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Research article

Inhibition of *Candida albicans* biofilm by lipopeptide AC7 coated medical-grade silicone in combination with farnesol

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Abstract: Biosurfactants affect interaction of microorganisms with material surfaces by altering interfacial properties, and have recently attract the attention of the scientific community for their use as anti-adhesive and anti-biofilm agents. The work studied the synergistic effect of a lipopeptide from *Bacillus subtilis* AC7 (AC7BS) combined with the quorum sensing molecule farnesol to counteract *Candida albicans* biofilms on silicone elastomer in simulated physiological conditions. The anti-adhesive and anti-biofilm properties of AC7BS, farnesol and their combination was evaluated after 1.5, 24 and 48 h by the viable count method on three *C. albicans* strains. Moreover, fungal biofilm was characterised by both scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). By combining the two molecules, a synergistic effect was observed with a significant reduction of *C. albicans* adhesion up to 74% at 1.5 h and of biofilm growth up to 93% at 24 h and 60% at 48 h. SEM and CLSM confirmed the synergistic anti-adhesive and anti-biofilm activity. Similar trends for the percentage of biofilm covered surface and biofilm mean thickness were observed. No cytotoxicity on eukaryotic cells was detected after exposures to AC7BS concentrations up to 0.5 mg ml^{-1}. Results demonstrated that the combination of the two molecules significantly inhibit both *C. albicans* initial adhesion and biofilm growth on silicone. Biosurfactant

AC7 in combination with farnesol is a hopeful coating to prevent *C. albicans* medical device-associated infection.

Keywords: *Candida albicans* biofilm; lipopeptide biosurfactant; farnesol; silicone elastomer; synergism

1. Introduction

Although *Candida albicans* is normally part of human microbiome, it can cause a wide range of infections including about 50% of cases of candidemia and 80% of cases of oropharyngeal and vulvovaginal candidiasis [1]. Human diseases caused by *C. albicans* are closely correlated to its ability to grow as biofilm [2]. Nowadays, *C. albicans* is the yeast most frequently associated with the formation of biofilms on a wide variety of medical devices, such as venous or urinary catheters, endotracheal tubes, dental prostheses, and other indwelling devices [3]. *In vitro* experiments showed that *C. albicans* biofilm is the result of a complex process in which several phases of development and multiple mechanisms of regulation are involved [4,5]. Initially, blastostores adhere to the surface and form distinct microcolonies. Afterwards, the development of filaments and the concomitant production of extracellular matrix lead to the formation of a structure with a three-dimensional architecture [6]. Biofilm protects the microorganism from host defenses as well as makes it more resistant to antifungal agents [7]. The development of new strategies to control and counteract the *Candida* spp. biofilms represents one of the main objectives in the clinical practice and preventive medicine [8–10].

It has been demonstrated that quorum sensing signaling regulates all the phases involved in biofilm development [11]. One quorum sensing molecule (QSM) secreted by *C. albicans* planktonic cells is E,E-farnesol [12,13]. Cell adhesion to a surface, biofilm growth and the cell dispersal are some of the crucial phases influenced by this molecule. In particular, biofilm formation is limited by farnesol, which inhibits filamentation regulating yeast-to-mycelium conversion thus leading to a decrease of biofilm size [14]. However, the use of farnesol alone is not sufficient in avoiding fungal adhesion and biofilm development on device surfaces, demanding for new or integrative approaches in prevention and treatment of *Candida* biofilm formation.

Recent studies have drawn attention to bacterial antagonistic bioproducts [15–17]. Among these, biosurfactants have gained the interest of the scientific community for their antibacterial, antifungal and anti-adhesive activities [18–22]. Biosurfactants are amphiphilic molecules, having both a hydrophilic and hydrophobic portion within the structure, that are able to reduce surface and interfacial tensions. Numerous investigations have highlighted the interesting bio-chemical properties of biosurfactants and several pharmaceutical and medical applications have been envisaged [22–26]. The ability to destabilise membranes by disturbing their integrity and permeability leading to metabolite leakage and cell lysis [27–30] has been seen as an important function of biosurfactants for their antimicrobial and anti-biofilm applications; as well as, their propensity to partition at the interfaces, modifying surface properties and thus affecting microorganisms adhesion [31,32].

Previous studies revealed an interesting anti-adhesive and anti-biofilm activity of a lipopeptide from *Bacillus subtilis* AC7 (AC7BS) on *C. albicans* strains, without affecting the viability of fungal

cells in both planktonic and sessile form [33]. The present work studied the efficacy of AC7BS in combination with farnesol to inhibit different stages of *Candida albicans* biofilm development on medical-grade silicone disks (SEDs) in physiological conditions. Adherent cells and biofilm were characterized by using the viable cell counting method, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

2. Materials and methods

2.1. Strains

The endophytic biosurfactant-producing strain *Bacillus subtilis* AC7 [33] and three *Candida* strains (the reference strain *C. albicans* IHEM 2894 and two clinically isolates *C. albicans* 40-DSM 29204 and 42-DSM 29205) were used in this study. Strains were stored at −80 ℃ in appropriate broth supplemented with 25% glycerol and grown on agar plate for 24 h before experimental assays.

2.2. Biosurfactant production and extraction

Biosurfactant was obtained as explained by Rivardo et al. [34]. Briefly, a loop of *B. subtilis* AC7 overnight culture was inoculated into 20 ml of LB broth and incubated at 28 ℃ for 4 h at 140 rpm. Thereafter, 2 ml of the seed culture were inoculated in 500 ml of the same medium and incubated for 24 h at the previously described conditions of growth. The bacterial cells were removed by centrifugation at 6000 × g for 20 min at 4 ℃. The supernatant was adjusted to pH 2.2 with 6 M HCl, stored overnight at 4 ℃ and extracted three times with EtOAc:MeOH (4:1) (Sigma-Aldrich). The organic phase was anhydrified and AC7 biosurfactant (AC7BS), composed by homologues of the surfactin (98%) and fengycin (2%) families [33], was concentrated by solvent evaporation.

2.3. Anti-adhesion and anti-biofilm assays

2.3.1. SEDs preparation

The cleaning and sterilization of SEDs- 15 mm in diameter, 1.5 mm in thickness (TECNOEXTR S.r.l., Italy) was carried out as explained in Ceresa et al. [35]. SEDs were submerged in 2 ml of AC7BS solution (2 mg ml^{-1}) or PBS only at 37 ℃ for 24 h at 140 rpm. Afterward, solutions were gently aspirated and SEDs were moved to a new 12-well plate for subsequent assays.

2.3.2. Biofilm development

C. albicans strains were cultured in Yeast Nitrogen Base with 50 mmol l^{-1} Dextrose (YNBD) at 37 ℃ for 24 h at 140 rpm. Cells were washed twice with PBS and pellets were standardized to 1×10^6 Colony Forming Unit per ml (CFU ml^{-1}). AC7BS coated and uncoated SEDs (6 disks per group) were inoculated with 2 ml of fungal suspensions (time 0) and incubated for 1.5 h in static condition at 37 ℃ (adhesion phase). Afterwards, SEDs were moved into a new plate with 2 ml of

YNBD in each well and incubated at 37 °C at 90 rpm for 24 h and 48 h for biofilm growth (intermediate and mature phases).

In order to evaluate the activity of farnesol alone or in combination with AC7BS against adhesion (1.5 h) and biofilm growth (24 h and 48 h) of *C. albicans* strains on SEDs, three sets of experiments were performed in 12-well plates. A scheme describing the three kind of treatments is included as supplementary material (Figure S1). In the first experiment, farnesol (Sigma-Aldrich) (stock solution 50 mmol l^{-1} in pure MeOH) was added to the fungal suspensions at time 0 in the wells containing AC7BS coated and uncoated SEDs (3 disks per group) to test a final concentration of 100 μmol l^{-1} (anti-adhesive activity—endpoint at 1.5 h). In the second assay, farnesol was added both to the fungal suspensions at time 0 and to the growth medium at 1.5 h to AC7BS coated and uncoated SEDs (3 disks per group) (anti-biofilm activity on intermediate phase—endpoint at 24 h). In the third experiment, *C. albicans* biofilms were first grown on AC7BS coated and uncoated SEDs for 24 h at 37 °C. Afterwards, farnesol was added into the wells of coated and uncoated SEDs (3 disks per group) and the plate was kept for additional 24 h at 37 °C (anti-biofilm activity on mature phase—endpoint at 48 h).

The anti-adhesive and anti-biofilm activity of AC7BS, farnesol and AC7BS + farnesol against *C. albicans* biofilms development was evaluated by viable counting method as described in Ceresa et al. [30] and results were expressed as $\log_{10}$ CFU $disk^{-1}$. In order to detach adherent cells and biofilms from silicone, disks were placed in 10 ml PBS (in 50 ml tubes) and subjected to four cycles of sonication (30 s) and stirring (30 s). Experiments were performed in triplicate and were repeated two times (n = 6).

2.4. Effect of farnesol on planktonic cells

The antifungal activity of farnesol (100 μmol l^{-1}) on *C. albicans* planktonic cells was carried out in 96-well microtiter plates. Briefly, fungal suspensions at the concentration of 1–5 $\times 10^5$ CFU ml^{-1} were prepared in sterile RPMI-1640 (Sigma-Aldrich) buffered with 3-(N-morpholino) propanesulfonic acid buffer (MOPS) (Sigma-Aldrich) and supplemented with D-glucose (2% final concentration) pH 7.0. One hundred microliters of a double-concentrated farnesol solution (200 μmol l^{-1}) prepared in RPMI-1640 were mixed to an equal volume of standardized *Candida* suspensions to a final concentration of 100 μmol l^{-1} and the 96-well microtiter plate (Bioster) was incubated in static conditions at 37 °C for 24 h. The antifungal activity of MeOH was also evaluated. Control wells (w/o farnesol and MeOH) contained 100 μl of sterile medium and 100 μl of standardized cellular suspensions. Blank wells (w/o cells) were also included. After incubation, the absorbance was measured at 450 nm (Ultramark microplate imaging system—Bio-Rad) and data were normalized with respect to the blank wells. Assays were performed in quadruplicate and the experiments were repeated two times (n = 8).

2.5. Hemolysis and cytotoxicity assays

For hemolysis assay, sheep red blood cells (SRBCs) (Biolife Italiana srl) were separated by centrifugation at 2000 $\times$ g, washed twice in PBS and then suspended at a cell density of 5 $\times 10^8$ cells ml^{-1}. One milliliter of AC7BS solutions (0.1, 0.2, 0.3, 0.4, 0.5, 1, 2 mg ml^{-1}) and 100 μl of SRBCs suspension were co-incubated at 25 °C for 30 minutes. Unaltered SRBCs were then removed by

centrifugation at 10000 $\times$g and the absorbance (Abs) of the supernatant was measured at 540 nm. In order to evaluate the percentage of hemolysis, the values of test samples were compared with the values of two different control samples. Positive control (100% hemolysis) contained 1 ml distilled water and 100 µl of SRBCs suspension; negative control (0% hemolysis) contained 1 ml PBS and 100 µl of SRBCs suspension.

The percentage of undamaged erythrocytes was calculated as follows:

$$\left(1 - \frac{\mathrm{Abs}_{\mathrm{AC7BS}} - \mathrm{Abs}_{\mathrm{neg.ctr}}}{\mathrm{Abs}_{\mathrm{pos.ctr}} - \mathrm{Abs}_{\mathrm{neg.ctr}}}\right) \times 100$$

Where:

$\mathrm{Abs}_{\mathrm{AC7BS}}$: Absorbance at 540 nm of sample containing AC7BS solution

$\mathrm{Abs}_{\mathrm{pos.ctr}}$: Absorbance at 540 nm of positive control containing PBS

$\mathrm{Abs}_{\mathrm{neg.ctr}}$: Absorbance at 540 nm of negative control containing distilled water

Each test was performed in triplicate (n = 3).

Cytotoxicity on human cell lines was evaluated by lactate dehydrogenase (LDH) assay (ISO 10993) (TOX7 Sigma-Aldrich), using normal lung fibroblasts (MRC5), according toTOX7 operative procedures.

Briefly, the cells were seeded in 96-well tissue culture plates and cultured in standard medium until about 70% confluence (24 h). Later, the cells were exposed for 48 h to the medium containing AC7BS solutions at different concentrations (2.0, 1.0, 0.5, 0.4, 0.3, 0.2, 0.1 mg ml^{-1}). Positive control for cytotoxicity was constituted by fully lysate cells (0.5% Triton X), while cells in reduced medium without surfactant constituted negative control. LDH level was evaluated by light absorbance at 490 nm (Tecan Spark 10 M) averaging the signal from 5 samples and calculating standard deviation.

The percentage of cell death was calculated as explained before for erythrocytes test. Each test performed out in quintuplicate (n = 5).

2.6. Scanning electron microscopy (SEM) analysis

A qualitative and quantitative analysis of *C. albicans* IHEM 2894 adherent cells and biofilms on SEDs was carried out as described in Ceresa et al. [35].

Briefly, biofilms on SEDs were fixed with a 2.5% glutaraldehyde solution in 0.1 mol l^{-1} phosphate buffer (at 4 ℃ for 24 h), washed twice in distilled water and dehydrated by immersion in 70%, 90% and 100% ethanol solutions for 10 min each. After overnight drying under a laminar flow, SEDs were glued to SEM sample holder by double bonding carbon tape and gold sputtered.

SEM analyses were carried out in a XL30 ESEM FEG (Fei-Eindhoven, The Netherland) scanning electron microscope at a 10 KV beam voltage. Images at 1000$\times$ magnification were acquired to detect fine morphological details of cells by collecting the secondary electrons signal. For quantitative outcomes, a set of nine different fields of view at 40$\times$magnification was obtained by collecting the backscattered electrons signal. To distinguish between biofilm covered surface and exposed silicone, high resolution digital images (1936 $\times$ 1452 pixels) were processed and binarized by semi-automated routine implemented in ImageJ (NIH, US). Percent area of the silicone disk covered by *Candida* biofilm, i.e. biofilm area percentage (BA%), was computed calculating the

percent ratio of dark pixels (corresponding to biofilm covered surface) over the whole pixel number of the image (corresponding to the total disk area).

2.7. Confocal laser scanning microscopy (CLSM) analysis

For the analysis, a set of *C. albicans* IHEM 2894 adherent cells and biofilms on SEDs was realised. After incubation, each SED was washed three times in PBS and incubated for 30 min at 37 ℃ in 2 ml of staining solution composed by 2 μl of 10 mmol l^{-1} FUN-1 solution (Life Technologies) and 10 μl of Concavalin A (CON-A, Life Technologies) 5 mg ml^{-1} solution in PBS. Observations were performed with an inverted confocal microscope (Nikon A1, Nikon Corporation, Japan) in wet conditions with the sample positioned upside down, to avoid the opaque silicone disk interference. Samples were scanned using 488 nm and 525 nm excitation wavelengths and collecting emissions at 525/25 nm and 650/100 nm respectively. FUN-1 is converted by metabolically active cells into red-orange cylindrical intravacuolar structures. CON-A binds to glucose and mannose residues of cell wall polysaccharides and results in green fluorescence. Z-stack pictures of approximately 0.5 mm^2 areas have been collected to observe the whole biofilm volume. The distance between the first and the last fluorescent confocal plane was defined as biofilm thickness.

2.8. Statistical analysis

Statistical analysis was elaborated by means of the statistical program R,3.1.2. (R Development Core Team, http://www.R-project.org). ANOVA was performed to study the effect of farnesol on planktonic cells on the three strains. ANOVA followed by Tukey's HSD test was performed to investigate the effect of AC7BS, farnesol or AC7BS + farnesol on the three *C. albicans* strains adhesion and biofilm growth. Wilcoxon signed rank test was used to compare the effects of the two compounds alone and of their combination. One-way ANOVA with Bonferroni correction was applied to evaluate the significance of data in hemolysis and LDH cytotoxicity assay. The R package dupiR was used to estimate $\log_{10}$ CFU $disk^{-1}$ from colony counts [36]. Results were considered to be statistically significant when $p < 0.05$.

3. Results

3.1. Anti-adhesive and anti-biofilm activity of AC7BS and farnesol

The anti-adhesive and anti-biofilm activity of farnesol, AC7BS and the combination of AC7BS and farnesol (AC7BS + farnesol) against adhesion and biofilm growth of *C. albicans* strains on SEDs was detected after fungal adhesion (at 1.5 h), intermediate (at 24 h) and mature stages (at 48 h) of biofilm development.

The efficacy of AC7BS alone, farnesol alone, and of AC7BS + farnesol in the inhibition of biofilm development of the three *C. albicans* strains is displayed in Figure 1. The comparative boxplots show that fungal adhesion (1.5 h) and biofilm growth (24 and 48 h) on treated SEDs were significantly lower than on control SEDs. The inhibition was more evident at 24 h (Figure 1b) rather than at 1.5 h and 48 h (Figures 1a, c). To be noted that, at 1.5 h, cells counts were lower as fungi are in the initial stage of biofilm development (Figure 1a). The highest performance of AC7BS pre-coating

alone was observed during *C. albicans* adhesion phase (Figure 1a) whereas during the biofilm growth phases the inhibition was lower but still significant (Figures 1b, c). Farnesol alone showed the highest inhibitory effect after 24 h, during the intermediate phase of biofilm formation (Figure 1b). A lower effect of farnesol was observed during the adhesion and mature phases of biofilm development (Figures 1a, c). The effect of AC7BS pre-coating alone and of farnesol alone was found to be similar at 1.5 h and 48 h (Figures 1a, c). On the contrary, at 24 h the two compounds were found to perform differently, where the activity of farnesol against biofilm growth was more than the double of that of AC7BS (Figure 1b). When AC7BS was used in combination with farnesol, their joint activity was greater than the performance of each molecule alone.

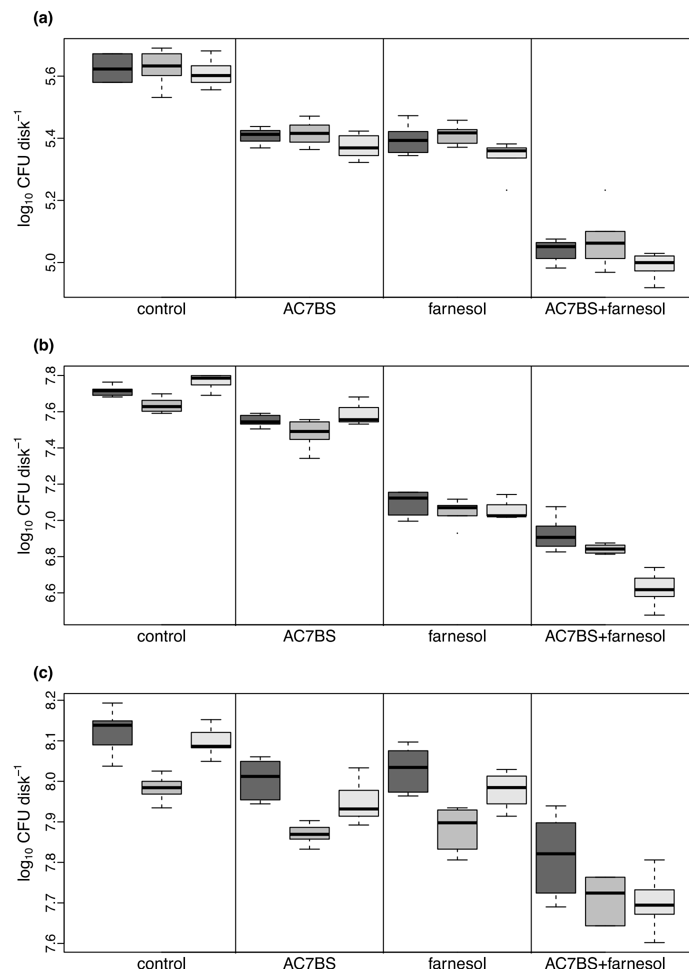

Figure 1. AC7BS and farnesol activity against *Candida albicans* biofilm formation. The inhibition of adhesion and biofilm growth of *Candida albicans* 40 (dark grey bars), *Candida albicans* 42 (grey bars), *Candida albicans* IHEM 2894 (light grey bars) was evaluated on SEDs at (a) 1.5 h, (b) 24 h, and (c) 48 h, by the viable cell counting method. For each condition (strain, treatment) minimum, maximum, median and interquartile range are illustrated using a box plot.

According to ANOVA analysis, *C. albicans* adhesion and biofilm growth are significantly dependent on the type of treatment ($p < 2 \times 10^{-16}$), incubation time ($p < 2 \times 10^{-16}$) and strain ($p = 2 \times 10^{-14}$).

The anti-adhesive and anti-biofilm effects obtained by the combination of the two compounds differed significantly from those observed when AC7BS and farnesol were applied alone ($p < 10^{-3}$).

The percentages of inhibition of *Candida* adhesion and biofilm growth were calculated as $(1-10^{\mu}) \times 100$, where μ is the difference in $\log_{10}$ CFU disks^{-1} between AC7BS, farnesol or AC7BS + farnesol and control samples.

Compared to controls, pre-coating with AC7BS significantly reduced the adhesion (1.5 h) in a range between 38.5% and 42.0% ($p < 5 \times 10^{-5}$). Biofilm growth was significantly inhibited in a range between 30.3% and 34.8% ($p < 3 \times 10^{-3}$) and between 22.9% and 29.1% ($p < 3 \times 10^{-3}$), respectively after 24 and 48 h. The treatment of SEDs with farnesol significantly reduced *C. albicans* adhesion and biofilm growth at 24 h in respect to control in a range between 39.0% and 46.2% ($p < 5 \times 10^{-5}$) and between 74.1% and 80.4% ($p < 3 \times 10^{-7}$), respectively. Furthermore, farnesol significantly affected the maturation of 24h-old biofilms in a range between 19.6% and 23.8% ($p < 1 \times 10^{-2}$).

AC7BS in combination with farnesol significantly reduced the adhesion of the three *C. albicans* strains in a range between 72.1% and 75.9% ($p < 9 \times 10^{-7}$) and biofilm growth at 24 h in a range between 83.8% and 92.9% ($p < 1 \times 10^{-6}$). When farnesol was added after 24 h of incubation to AC7BS pre-coated disks, the maturation of 24h-old biofilms was affected in a range between 46.6% and 59.8% ($p < 3 \times 10^{-4}$).

In order to evaluate whether a synergistic effect of AC7BS and farnesol was present, the effects (E) of the two compounds alone and of their combination were calculated as the difference in log10 CFU disk^{-1} between controls and treated samples (log10 CFU $\text{disk}_{\text{control}}^{-1}$–log10 CFU $\text{disk}_{\text{treated}}^{-1}$). Synergism is referred to the interaction between two or more molecules when their combined effect is higher than the sum of the effects of the single compounds.

Table 1 shows the sum of the single effects of AC7 and farnesol compared with the effects of their combination. When AC7BS was combined with farnesol, their joint effect was greater than the sum of the single effects during all the *C. albicans* biofilm development steps (with the exception of *C. albicans* 40 at 24 h), indicating a synergistic activity of the two compounds ($p = 0.003906$).

Table 1. Synergism of AC7BS and farnesol in anti-adhesion (1.5 h) and anti-biofilm (24 and 48 h) assays. Synergistic activity of the two compounds is defined when E(AC7BS + farnesol) > E(AC7BS) + E(farnesol).

Time (h)	Strain	E(AC7BS) + E(farnesol)	E(AC7BS + farnesol)
1.5	*C. albicans* 40	0.45	0.59
	C. albicans 42	0.42	0.55
	C. albicans IHEM 2894	0.51	0.62
24	*C. albicans* 40	0.79	0.79
	C. albicans 42	0.75	0.79
	C. albicans IHEM 2894	0.90	1.15
48	*C. albicans* 40	0.21	0.31
	C. albicans 42	0.21	0.27
	C. albicans IHEM 2894	0.27	0.40

3.2. SEM and CLSM analyses

Qualitative analysis of *C. albicans* IHEM 2894 biofilm microstructure on high magnification SEM images (Figure 2) and CLSM images (Figure 3) revealed a complex multilayer structure characterized by the presence of true long hyphae on control SEDs at 24 and 48 h. Conversely, biofilms with a less compact architecture and a thin hyphal network were evidenced on SEDs treated with AC7BS alone, farnesol alone, AC7BS + farnesol in comparison to controls.

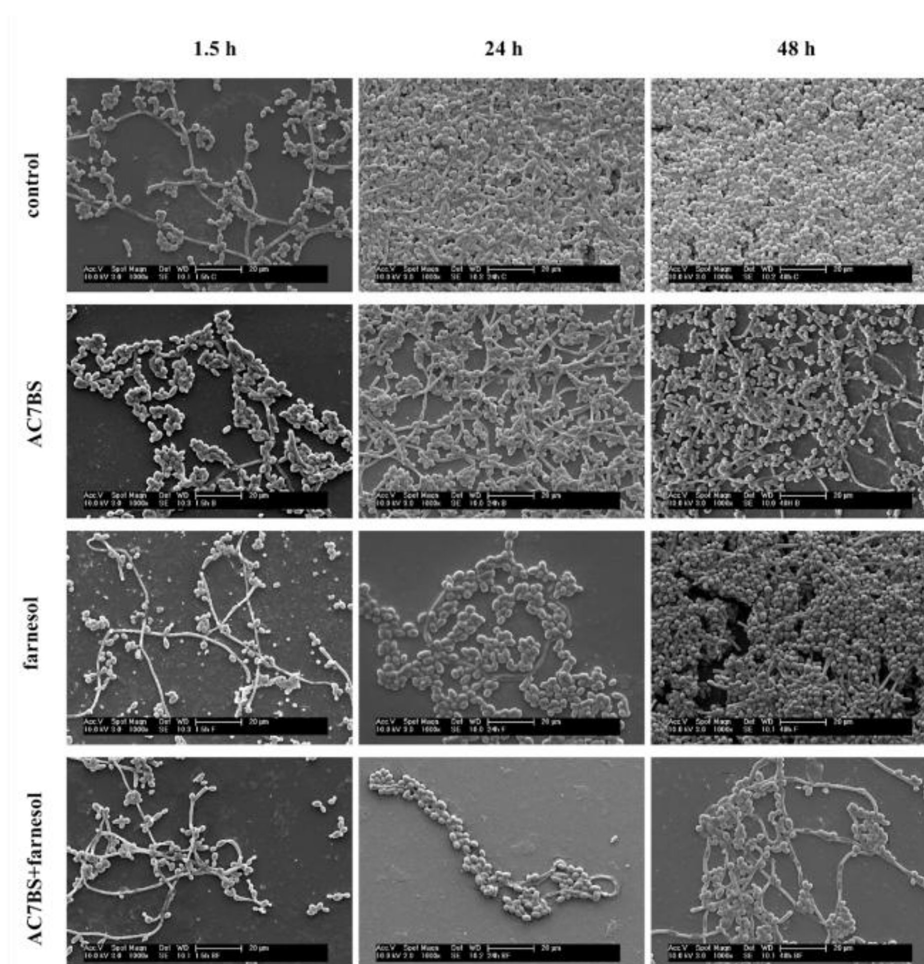

Figure 2. Scanning electron micrographs of treated and untreated SEDs. Images were acquired after *C. albicans* IHEM 2894 adhesion phase (1.5 h), intermediate (24 h) and mature (48 h) growth phases of biofilm formation. Original magnification 1000×.

No phenotypic differences were found between *Candida* cells grown on control or treated SEDs, in which ovoid spherical yeasts with budding and long hyphae were observed.

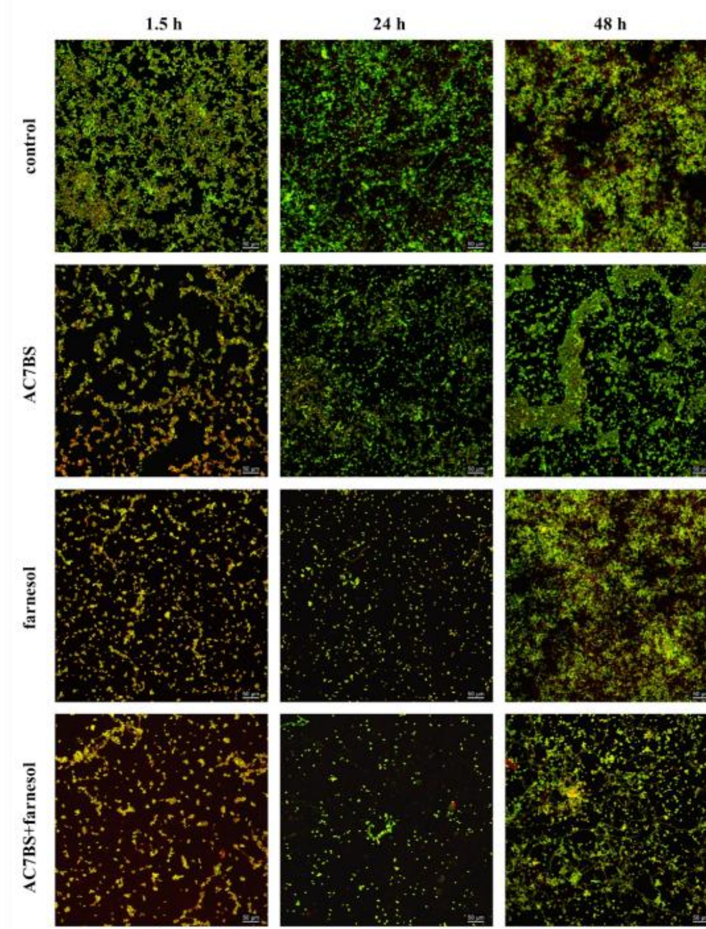

Figure 3. Confocal laser scanning micrographs of treated and untreated silicone elastomeric disks. Images were acquired after *C. albicans* IHEM 2894 adhesion phase (1.5 h), intermediate (24 h) and mature (48 h) growth phases of biofilm formation. FUN-1 staining results in red fluorescence whereas CON-A results in intense green fluorescence. Yellow areas represent dual FUN-1+CON-A staining. Scale bar is 100 μm.

The percentage of the silicone disk area covered by *C. albicans* IHEM 2894 biofilm (BA%) showed a trend of reduction in agreement with viable count data. Differences in BA% between SEDs treated with AC7BS alone, farnesol alone or AC7BS + farnesol and controls were observed. After 1.5 h of incubation, 24% of the untreated surfaces was covered by *C. albicans* cells whereas, cells were found only on the 11.2% of the farnesol treated surfaces, 11.5% of the AC7BS treated surfaces, and 8.6% of AC7BS + farnesol treated surfaces. After the biofilm formation phase, control SEDs were almost completely covered (BA% = 97%) by *C. albicans* IHEM 2894 biofilms both at 24 and 48 h, whereas SEDs treated with AC7BS displayed a BA% of 67% and 39%, SEDs treated with farnesol of 13% and 68%, SEDs treated with AC7BS + farnesol of 7% and 25%, respectively at 24 and 48 h.

From a comparative evaluation of the fluorescence from FUN-1 (addressing membrane integrity and metabolic capability) and CON-A (addressing cell membrane), it was possible to assess that the biofilm of all samples was highly viable (Figure 3). Metabolically active cells are characterized by red fluorescent areas whereas the presence of cell wall-like polysaccharides is indicated by green fluorescence. Yellow areas represent dual FUN-1+CON-A staining.

A preliminary evaluation of the film thickness, educible from the multi-stack images acquired from confocal microscope, evidences that after 24 h the AC7BS + farnesol treated samples are capable of a reduction in the biofilm thickness (Table 2). Both farnesol and AC7BS alone have a similar behavior with respect to the control sample, while they seem to express a detectable capacity in controlling the biofilm thickness only after 48 h.

Table 2. *C. albicans* IHEM 2894 biofilm thickness after 24 and 48 h evaluated by confocal microscopy.

	Biofilm thickness (μm)*	
Sample	24 h	48 h
Control	56 ± 2	58 ± 2
AC7BS	54 ± 2	41 ± 2
Farnesol	56 ± 2	46 ± 2
AC7BS + farnesol	36 ± 2	38 ± 2

*Data are represented as mean ± instrumental error.

3.3. Effect of farnesol on planktonic cells

ANOVA indicated $O.D._{450nm}$ was not significantly associated with the presence of farnesol ($p = 0.98$), showing that this molecule did not have an antifungal activity on *C. albicans* planktonic cells at the tested concentration. Additionally, preliminary experiments showed that methanol did not interfere with fungal viability at the concentrations used in this study.

3.4. Cytotoxicity assays

A low concentration-dependent hemolytic activity was observed ($p < 0.0001$). In particular, the percentage of hemolysis of SRBCs after incubation with AC7BS solutions ranged from 2% (0.1 mg ml^{-1}) to a maximum of 11% (2 mg ml^{-1}) compared to controls (Figure 4a). Cytotoxicity assays on MRC5-human normal lung fibroblasts cell lines indicated limited cytotoxic activity of AC7BS starting from exposure to AC7BS concentrations of 0.5 mg ml^{-1} (about 18%) and 0.4 mg ml^{-1} (10.4 ± 1.0%) (Figure 4b). For lower concentrations cytotoxicity drops down to negligible values (lower than 2.3%).

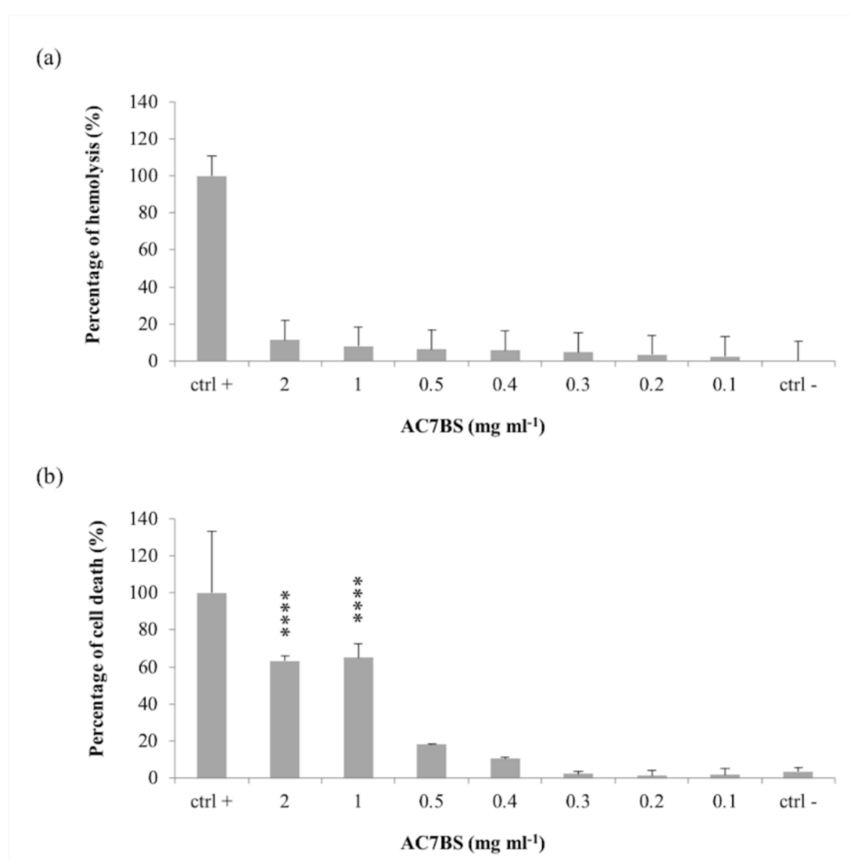

Figure 4. Hemolysis and cytotoxicity assays. (a) Hemolytic activity of different concentrations of AC7BS on sheep red blood cells (SRBCs). Negative control (ctrl-) is represented by cells in PBS, positive control (ctrl+) is represented by cells in distilled water. Statistical significance was tested with one-way ANOVA with Bonferroni correction. All the samples resulted significantly different in comparison to each other $p < 0.0001$. (b) Cytotoxicity of different concentrations of AC7BS on human normal lung fibroblasts (MRC5). Negative control (ctrl-) is represented by cells in growth medium, positive control (ctrl+) is represented by fully lysate cells (0.5% Triton X). Statistical significance was tested with one-way ANOVA with Bonferroni correction ****$p < 0.0001$.

4. Discussion

The continuous increase in the use of medical devices is associated with important and mostly hazardous *C. albicans* infections, usually due to the formation of biofilms. Biosurfactants form a group of natural biocontrol molecules that interfere with microbial adhesion and biofilm growth thanks to their ability to modulate the interaction of cells with surfaces, altering the chemical and physical condition of the developing biofilms environments [37,38].

In a previous study, it was demonstrated that the lipopeptide AC7BS significantly reduced biofilms of *C. albicans* strains on SEDs both in co-incubation and pre-coating conditions. Furthermore, this lipopeptide displayed no antifungal activity against planktonic or sessile forms of *C. albicans* strains at concentrations from 0.06 mg ml^{-1} to 3 mg ml^{-1} [33]. AC7BS was also tested in association with common antifungal drugs (amphotericin B and fluconazole) against *C. albicans*

biofilms. In particular, when amphotericin B was added to AC7BS pre-coated disks a synergistic effect was observed at different stages of biofilm development. This result could be explained by the AC7BS anti-adhesive activity and ability to change membrane permeability, increasing the entry rate of amphotericin B and thus its antifungal effect [39].

In the present work, the possibility to enhance and prolong the efficacy of AC7BS against *C. albicans* biofilm formation on medical-grade silicone was evaluated by the addition of a natural compound, the quorum-sensing molecule farnesol. Farnesol is getting increased attention as promising compound capable of interfering with some crucial stages of biofilm development by inhibiting *C. albicans* filaments growth and the expression of hyphae-specific genes [11]. The activity of both AC7BS and farnesol, alone or in combination, was assessed in simulated physiological conditions, with the addition of FBS to simulate silicone contact with biological fluids during clinical use. The assays were carried out using two clinically relevant wild strains (*C. albicans* 40 and *C. albicans* 42) isolated from central venous catheter or urinary tract catheter respectively and a standard strain (*C. albicans* IHEM 2894) isolated from tongue.

SEDs pre-coated with AC7BS caused a significant reduction of cell adhesion and biofilm growth of *C. albicans* strains. AC7BS pre-coating showed a higher efficacy during the adhesion phase and a lower effect during the growth and mature phases. This loss of activity can be explained by the fact that the biosurfactant film might have been gradually removed from the silicone surfaces during SEDs transfering procedures, being attached to the silicone surfaces by weak bonds.

The treatment with farnesol alone resulted in a higher inhibitory effect after the intermediate phase of biofilm formation. A less marked, but still significant, activity was observed after the adhesion and mature phases. Similar findings were observed by Jabra-Rizk et al. [40] and by Ramage et al. [11]. In both studies, the addition of farnesol at the concentration of 100–300 μM to the initial fungal suspension resulted in a higher reduction of biofilm formation whereas a lower effect, although still significant, was revealed when farnesol was added on 24h-old biofilm. As noted by Ramage et al. [11] the addition of farnesol, prior to initial adhesion phase, was crucial in terms of biofilm reduction. Cells that began yeast-to-hyphae conversion during the initial attachment resulted not sensitive to this QSM. The effect of farnesol on 24h-old biofilm was thus related to the ability of this molecule to inhibit mycelial development in newly produced cells without affecting the already formed biofilm [11,40]. Furthermore, this molecule at the concentration of 100 μmol l^{-1} did not affect cell viability of *C. albicans* strains, as also observed by Jabra-Rizk et al. ($MIC_{farnesol}$ = 300 μmol l^{-1}) [40].

Interestingly, when AC7BS was used in combination with farnesol, a synergistic activity of the two molecules against *C. albicans* biofilm formation was observed. The term synergism, meaning working together, is referred to the interaction between two or more molecules when their combined effect is greater than the sum of the effects of the individual compounds. In particular, the synergistic effect was more evident during the adhesion and mature phases of biofilm formation, respectively at 1.5 and 48 h. Most probably, during the adhesion phase the synergistic effect was due to the combined antiadhesive and anti-germination activities of AC7BS and farnesol respectively. At 48 h, the inhibition of cell adhesion and biofilm growth the AC7BS coating was probably enhanced by the inhibition of mycelial formation in newly produced *Candida* cells consequent to the addition of farnesol after 24 h of biofilm growth. Many crucial phases of the biofilm development are, in fact, influenced by this QSM, in particular, the inhibition of filaments growth and the repression of hyphae-specific genes expression, regulating yeast-to-mycelium conversion thus leading to a decrease of biofilm size.

Microscopic investigation of *C. albicans* IHEM 2894 by SEM and CLSM showed that the percentage of biofilm coated surface and the biofilm mean thickness are qualitatively in agreement with cultural data. The use of the sole AC7BS coating resulted in an effective limitation of cell adherence, but the efficacy in limiting biofilm growth at 24 h and 48 h was less predictive with a more scattered and uneven inhibitory effect, possibly due to partial removal of the compound from the silicon surface. Conversely, farnesol alone allowed inhibition of biofilm growth at 24 h, but showed a limited effect on mature biofilm at 48h. Silicone disks treated by the combination of AC7BS and farnesol resulted in the lowest percentage of biofilm covered surface and biofilm thickness. These data document an overall minimization of the biofilm volume. However, major changes in the surface appearance of cell wall previously reported for silver-based compounds [41] or bacterial metabolites [42], were not found. This is in agreement with the documented anti-biofilm but non-fungicidal properties of the tested compounds.

Additionally, a low hemolytic activity was detected for AC7BS, suggesting that the cytotoxicity of this compound could be low or absent. In a previous work, a low cytotoxicity of lipopeptides produced by endophytic bacteria against mouse fibroblasts (99–82% survival) and human keratinocytes (97–79% survival) was detected up to 312.5 μg ml^{-1}, compared with cells treated with 0.3% chlorhexidine (60% survival) and controls [43]. LDH test performed on MRC5 cell line confirmed low to absent cytotoxic effect of AC7BS concentrations up to 0.5 mg ml^{-1}, which indicates the relatively good tolerance of eukaryotic cells towards this biosurfactant.

5. Conclusions

These findings showed the efficacy of the synergistic activity of lipopeptide AC7BS and farnesol in inhibiting the attachment and biofilm growth of *C. albicans* on silicone. Although additional studies are necessary to clarify the molecular basis for the observed synergistic effect, the obtained results suggest a potential applicability for these two combined compounds with different anti-biofilm mechanism of action to counteract *C. albicans* adhesion and biofilm formation on materials for medical use, thus limiting the onset of infections.

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Conflict of interest

All authors declare no conflicts of interest in this paper.

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