table 1
Gene name, acronym, primer name, sequence and amplicon length of genes used in this study.

Gene name	Acronym	Primer name	Sequence (5′→3′)	Amplicon length (bp)
S-ribosylhomocysteine lyase	*luxS*	K.pneumoniae_luxS_F	ATCGACATTTCGCCAATGGG	157
S-ribosylhomocysteine lyase	*luxS*	K.pneumoniae_luxS_R	ACTGGTAGACGTTGAGCTCC	157
Type 3 fimbrial shaft	*mrkA*	K.pneumoniae_mrkA_F	ACGTCTCTAACTGCCAGGC	115
Type 3 fimbrial shaft	*mrkA*	K.pneumoniae_mrkA_R	TAGCCCTGTTGTTTGCTGGT	115
16S ribosomial RNA	*16S rRNA*	K.pneumoniae_16S_F	AGCACAGAGAGCTTG	126
16S ribosomial RNA	*16S rRNA*	K.pneumoniae_16S_R	ACTTTGGTCTTGCGAC	126
Hyphal wall protein 1	*HWP1*	C.albicans_HWP1_F	CAGCCACTGAAACACCAACT	135
Hyphal wall protein 1	*HWP1*	C.albicans_HWP1_R	CAGAAGTAACAACAACAACACCAG	135
Agglutinin like-sequence 3	*ALS3*	C.albicans_ALS3_F	CTAATGCTGCTACGTATAATT	201
Agglutinin like-sequence 3	*ALS3*	C.albicans_ALS3_R	CCTGAAATTGACATGTAGCA	201
Actin	*actin*	C.albicans_actin_F	AGCCCAATCCAAAAGAGGTATT	153
Actin	*actin*	C.albicans_actin_R	GCTTCGGTCAACAAAACTGG	153

propidium iodide (PI), the red-fluorescent nucleic acid stain (60 μM), following the manufacturer's instructions.

A qualitative analysis to identify the relative percentage of both species in the mixed biofilm, with and without treatment, was also performed. Calcofluor white stain (CFW), which binds to cellulose and chitin in cell walls, and SYTO® 9 were used to differently detect *C. albicans* and *K. pneumoniae* cells.

All microscopic observations and image acquisitions were performed with a confocal laser scanning microscope (CLSM; LSM700-Zeiss, Jena, Germany) equipped with an Ar laser (488 nm), and a He−Ne laser (555 nm). Images were obtained using a 20×/0.8 objective. The excitation/emission maxima for these dyes are 355/433 nm for CFW, 480/500 nm for SYTO® 9, 490/635 nm for PI. Z-stacks were obtained by driving the microscope to a point just out of focus on both the top and bottom of the biofilms. Images were recorded as a series of .tif files with a file-depth of 16 bits.

2.8. Preparation of the anti-biofilm PDMS coating

PDMS surfaces were prepared using SYLGARD® 184 silicone elastomer kit (Dow Corning Corporation, Midland, MI). PDMS substrates were fabricated using a ratio base to curing agent 10:1 w/w. The base and curing agent were mixed and then degassed until all air was removed. For the use of PDMS in the convertible flow cells, the polymer mixture was poured into the bottom of the flow cell and then cured at 65 °C for at least 5 h.

PDMS surfaces were then sterilized by soaking in 70% ethanol for 30 min, washed with sterile distilled water and dried at room temperature under sterile conditions. In this work, the commercial pentadecanoic acid (Sigma−Aldrich, Inc., St. Louis, MO # 2018 Merck KGaA, Darmstadt, Germany) was used to coat PDMS by drop-casting. Briefly, a solution of pentadecanoic acid in acetone (4 mg mL^{-1}) was deposited dropwise onto the PDMS surface and dried under ambient sterile conditions until complete solvent evaporation. Then the coated surface was washed with sterile distilled water, dried at room temperature under sterile conditions and stored until use.

2.9. Anti-biofilm PDMS coating effect on mixed biofilm formation in a flow cell system

The effect of pentadecanoic acid on *C. albicans* and *K. pneumoniae* mixed biofilm was also evaluated using flow cell methods, that allow non-invasive and non-destructive examination of biofilms. In this study, the analysis of the mixed biofilms was performed using convertible flow cells (Stovall Life Science, Inc., Greensboro, NC, USA), assembled as per the manufacturer's instructions. The single chamber (7.7 cm^3, 24 mm × 40 mm × 8 mm) has a detachable, re-attachable top, which allowed the polymerization of the PDMS directly into the flow chamber and then coated with pentadecanoic acid, as previously described [29]. Culture medium or buffer was flowed through the cell at a controlled flow rate of 160 μL min^{-1} using a Ismatec IPC 4 Peristaltic Pump (Cole−Parmer GmbH, Germany). The flow system was kept free of air bubbles using a bubble trap, which created a low positive pressure with medium or buffer flow, thus mitigating undesirable peristaltic pulsation in liquid delivery to the flow cell. The efficacy of the anti-biofilm coating was evaluated on *C. albicans*/*K. pneumoniae* biofilm. In detail, a *C. albicans* and *K. pneumoniae* mixed suspension, diluted at 1 × 10^6 colony forming units (CFU) mL^{-1} in a solution of TSB 2% (v/v) in PBS, was flowed into two convertible flow cells: a cell with the anti-biofilm coating and a cell with the uncoated PDMS. The mixed suspension was circulated through the system for 2 h to allow cell adhesion, then non-adherent cells were washed away with sterile PBS for 30 min. Finally, a fresh medium (TSB 50% v/v in PBS) was circulated for 24 h through the system to allow the biofilm formation on the coated and uncoated PDMS surfaces. Biofilms were analyzed by confocal laser scanning microscopy. The cell viability in the biofilms was determined by live/dead stain, which was injected with a syringe into the convertible flow cells, without removing them from the flow system, and incubated for 20−30 min at room temperature, protected from light. Then, fresh PBS was flowed to remove the excess stain. All microscopic observations and image acquisitions were performed as previously described.

2.10. Statistical analysis

Statistical analysis was performed using GraPhad Prism Software (version 8.02 for Windows, GraPhad Software, La Jolla California USA). Data are shown as the mean of three biologically independent experiments ± standard deviation (SD). For inhibition and eradication biofilm assay, the analysis of variance (ANOVA) with Tukey's correction for multiple comparisons was used to compare control with groups of treatments.

3. Results

3.1. The effect of pentadecanal and pentadecanoic acid on C. albicans and K. pneumoniae single and mixed biofilms

Before investigating the anti-biofilm activity of both pentadecanal and pentadecanoic acid, we determined the susceptibility of planktonic *K. pneumoniae* and *C. albicans* cells towards the two molecules, expressed in terms of minimal inhibition concentration (MIC). The MIC values of both compounds for *K. pneumoniae* were 150 μg mL^{-1}, whereas no growth inhibition was evidenced for *C. albicans* at all the tested concentrations.

C. albicans and *K. pneumoniae* formed biofilms individually as well as when grew together in static conditions [21], therefore, a detailed biofilm study was performed to evaluate the effect of pentadecanal and pentadecanoic acid on *C. albicans* and *K. pneumoniae* single and mixed biofilms, both in prevention and eradication assays.

The results obtained from the prevention assay (Fig. 1) showed that both pentadecanal and pentadecanoic acid inhibited *C. albicans* and *K. pneumoniae* single and mixed biofilm formation in a dose-dependent manner. In particular, pentadecanal inhibited *C. albicans* biofilm formation of about 30% at the maximal concentration used (200 μg mL^{-1}) (Fig. 1a), and, at the same concentration, the inhibitory effect was more pronounced in the case of *K. pneumoniae* single biofilm, that was reduced by more than 70%. Pentadecanoic acid appeared to be more active than pentadecanal in preventing single biofilm formation of both *C. albicans*, showing more than 20% inhibition at 100 μg mL^{-1}, and *K. pneumoniae*, inducing a 30% inhibition already at 50 μg mL^{-1} and reaching 90% inhibition at 200 μg mL^{-1} (Fig. 1b). Whereas, on the dual-species biofilm, a comparable effect was exerted by both the compounds, which induced a reduction of about 20% at the highest concentration.

As for the eradication assay, the effect of pentadecanal and pentadecanoic acid on preformed single and mixed biofilms was reported in Fig. 2. The eradication activity of both the molecules was the same, and it was more significant in the case of *K. pneumoniae* than *C. albicans* single biofilms. It is interesting to note that both compounds were able to eradicate up to 40–50% of the total biomass of the polymicrobial biofilm when used at the concentration of 200 μg mL^{-1}.

Since pentadecanoic acid exhibited a slightly better inhibition than pentadecanal and it is more chemically stable (the pentadecanal is an aldehyde that could easily undergo oxidation reactions), we focused on the acid in further experiments.

To evaluate the species-specific efficacy of pentadecanoic acid against the two microbial components of the mixed biofilm, treated and untreated mixed biofilm composition was evaluated by the plate counting technique on selective media. Results demonstrated that the 24 h-old untreated mixed biofilm was formed by more than 70% of *C. albicans* cells, instead, in the 48 h-old mixed biofilm the amount of each species was almost the same (Table 2). Whereas, in both prevention and eradication assays, pentadecanoic acid treatment induced a reduction of the total CFU of both

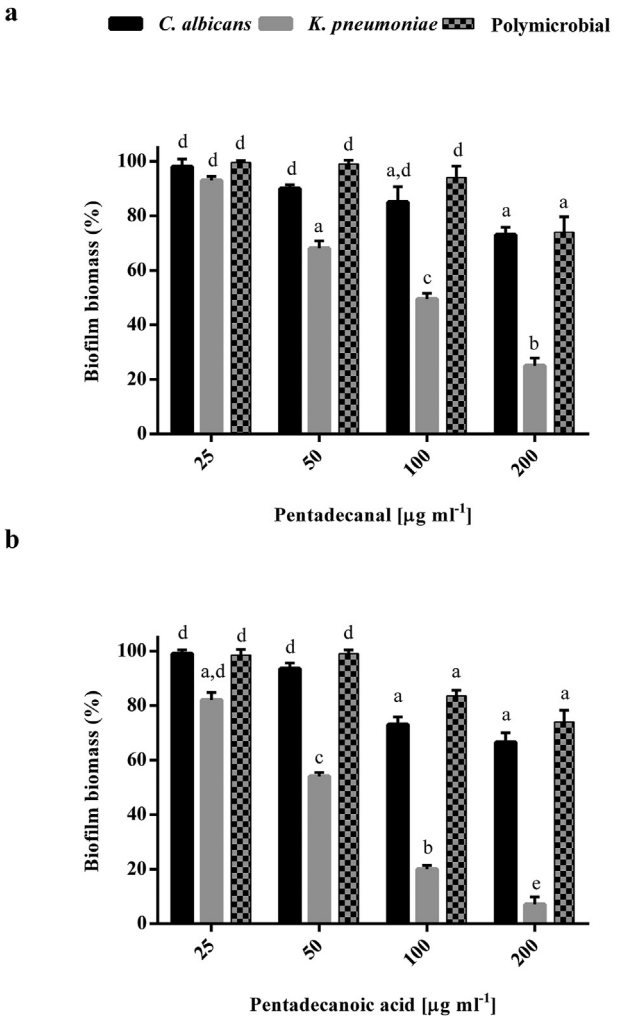

Fig. 1. Inhibition effects of pentadecanal (a) and pentadecanoic acid (b) on single and dual-species biofilms of *C. albicans* and *K. pneumoniae* in 96-well microplate. Microbial cells were co-cultured with pentadecanal or pentadecanoic acid at different concentrations (25, 50, 100 and 200 μg mL^{-1}) for 24 h. Data shown represent as mean of biofilm biomass reduction (%) ± SD. $n = 3$. Data with different letters (a–e) are significantly different (two-way ANOVA followed by Tukey's post hoc test), $p < 0.05$.

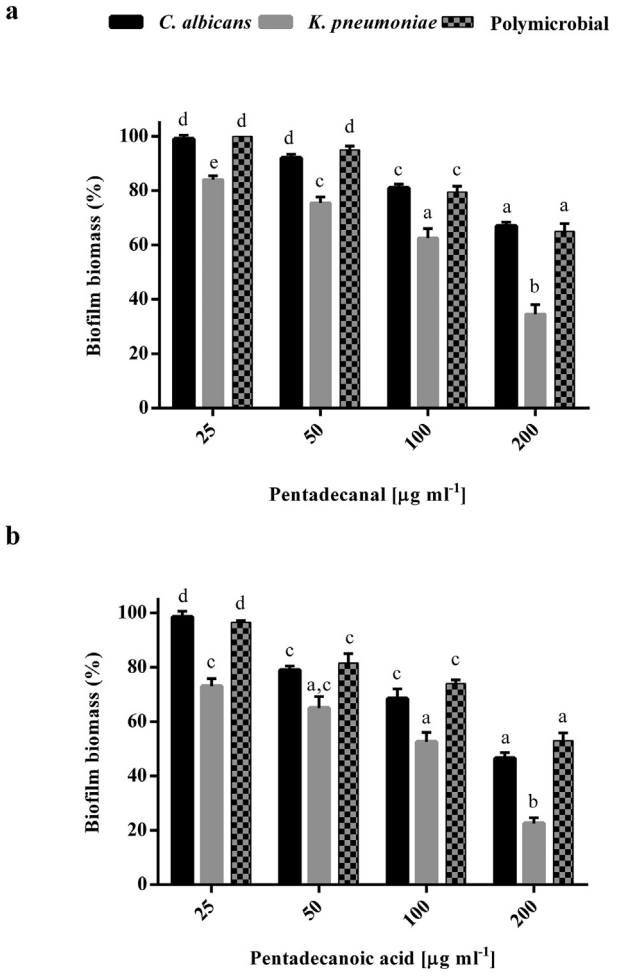

Fig. 2. Eradication effects of pentadecanal (a) and pentadecanoic acid (b) on single and dual-species biofilm of *C. albicans* and *K. pneumoniae* in 96-well microplate. Microbial cells were co-cultured with pentadecanal or pentadecanoic acid at different concentrations (25, 50, 100 and 200 μg mL^{-1}) for 24 h. Data shown represent as mean of biofilm biomass reduction (%) ± SD. $n = 3$. Data with different letters (a–e) are significantly different (two-way ANOVA followed by Tukey's post hoc test), $p < 0.05$.

Table 2
Percentage composition of *C. albicans*/*K. pneumoniae* mixed biofilm with and without treatment with pentadecanoic acid at 24 h (inhibition assay) and 48 h (eradication assay). Values were expressed as the mean of percentage ± standard deviation ($n = 3 \pm SD$).

Composition of *C. albicans*/*K. pneumoniae* mixed biofilm (%)				
	24 h		48 h	
	Control	Pentadecanoic acid 100 μg mL^{-1}	Control	Pentadecanoic acid 100 μg mL^{-1}
C. albicans	71.0 ± 4.3	90.0 ± 2.6	52.00 ± 2.8	93.00 ± 1.5
K. pneumoniae	29.0 ± 4.3	10.0 ± 2.6	48.00 ± 2.8	9.00 ± 1.5

C. albicans and *K. pneumoniae* (data not shown), correlated with the reduction of the biofilm biomass, with a drastic reduction in the percentage of *K. pneumoniae* in the residual biofilms (Table 2), suggesting a more specific activity against the bacterial species present in the mixed biofilm.

3.2. Confocal laser scanning microscopy analysis of the pentadecanoic effect on dual-species biofilm

The anti-biofilm activity of pentadecanoic acid on dual-species biofilm formation and eradication was also evaluated by confocal laser scanning microscopy (CLSM) (Fig. 3). In detail, the *C. albicans* and *K. pneumoniae* mixed biofilm was obtained in the presence of the anti-biofilm molecule at sub-MIC concentration (100 μg mL^{-1}) and then observed by CLSM; the analysis was performed using SYTO® 9 to reveal both *C. albicans* and *K. pneumoniae* cells, and CFW to better discriminate *C. albicans* cells [38]. As shown in Fig. 3, the CLMS analysis confirmed the pentadecanoic acid capability to inhibit and eradicate the mixed biofilm. Moreover, the dual-species biofilm developed in presence of pentadecanoic acid resulted to be mainly composed of *C. albicans* cells, confirming that the pentadecanoic acid biofilm inhibition in prevention was more efficient on *K. pneumoniae* than on *C. albicans*.

The effect of pentadecanoic acid on the eradication of mature dual-species biofilm was also explored using CLMS and reported data demonstrated reduction of both cell types (Fig. 4). Furthermore, the preformed mixed biofilm, with and without the anti-biofilm treatment, was also characterized by live-dead stain (Fig. 4).

The eradication obtained by pentadecanoic acid treatment let to a reduction of biofilm biomass and thickness (Fig. 4A and B). Moreover, cells exposed to the pentadecanoic acid were alive (green indicates viable cells and red indicates dead or damaged cells), confirming that the molecule had no bactericidal activity on both *C. albicans* and *K. pneumoniae* living in the mixed biofilm, at the tested concentration. To obtain more detailed information on the dual-species biofilm structure and on the effect of anti-biofilm acid treatment, the three-dimensional images, collected by CLSM analysis, were analyzed using the COMSTAT image analysis software package [39]. An increased roughness coefficient is observed for the treated sample (Fig. 4B). This dimensionless factor provides a measure of the thickness variation of a biofilm, and thus it is used as an indirect indicator of biofilm heterogeneity. The analysis

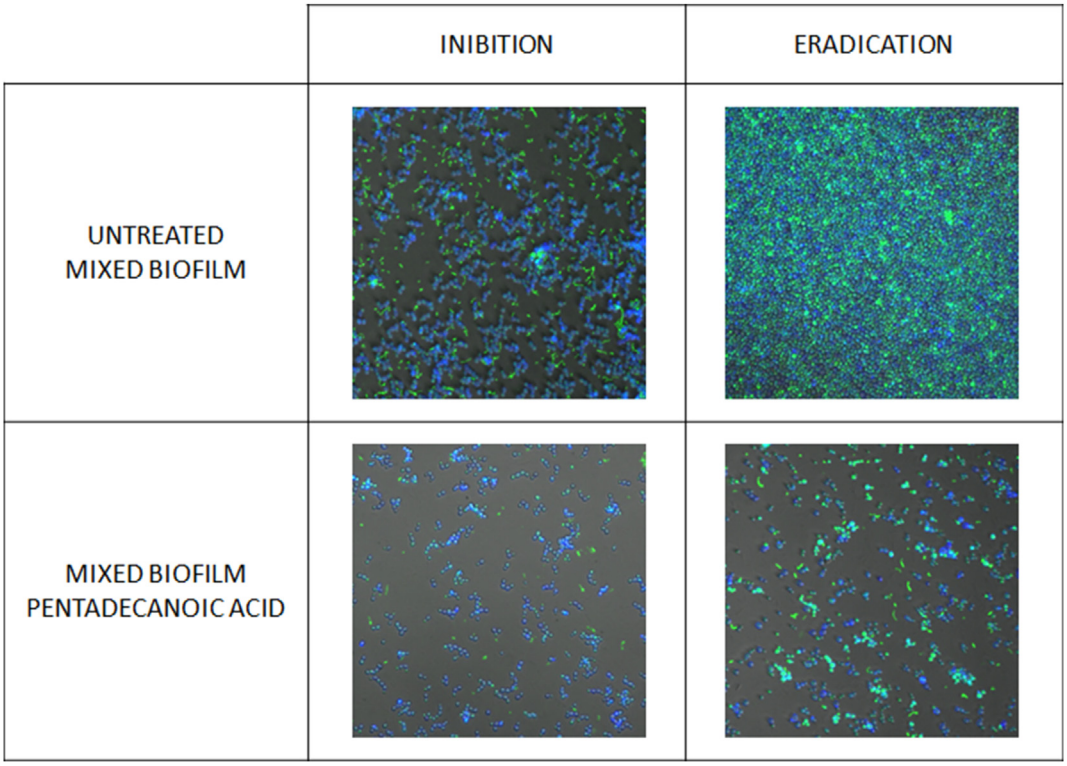

Fig. 3. CLSM analysis of the *C. albicans*/*K. pneumoniae* mixed biofilms untreated (upper part) and treated with pentadecanoic acid (lower part), in inhibition (left part) and eradication biofilm assays (right part). Biofilms were stained with SYTO9 (in green), that binds both *C. albicans* and *K. pneumoniae cells*, and CFW (in blue) to discriminate *C. albicans* cells.

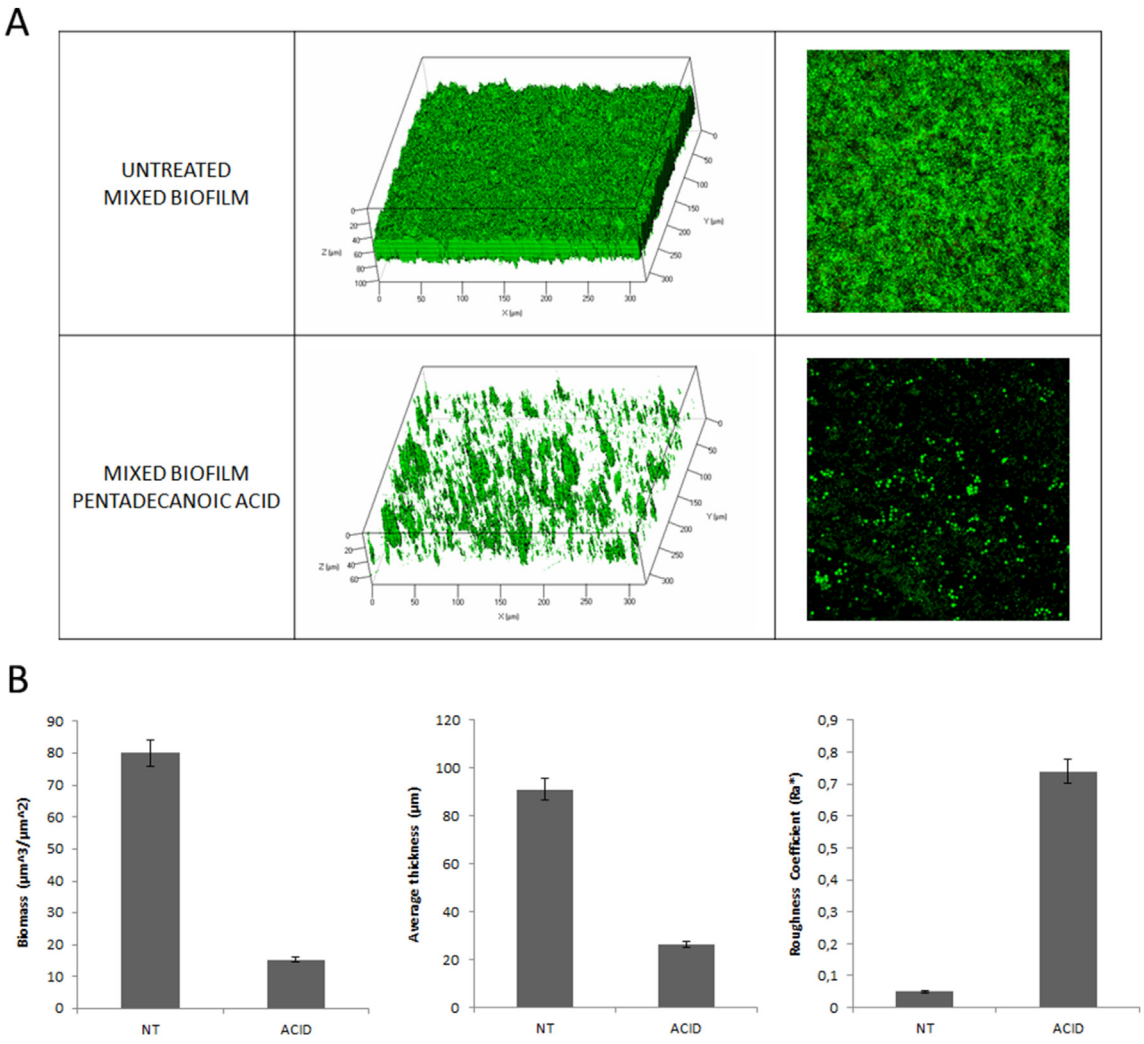

Fig. 4. Analysis of the pentadecanoic acid effect on the *C. albicans*/*K. pneumoniae* mixed biofilm structure. (A) CLSM analysis of mature mixed biofilms formed after 24 h incubation in the presence and in absence of pentadecanoic acid (eradication biofilm assay). Bi-dimensional and three-dimensional biofilm structures were obtained using the LIVE/DEAD® Biofilm Viability Kit. (B) COMSTAT quantitative analysis of biomass, average thickness and roughness coefficient of treated (ACID) and untreated (NT) mixed biofilms.

revealed that the treatment resulted in an inhomogeneous and unstructured mixed biofilm.

3.3. Evaluation of dual-species biofilm formation on the pentadecanoic PDMS coating in a flow cell system

To obtain an anti-biofilm coating for medical devices able to prevent *C. albicans* and *K. pneumoniae* polymicrobial infections, a surface of PDMS was coated with the pentadecanoic acid. The coating was made by physical adsorption of the anti-biofilm molecule onto the PDMS surface using the drop-casting method. In detail, a solution of pentadecanoic acid in acetone was deposited dropwise on the PDMS surface and the coating was obtained after subsequent spontaneous solvent evaporation.

The biofilm formation of *C. albicans* and *K. pneumoniae* on PDMS with and without the anti-biofilm coatings was evaluated in a convertible flow cell, that allowed a non-invasive and non-destructive examination of biofilms.

A flow system based on the use of convertible flow cells was set up to perform functional studies on the efficacy of pentadecanoic acid anti-biofilm coating in preventing the dual-species biofilm formation. In detail, the system was assembled connecting a container for the input solution buffer, a peristaltic pump, set at a flow rate of 160 µL min^{-1}, a bubble-trap, to prevent air bubbles to disturb the coating, a convertible flow cell containing PDMS coated with pentadecanoic acid, a container for the output solution buffer and connecting tubing. Briefly, a predetermined volume of mixed culture was flown for 1 h into the convertible flow cells, connected in parallel to the flow system, the first containing the uncoated PDMS and the second containing the PDMS coated with pentadecanoic acid.

Then, the mixed biofilms were grown for 24 h and the biofilm structures and cell viability were analyzed by CLSM (Fig. 5A). Bi-dimensional (SNAP) and three-dimensional (Z-STACK) biofilm structures were obtained using the live/dead staining, indicating viable cells by green fluorescence and red for dead (cell membrane damaged) bacteria. CLSM analysis demonstrated the capability of the coating to reduce the dual-species biofilm formation. To get more detailed information about the biofilm structure, the collected three-dimensional images were analyzed using the COMSTAT image analysis software package [39]. Mixed biofilms formed on the uncoated PDMS surface were thick and showed a more compact structure, compared to those formed on the pentadecanoic acid coating, which appeared less homogeneous and thinner (Fig. 5B). Moreover, it is interesting to note the higher number of dead cells present in the biofilm formed on coating, compared to that formed on the uncoated surface (Fig. 5A and B).

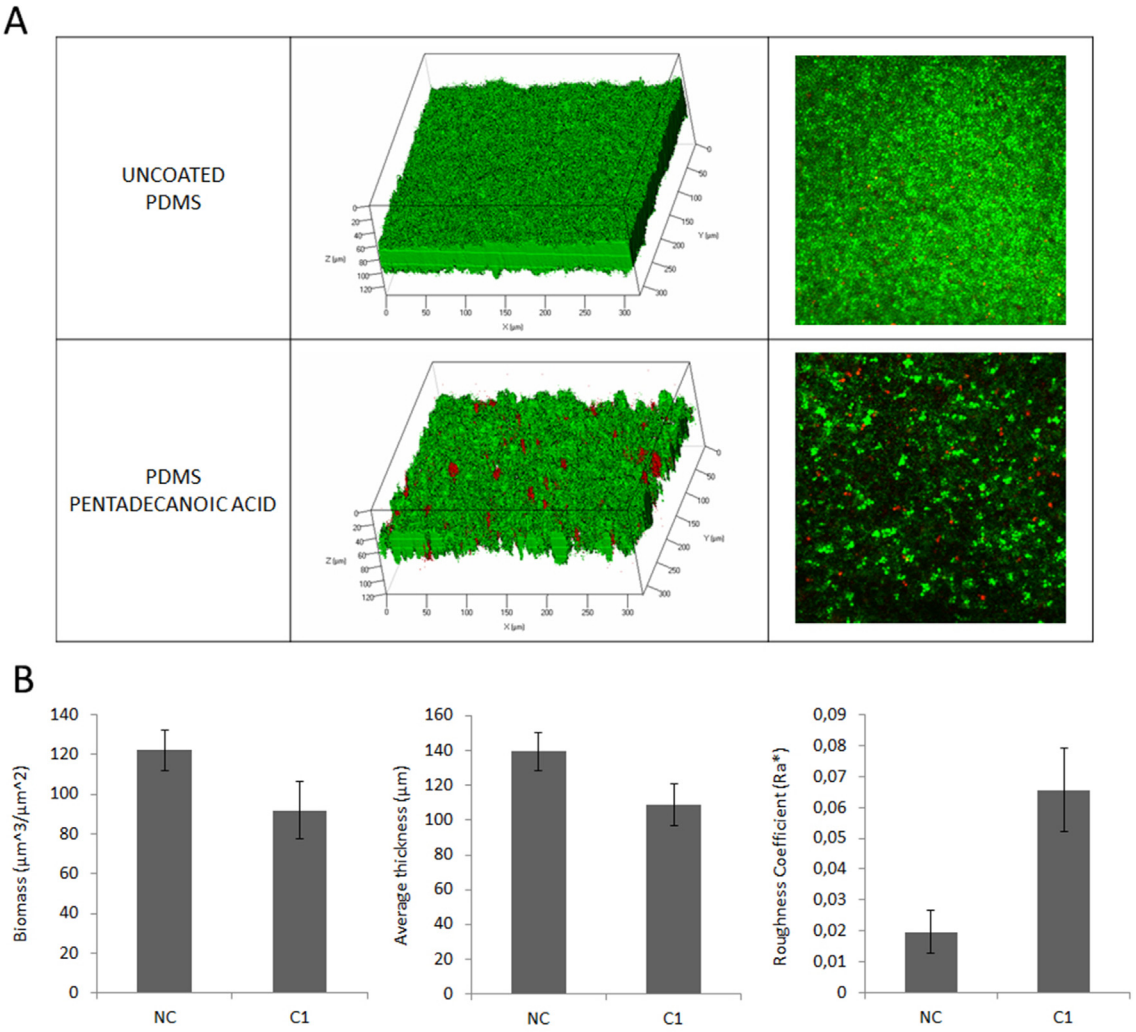

Fig. 5. Analysis of the biofilm-inhibiting effect of the pentadecanoic acid coating on *C. albicans*/*K. pneumoniae* mixed biofilm structures. (A) CLSM analysis of mixed biofilms formed after 24 h on uncoated PDMS and on pentadecanoic acid coating. Bi-dimensional and three-dimensional biofilm structures were obtained using the LIVE/DEAD® Biofilm Viability Kit. (B) COMSTAT quantitative analysis of biomass, average thickness and roughness coefficient of mixed biofilms formed on uncoated PDMS (NC) and on pentadecanoic acid coating (C1).

3.4. Effect of pentadecanoic acid on biofilm-associated genes expression in C. albicans *and* K. pneumonia

Transcriptional level of biofilm-related genes in *C. albicans* and *K. pneumoniae* in the presence of pentadecanoic acid at sub-MIC concentration (100 μg mL^{-1}) was quantified by qPCR (Fig. 6). The relative fold change in gene expressions of *C. albicans* and *K. pneumoniae* were normalized to each housekeeping gene *Actin* and *16S rRNA*, respectively and calculated by the Rest method. Comparing gene expression changes in dual-species biofilms between untreated and treated samples showed remarkable down-regulated expression of both *lux*S and *mrk*A after 24 h. By contrast *HWP1* and *ALS3* genes were not significantly affected by pentadecanoic acid at 100 μg mL^{-1}.

4. Discussion

Mixed-species biofilms cause infections that are considerably more difficult to treat compared to single-species counterparts and require complex multi-drug treatment strategies [40]. Both *C. albicans* and *K. pneumoniae* can colonize medical devices alone or together forming single or dual-species biofilm.

In the first part of this study, we analyzed the composition of *C. albicans*/*K. pneumoniae* biofilm. The strict interconnection between *C. albicans* and *K. pneumoniae* gave rise to the formation of a strong sessile community, where the fungus was initially (after 24 h) dominant with respect to the bacterium. Whereas, in the biofilm at 48 h, the two species were equally present. It is possible to speculate that in the experimental condition adopted in this work, *C. albicans* adherence and growth might be favoured due to the initial aerobic conditions, in addition to the competition for glucose present in the culture medium. Later, once the early biofilm was established, and the microenvironment would progressively become more anaerobic, *K. pneumoniae* proliferated until the two species ended up co-existing in the same amount. Indeed, it has been reported that the establishment of *C. albicans* biofilm provides a hypoxic microenvironment that supports the growth of gastrointestinal facultative (including *K. pneumoniae*) and strict anaerobic bacteria [41].

Then, to identify an anti-biofilm molecule able to interfere with the mixed biofilm formation and stability, we tested the activity of pentadecanal and pentadecanoic acid. These two molecules resulted to be effective against other pathogens [28,42] and a coating system of these two compounds on PDMS surface has been recently

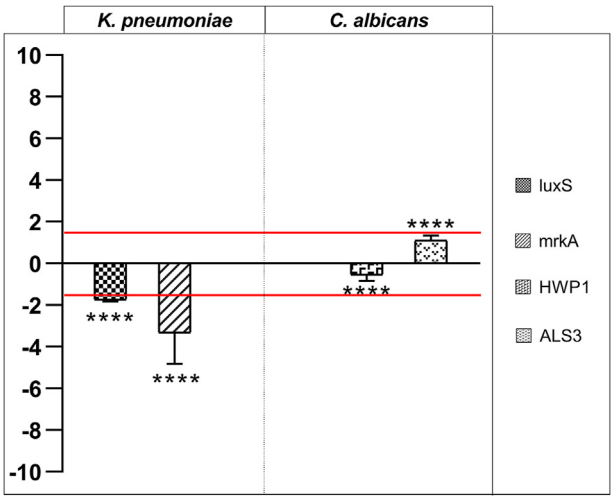

Fig. 6. Expression changes of *luxS*, *mrkA* in *K. pneumoniae* and *HWP1*, *ALS3* in *C. albicans* after exposure to 100 μg mL^{-1} pentadecanoic acid. The expression levels of all these genes are statistically significant ($p < 0.0001$) when compared with each other. Relative expression ratios greater than ±1.5 (red lines) were considered significant.

set up [29]. Moreover, their chemical structure is similar to that of diffusible signal factor molecules (DSF) and long-chain fatty acids that resulted to be effective against *C. albicans* [33,34] and *K. pneumoniae* [43,44]. Indeed, fatty acids have been widely proposed as safe antimicrobials in alternatives to conventional drugs [45,46] and as anti-biofilm molecules when used at low, sub-MIC concentrations [34]. But so far, their use as anti-biofilm molecules has been restricted to single-species biofilms.

Results reported in this paper demonstrated that pentadecanal and pentadecanoic acid were able to destabilize both single and dual-species biofilms of *C. albicans* and *K. pneumoniae*. Although both molecules can prevent the mixed biofilm formation, we choose to focus the study on pentadecanoic acid because of its higher chemical stability.

The pentadecanoic acid effect on dual-species biofilm was examined in detail, the anti-biofilm molecule had a preferential action against the bacterial cells, indeed the *K. pneumoniae* cells in the mixed biofilm were drastically reduced by exposure to pentadecanoic acid. This preferential action effect was demonstrated by the quantification of cells in treated mixed biofilm and confirmed by CSLM analysis. Indeed, the mixed biofilm formed in the presence of pentadecanoic acid had a composition different from the dual-species biofilm formed in absence of the molecule. Pentadecanoic acid had also a deep effect on biofilm structure either if added to a mature biofilm (Fig. 4) or if present during biofilm development (Fig. 5). In both cases the biofilm was characterized by lower biomass and an increased roughness coefficient, which is a direct indicator of biofilm heterogeneity, demonstrating that the treatment with pentadecanoic acid led to the formation of an inhomogeneous and unstructured biofilm.

The pentadecanoic acid is a saturated long fatty acid (FA), and saturated FAs (C8 to C18) are quite commonly found in nature and work as active agents that control biofilms of different microorganisms [34]. Of course, the specific mechanisms underlying fatty acids action depends on the type of cells forming the biofilm, and include changing cell-membrane fluidity, reducing EPS, fimbriae or hyphae formation, and modulating QS systems [34].

In a recent report, caprylic acid (octanoic acid) showed anti-biofilm activity against multi-drug resistant *K. pneumoniae* and was demonstrated to prevent, as well as eradicate, preformed *K. pneumoniae* biofilms [47]. *In silico* interaction studies revealed that caprylic acid, in *K. pneumoniae*, interfered with MrkA and GalF proteins, which are related to biofilm and capsule formation, respectively [43].

The capric acid (decanoic acid) resulted to be able to inhibit yeast-to-hyphae transition, adhesion, and biofilm formation of *C. albicans* [48]. The capric acid works on *C. albicans* cells as an analogue of farnesol (a well-known biofilm inhibitor) by repressing *HWP1* (the hypha-specific cell wall protein 1) [48], while myristic acid (tetradecanoic acid) affects several virulence pathways of *C. albicans* independent of QS, such as ergosterol synthesis, oxidative stress, and sphingolipid metabolism [49].

To collect information on the molecular mechanisms involved in pentadecanoic acid action, the transcriptional responses of *C. albicans* and *K. pneumoniae* in dual-species biofilms were explored after pentadecanoic acid treatment, in particular the relative expressions of *luxS* and *mrkA*, and *HWP1* and *ALS3* genes were evaluated in the presence of pentadecanoic acid. In the tested condition, the expression of *HWP1* and *ALS3* genes were not affected by the pentadecanoic acid, whereas a downregulation of *lux*S and *mrk*A genes was observed. The enzyme LuxS synthesizes the signal molecule of Type II quorum sensing system (the AI-2 molecule). In *K. pneumoniae* the LuxS-dependent signal plays a key role in the early stages of biofilm formation [50]. *mrk*A encodes the major subunit of type 3 fimbriae that are essential in the initial stage of biofilm formation in *Klebsiella pneumonia* [43]. Therefore, the downregulation of *lux*S and *mrk*A genes may explain the efficacy of pentadecanoic acid not only in preventing the formation of the single biofilm of *K. pneumoniae* but also impairing that of the dual-species one.

Although the detailed molecular mechanisms that underpin the anti-biofilm activity of pentadecanoic acid is not completely elucidated, the pentadecanoic acid exploitation as anti-biofilm agent just overcame some challenges common to several fatty acids related to solubility, delivery strategies, and toxicity. Indeed, the pentadecanoic acid toxicity was recently evaluated [28] and a possible solution for its use in biofilm prevention was proposed by the development of a coating system by physical adsorption on PDMS surface [29]. The poor water solubility of the pentadecanoic acid is the key factor in obtaining a gradual release of the acid in a water solution, and this slow release allows durable efficacy overtime of the developed coatings. To demonstrate the coating biofilm-inhibiting properties against *C. albicans/K. pneumoniae* mixed biofilm, a flow system was set up, which provide culture chambers for the real-time non-destructive study of biofilms under continuous hydrodynamic conditions at a controlled flow rate. CLSM analysis on biofilm formed on coated surface confirmed the biofilm-inhibiting effect of the pentadecanoic acid coating; besides showing a clear reduction of dual-species biofilm, it also induced a toxic effect on the bacterial cells, clearly indicated by the presence of dead cells zones (Fig. S1 and Fig. 5). This effect could be probably due to the rough and inhomogeneous distribution of the pentadecanoic acid on the surface after drop-casting. This effect could be avoided by improving the deposition method, spray-coating or spin coating will be evaluated to obtain more uniform adsorption of pentadecanoic onto the PDMS surface. An improved deposition could also have an advantage of increased efficacy of the coating on dual -species biofilm and a reduction in the quantity of molecule used per unit area.

5. Conclusions

The data described in this paper demonstrate the inhibiting and dispersion action of pentadecanoic acid towards mixed *Candida/Klebsiella* biofilm, with a major propensity to act against bacterial

than fungal cells in the consortium. We used this activity to develop an anti-biofilm surface by coating the acid on PDMS to prevent a *C. albicans* and *K. pneumoniae* polymicrobial biofilm. Therefore, further investigation will be focused to enhance pentadecanoic acid activity in combination with traditional antifungals, aiming at reducing their dosage and toxicity. Further, these data together with the previously reported efficacy of pentadecanoic acid on *S. epidermidis* [28] and *X. campestris* [42] endorse the pentadecanoic acid coating as a very promising strategy to prevent polymicrobial infections on medical devices.

Declaration of competing interest

All authors declare that they have no conflict of interest.

Acknowledgements

None.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.resmic.2021.103880.

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