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Research article

Synergistic activity of antifungal drugs and lipopeptide AC7 against *Candida albicans* biofilm on silicone

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Abstract: The occurrence of *Candida albicans* device-associated infections is tightly correlated to the ability of this fungus to form biofilms. The presence of this three-dimensional structure protects cells from host defenses, and significantly increases their resistance to antifungal agents. Lipopeptide biosurfactants are microbial products with interesting antibacterial, antifungal and anti-adhesive properties. Aim of the present study was to investigate a possible synergistic effect of lipopeptide AC7BS in combination with amphotericin B or fluconazole against *C. albicans* planktonic cells, biofilm formation and 24 h-old biofilms on medical-grade silicone elastomer disks, in simulated physiological conditions. In co-incubation experiments, AC7BS alone was not effective. However, the combination of AC7BS with the antifungal compounds resulted in a synergistic increase in the efficacy of the drugs against planktonic cells and biofilm, leading to a reduction of MICs and $SMICs_{50}$. In pre-coating conditions, amphotericin B alone and AC7BS alone significantly inhibited *C. albicans* biofilms. When the two molecules were tested in association, a synergistic effect was observed on different phases of biofilm formation and a lower $SMIC_{50}$ was detected. The observed synergism could be related to the combination of the AC7BS anti-adhesive activity and the AMB antifungal effect, but also to the ability of the biosurfactant to affect membranes, thus facilitating AMB entry in the cells. These results suggest that AC7BS can be considered a potential inhibitor of *C. albicans* biofilm on medical insertional materials and its use as coating agent may potentiate the effect of antifungal compounds such as AMB, when applied in combination.

Keywords: amphotericin; antifungals; biofilm; biosurfactant; *Candida albicans*; coating; fluconazole; lipopeptide; silicone elastomer; synergy

1. Introduction

Biofilms are communities of microorganisms attached to biotic and abiotic surfaces surrounded by an extracellular polymeric substance (EPS) and often involved in chronic infections and medical device contamination [1,2]. Host defense systems typically eliminate transient bacterial contamination, however, the presence of biofilm may protect the microorganisms, and significantly reduce its susceptibility to antimicrobial agents [3,4].

The dimorphic yeast *Candida albicans* is the fungal species most frequently isolated from medical devices, such as catheters, heart valves and urinary devices [5,6]. When host immune functions are decreased or the competitive commensal flora is perturbed, *C. albicans* can be responsible for superficial or life threatening systemic infections [7]. A study performed in the United States showed that *C. albicans* infections are the fourth most common hospital acquired systemic infections with a high mortality rate [8]. Risk factors for infection include neutropenia, damage of mucosae and the use of broad-spectrum antimicrobials. Moreover, the application of central venous catheters represents a cause of systemic infections because of direct contact with the bloodstream [9].

A major problem in the eradication of nosocomial *C. albicans* infections is the resistance of the biofilms to established antifungal agents like polyenes and azoles. The resistance is found to be multifactorial. Ramage et al. [10] describe the resistance mechanisms, which include general physiological state of sessile cells, cell density, over-expression of drug targets, efflux pump mediated resistance, extracellular matrix, persister cells and tolerance against stress. In almost all the cases, the presence of *C. albicans* biofilms often requires implant removal, with significant increase of morbidity, mortality and hospital costs [11]. As frequent replacement is uncomfortable, costly, time consuming and may lead to damage of the cellular tissue in patients, alternative approaches are highly desirable. Current strategies to prevent biofilm formation provide medical devices coated with antimicrobials [1]. From this point of view, it could be useful to increase the efficacy of known antifungal drugs.

Biosurfactants, amphiphilic metabolites produced by a wide group of microorganisms, can represent a useful approach to counteract biofilms. In particular, lipopeptides exhibit interesting biological properties such as high surface activity and antimicrobial potential [12]. We previously demonstrated that the coating of silicone elastomer disks with a lipopeptide biosurfactant from *Bacillus subtilis* AC7 led to the reduction of *C. albicans* biofilm formation [13]. Other studies on lipopeptides demonstrated their efficacy against *Escherichia coli* CFT073 pre-formed biofilms in combination with antibiotics [14] or silver [15]. In particular, results indicated that the V9T14 lipopeptide alone was not able to remove pre-formed biofilms but its association with antibiotics led to a synergistic increase in their efficacy up to a total eradication of biofilm, in some combinations [14]. In addition, the activity of silver was synergistically enhanced by the presence of V9T14 lipopeptide, leading to a significant reduction of the amount of $AgNO_3$ used and to an increase of its antimicrobial activity [15].

In the present study, the efficacy of AC7 lipopeptide biosurfactant in association with two clinically used antifungal agents, amphotericin B and fluconazole, against *C. albicans* planktonic cells and biofilm formation was assessed on silicone elastomer, with the aim to identify a synergistic combination of molecules with different origin and mechanism of action to treat or prevent *Candida* biofilms.

2. Materials and Methods

2.1. Strains and growth condition

The biosurfactant-producing strain *Bacillus subtilis* AC7, isolated from the inside of stems of *Robinia* pseudoacacia, was cultivated in Luria–Bertani (LB) broth (Sigma-Aldrich) and stored at –80 °C in the same medium supplemented with 25% glycerol. For biofilm assays, *Candida albicans* 40 (DSM 29204), a wild strain clinically isolated from a central venous catheter (courtesy of Hospital ‘‘Maggiore della Carità’’, Novara, Italy), was cultivated in Yeast Nitrogen Base broth (Sigma-Aldrich) with 50 mmol l^{-1} dextrose (Biolife Italiana Srl, Milan, Italy) (YNBD) and stored at –80 °C in sabouraud dextrose broth (Sigma-Aldrich) supplemented with 25% glycerol.

2.2. Biosurfactant production

A loop of *B. subtilis* AC7, from a LB agar overnight culture, was inoculated into 20 ml of LB broth and incubated at 28 °C for 4 h at 140 rev min^{-1}. Two milliliters of the seed culture were inoculated in 500 ml of the same medium, and incubated for 24 h at the previously described growth conditions. AC7BS was extracted according to the method described by Rivardo et al. [16].

2.3. Medical-grade silicone elastomeric disks preparation

Two different sizes of medical-grade silicone elastomeric disks (SEDs) (TECNOEXTR S.r.l., Italy) were used: 5 mm in diameter and 1.5 mm in thickness for experiments in 96-well plates, and 10 mm in diameter and 1.5 mm in thickness for experiments in 24-well plates. Cleaning and sterilisation of SEDs were carried out as described by Busscher et al. [17]. Briefly, disks were immersed in 200 ml of distilled water supplemented with 1.4% (v/v) of RBS^{TM} 50 solution (Sigma-Aldrich), sonicated for 5 min at 60 kHz using Elma S30H (Elmasonic, VWR International) and rinsed twice in 1 l of MilliQ water. Then, disks were submerged in 20 ml of methanol (99%) (Sigma-Aldrich), rinsed twice and autoclaved for 15 min at 121 °C.

2.4. Antifungal activity on planktonic cells

The antifungal activity of AMB (Sigma-aldrich), FLC (Sigma-aldrich) and AC7BS towards planktonic cells of *C. albicans* 40 was assessed according to EUCAST guidelines [18]. Briefly, 100 µl AC7BS 2× (2 mg ml^{-1} in phosphate buffered saline-PBS), AMB 2× (0.25, 0.5, 1 µg ml^{-1} in PBS) or FLC 2× (0.25, 0.5, 1 µg ml^{-1} in PBS) were added in a 96-well plate (Bioster). When the joint activity of AC7BS and the antifungal drugs was evaluated, 50 µl AC7BS 4× (4 mg ml^{-1} in PBS) were mixed with 50 µl of AMB 4× (0.5, 1, 2 µg ml^{-1} in PBS) or FLC 4× (0.5, 1, 2 µg ml^{-1} in PBS). In control wells (no biosurfactant or antifungal drugs added), 100 µl of sterile PBS were used. A standardized *C. albicans* suspension at the concentration of 1–5 × 10^5 Colony Forming Units (CFU ml^{-1}) was prepared in sterile double-strength Roswell Park Memorial Institute (RPMI) 1640 medium (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 this suspension were added to test wells, to obtain final concentrations of 1 mg ml^{-1} AC7 BS, 0.125, 0.25, 0.5 µg ml^{-1} AMB and

0.125, 0.25, 0.5 μg ml^{-1} FLC, and to control wells. Corresponding blank wells (without planktonic cells) were also prepared. The plate was incubated at 37 °C for 24 h in static conditions. Finally, OD_{450} was measured in each well using an Ultramark Microplate Imaging System (Bio-Rad). The data were normalized with respect to the value of the corresponding blank wells. The percentage of inhibition in each well, compared to control wells (containing disks not treated with biosurfactant or AMB), was determined as:

$$[1–(OD_{treat}/OD_{ctrl})] \times 100 \quad (1)$$

Where: OD_{treat}, optical density of treated samples; OD_{ctrl}, optical density of controls.

The minimal inhibitory concentration of AMB was defined as the lowest concentration leading to a growth inhibition ≥ 90% in comparison to control (MIC_{90}), while that of FLC as the lowest concentration giving inhibition ≥ 50% (MIC_{50}). Assays were carried out in triplicate, and repeated in two different days.

2.5. Anti-biofilm assays

2.5.1. Co-incubation

The susceptibility of *C. albicans* 40 sessile cells to AMB, FLC and AC7BS was performed in 96-well plates as described by Nweze et al. [19] with some modification. SEDs were pre-coated with 3 ml fetal bovine serum (FBS) (Sigma-Aldrich) in 12-well plates (eight disks per well) at 37 °C for 24 h at 140 rev min^{-1} and, then, inoculated with 4 ml standardized fungal suspension containing 1×10^7 CFU ml^{-1} in PBS.

To evaluate the susceptibility of *C. albicans* in the intermediate phase of biofilm formation, after 1.5 h at 37 °C (adhesion phase), SEDs were transferred in a 96-well plate (Bioster) and incubated in 200 μl YNBD supplemented with AC7BS alone (final concentration 1 mg ml^{-1}) or AMB (final concentrations 0.5, 1, 2 μg ml^{-1}) and FLC (final concentrations 64, 128, 256 μg ml^{-1}) alone or in combination with AC7BS. Control wells consisted of YNBD supplemented by an equal volume of the antifungal/AC7BS diluent (i.e. PBS). The plates were incubated at 37 °C for 24 h for biofilm growth.

Furthermore, the effect of AC7BS, AMB and FLC alone or in combination was also evaluated on 24 h-old biofilms (mature phase). In this case, *C. albicans* biofilms were grown for 24 h at 37 °C using the protocol described above. Subsequently, 200 μl YNBD supplemented with AC7BS (final concentration 1 mg ml^{-1}), AMB (final concentrations 2, 4, 8 μg ml^{-1}) and FLC (final concentrations 64, 128, 256 μg ml^{-1}) alone or in combination were added and the plates incubated for additional 24 h at 37 °C. Control wells consisted of YNBD supplemented by an equal volume of the antifungal/AC7BS diluent (i.e. PBS).

2.5.2. Pre-coating

C. albicans biofilms on AC7BS pre-coated SEDs were prepared as described by Ceresa et al. [13]. Briefly, SEDs were dipped into 1 ml of a 2 mg ml^{-1} AC7BS solution or in PBS only and incubated at 37 °C for 24 h at 140 rpm. SEDs were then placed in a new 24-well plate and 1 ml of standardized *C. albicans* suspension at the concentration of 1×10^7 CFU ml^{-1} in PBS + 10% FBS was added in each well (t = 0). After 1.5 h incubation, the disks were transferred into 1 ml YNBD + 10% FBS and

incubated at 37 °C for 24 h at 100 rpm.

The activity of AMB was evaluated at different times of biofilm formation on SEDs pre-coated or not with AC7BS. In particular, the antifungal drug was added both to the standardized fungal suspensions at t = 0 and to the growth medium at t = 1.5 h to evaluate its efficacy on both adhesion and intermediate phase of biofilm formation, at the concentrations of 0.125, 0.25, 0.5 μg ml^{-1} (pre-coating type 1), or only to the growth medium at t = 1.5 h, to test its activity in the intermediate phase only, at the concentrations of 0.5, 1, 2 μg ml^{-1} (pre-coating type 2). Control wells (containing disks not treated with biosurfactant or AMB) and AC7BS alone wells (containing AC7BS pre-coated disks) consisted of 1 ml YNBD + 10% FBS, supplemented by an equal volume of the antifungal diluent (i.e PBS).

Furthermore, the effect of AMB alone or in combination with AC7BS was also evaluated on 24 h-old biofilms (pre coating type 3). Biofilms were formed for 24 h at 37 °C by using the protocol described above. Afterwards, 1 ml YNBD + 10% FBS supplemented with AMB at the final concentrations of 2, 4, 8 μg ml^{-1} was added and the plate incubated for additional 24 h at 37 °C. Control and AC7BS alone wells consisted of YNBD + 10% FBS, supplemented by an equal volume of the antifungal diluent (i.e. PBS).

2.6. Quantification of biofilm

The quantification of *C. albicans* 40 biofilms was performed by the {2,3 bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide} (XTT, Sigma-Aldrich) colorimetric assay. A working solution was prepared by adding 12.5 μl XTT solution (1 mg ml^{-1}) and 1 μl of 1 mmol l^{-1} menadione solution to 1 ml PBS. Biofilm growth medium was carefully removed by aspiration and replaced with XTT working solution. The plates were covered with aluminum foil and incubated for 5 h at 37 °C. Blank wells containing disks without biofilm were also included. Afterwards, the supernatant was carefully transferred into a new plate and the absorbance was measured at 490 nm (OD_{490}) using an Ultramark Microplate Imaging System (Bio-Rad). Each tested condition was carried out in triplicate and experiments were repeated at least two times in different days. The data were normalized with respect to the blank values. The percentage of inhibition in each well, compared to control wells, was determined as indicated previously.

The sessile minimal inhibitory concentration (SMIC) of AMB and FLC was defined as the lowest concentration leading to a metabolic activity inhibition ≥ 50% compared to control wells ($SMIC_{50}$).

2.7. Evaluation of synergism

Synergism is generally defined as the interaction between two or more molecules whose effect, in association, is greater than the combination of the effects of the individual compounds. Usually, the combination under consideration consists in an additive effect. However, if the effect is a percentage (in our case, a survival percentage), this is not additive but multiplicative; the combination of the effects of two compounds (assuming they act independently) is, thus, the product of the percentages. Synergy, in this context, amounts to say that the survival percentage due to the two compounds acting simultaneously is less than the product of survival percentages due to the two compounds by themselves.

In this work, the effect (E) of a treatment (T) (i.e. survival) is calculated as OD*T = ODT/ODctrl.

The synergism is evaluated by comparing the multiplicative effect $x_1 = OD^*_{(AC7)} \times OD^*_{(antifungal)}$ with the experimental effect $x_2 = OD^*_{(AC7\ \&\ antifungal\ agent)}$. If $x_1 > x_2$ in a statistical significant way, then we are in presence of synergism.

2.8. Statistical analysis

Statistical analysis and graphs were elaborated by means of the statistical program R,3.1.2. (R Development Core Team, http//www.R-project.org). Two-way ANOVA was performed to investigate the effect of AC7BS and antifungal agents alone or in combination on *C. albicans* planktonic or sessile cells both in co-incubation and pre-coating conditions. Results were considered to be statistically significant when $P < 5 \times 10^{-2}$.

3. Results

3.1. Joint activity of amphotericin B, fluconazole and lipopeptide biosurfactant AC7 on planktonic cells of Candida albicans 40

To define a possible joint activity against *C. albicans* 40 planktonic cells, the two antifungal agents were tested in association with the lipopeptide biosurfactant AC7 (AC7BS) at two sub-MIC concentrations (0.125 µg ml^{-1}, 0.25 µg ml^{-1}) and at the MIC (0.5 µg ml^{-1}). These concentrations were chosen based on previously calculated MIC_{90} for AMB and MIC_{50} for FLC (data not shown).

AC7BS was tested at the concentration of 1 mg ml^{-1}. The optical density at 450 nm (OD_{450}) of planktonic cells in co-incubation with or without AC7BS is displayed as a function of the concentration of AMB or FLC (Figure 1).

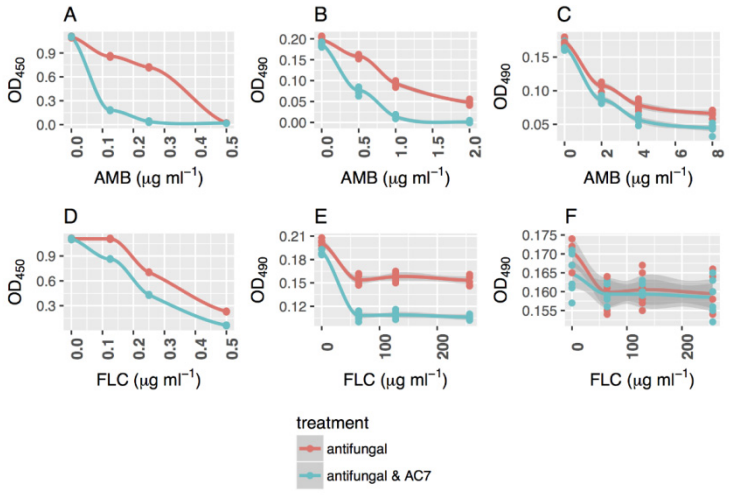

Figure 1. Dose-response (OD) curves of antifungal agents (AMB or FLC) alone or in association with AC7BS (1 mg ml^{-1}) on *Candida albicans* 40 planktonic (A, D) or sessile cells in the intermediate (B, E) and mature (C, F) phases of biofilm formation, in co-incubation condition. Dots (6 for each concentration) represent the experimental data of dose-response curve.

For AMB, the net OD_{450} value of control (no BS or antifungal drug added) was 1.103 ± 0.007 and decreased to 0.859 ± 0.005, 0.719 ± 0.005 and 0.022 ± 0.001 when planktonic cells were treated with 0.125, 0.25 and 0.5 μg ml^{-1} AMB, respectively. Cells co-incubated with AC7BS alone showed a net OD_{450} value of 1.105 ± 0.005 that decreased to 0.180 ± 0.001, 0.039 ± 0.008 and 0.018 ± 0.001 when the biosurfactant was associated with the three concentrations of AMB (Figure 1A).

For FLC, the net OD_{450} value of control was 1.108 ± 0.001 and decreased to 1.107 ± 0.002, 0.702 ± 0.001 and 0.231 ± 0.002 when planktonic cells were treated with 0.125, 0.25 and 0.5 μg ml^{-1} FLC, respectively. Cells co-incubated with AC7BS showed a net OD_{450} value of 1.108 ± 0.002 that decreased to 0.864 ± 0.002, 0.432 ± 0.003 and 0.064 ± 0.003 when the biosurfactant was associated with the tree concentrations of FLC (Figure 1D).

According to ANOVA analysis, the survival of *C. albicans* 40 planktonic cells was significantly dependent on the concentration of antifungal agents ($P < 1 \times 10^{-14}$) and on the type of treatment ($P < 1 \times 10^{-5}$) both for AMB and FLC.

The percentage of inhibition of *C. albicans* 40 planktonic cells are reported in Table 1. With respect to controls, the growth of *C. albicans* 40 planktonic cells was significantly inhibited up to 98.0% by 0.5 μg ml^{-1} AMB and up to 79.1% by 0.5 μg ml^{-1} FLC. AC7BS alone resulted to be ineffective, suggesting that biosurfactant had no antifungal activity. The joint application of AC7BS and the antifungals significantly reduced *Candida* growth up to 98.4% at 0.5 μg ml^{-1} AMB and up to 94.3% at 0.5 μg ml^{-1} FLC.

Table 1. Inhibition of *Candida albicans* 40 planktonic and sessile cells by treatment with antifungal drugs alone or in combination with AC7BS in co-incubation conditions.

	Antifungal	Concentration (μg ml^{-1})	Percentage of inhibition (%)	
			Antifungal	AC7BS&antifungal
Planktonic cells	AMB	0.125	22.2	83.7
		0.25	34.7	96.5
		0.5	98.0	98.4
	FLC	0.125	–	22.0
		0.25	36.5	61.0
		0.5	79.1	94.3
Sessile cells intermediate phase	AMB	0.5	20.0	61.2
		1	52.8	93.0
		2	75.7	99.6
	FLC	64	23.7	46.3
		128	22.0	46.0
		256	23.8	47.4
Sessile cells mature phase	AMB	2	37.9	50.0
		4	54.5	67.3
		8	61.8	74.3
	FLC	64	6.0	7.0
		128	6.0	7.0
		256	6.0	7.0

Furthermore, in both cases, the MIC value of each antibiotic was decreased by the presence of AC7BS from 0.5 μg ml^{-1} to 0.25 μg ml^{-1}.

Figure 2 shows the experimental and multiplicative effect of AC7BS and the antifungal drugs against *C. albicans* 40 planktonic cells, calculated as described in the Materials and Methods section. In particular, when the two sub-MIC concentrations of AMB (Figure 2A) or the three concentrations of FLC (Figure 2D) were associated with AC7BS, the experimental effect $OD^*_{(AC7\ \&\ antifungal)}$ was less than the multiplicative effect $OD^*_{(AC7)} \times OD^*_{(antifungal)}$, indicating a strong synergistic association. ANOVA analysis, confirms synergy ($P < 1 \times 10^{-5}$) and its dependence on concentrations ($P < 1 \times 10^{-14}$).

3.2. Anti-biofilm assays

3.2.1. Co-incubation

The two antifungal agents were tested in association with AC7BS to evaluate a possible joint activity against *C. albicans* 40 sessile cells at different phases of biofilm formation. AMB was applied at a sub-$SMIC_{50}$ concentration (0.5 μg ml^{-1}), at the $SMIC_{50}$ (1 μg ml^{-1}) and at 2 μg ml^{-1} in the intermediate phase whereas at 2 μg ml^{-1} (sub-$SMIC_{50}$), 4 μg ml^{-1} ($SMIC_{50}$) and 8 μg ml^{-1} in the mature phase of biofilm formation. FLC was tested in both cases at three sub-$SMIC_{50}$ concentrations (64, 128, 256 μg ml^{-1}). These concentrations were chosen based on previously calculated $SMICs_{50}$ for AMB and FLC on intermediate and mature phases of biofilm formation (data not shown). AC7BS was used at the concentration of 1 mg ml^{-1}. The metabolic activity (OD_{490}) of sessile cells in co-incubation with or without AC7BS is shown as a function of the concentration of AMB or FLC (Figure 1).

For AMB, in the intermediate phase of biofilm formation, the OD_{490} value of control was 0.198 ± 0.006 and decreased to 0.158 ± 0.001, 0.093 ± 0.005 and 0.048 ± 0.006 when sessile cells were treated with respectively 0.5, 1, 2 μg ml^{-1}AMB. When cells were co-incubated with AC7BS, the value was 0.188 ± 0.003 and decreased to 0.077 ± 0.006, 0.014 ± 0.002 and 0.001 ± 0.001 when the two molecules were associated (Figure 1B). In the mature phase, the OD_{490} value of control was 0.173 ± 0.004 and decreased to 0.108 ± 0.003, 0.079 ± 0.008 and 0.066 ± 0.004 when sessile cells were treated with 2, 4, 8 μg ml^{-1} AMB. In the presence of AC7BS, the OD_{490} value was 0.166 ± 0.005 and decreased to 0.087 ± 0.004, 0.057 ± 0.004 and 0.045 ± 0.006 when AC7BS was associated with AMB (Figure 1C).

For FLC, in the intermediate phase of biofilm formation, the OD_{490} value of control was 0.201 ± 0.005 and decreased to a mean value of 0.155 ± 0.007 at the three tested concentrations. Sessile cells co-incubated with AC7BS showed a value of 0.190 ± 0.002 that decreased to a mean value of 0.108 ± 0.005 when biosurfactant was associated with FLC (Figure 1E). In the mature phase, the value of control was 0.171 ± 0.004 and decreased to a mean value of 0.160 ± 0.005 in the presence of FLC. Sessile cells co-incubated with AC7BS showed a value of 0.165 ± 0.007 that decreased to a mean value of 0.159 ± 0.003 when biosurfactant was associated with FLC (Figure 1F).

In the intermediate phase, sessile cells survival was significantly dependent on the concentration of antifungal agent ($P < 1 \times 10^{-6}$) and on the type of treatment ($P < 1 \times 10^{-6}$) both for AMB and FLC whereas on 24 h-old biofilms, it was significantly dependent on the concentration of antifungal agent ($P < 1 \times 10^{-3}$) and on the type of treatment ($P = 1 \times 10^{-2}$) only in the case AMB.

The percentages of inhibition of *C. albicans* 40 sessile cells are reported in Table 2. In the intermediate and mature phases, the metabolic activity of cells co-incubated with AC7BS was

comparable to that of control (no BS or antifungal drug added) indicating that biosurfactant had no antifungal activity on biofilm. AMB significantly counteracted *C. albicans* 40 biofilm in the intermediate phase up to 75.7% at 2 μg ml^{-1} whereas significantly reduced 24 h-old biofilm up to 61.8% at 8 μg ml^{-1}. Regarding FLC, in the intermediate phase, *C. albicans* 40 biofilm formation was significantly inhibited of about 23% at 64, 128, 256 μg ml^{-1} but no relevant activity on 24 h-old biofilm was detected. The simultaneous use of AC7BS and AMB significantly inhibited *C. albicans* 40 intermediate phase biofilm up to 99.6% (at 2 μg ml^{-1}) whereas significantly reduced 24 h-old biofilms up to 74.3% (at 8 μg ml^{-1}). The simultaneous use of AC7BS and FLC at all the tested concentrations significantly inhibited *C. albicans* 40 in the intermediate phase of biofilm formation of about 46.6% whereas no joint activity on 24 h-old biofilms was observed.

Furthermore, the $SMIC_{50}$ values of AMB were decreased by the presence of AC7BS from 1 μg ml^{-1} to 0,5 μg ml^{-1} in the intermediate phase and from 4 μg ml^{-1} to 2 μg ml^{-1} in the mature phase of biofilm formation.

The synergistic activity of AC7BS and the antifungal drugs against *C. albicans* 40 biofilm formation was evaluated (Figure 2). In the intermediate phase, both for AMB and FLC, the occurrence of synergism is visualized by the higher position of the blue curve in comparison to the red curve (Figure 2B and Figure 2E). In the mature phase (Figure 2C and 2F), the synergism of AC7BS and antifungal drugs was observed only in the case of AMB (Figure 2C). Globally, the synergistic association and its dependence on the concentration of antifungal agents are significantly confirmed by ANOVA analysis ($P < 1 \times 10^{-2}$ and $P < 1 \times 10^{-14}$, respectively).

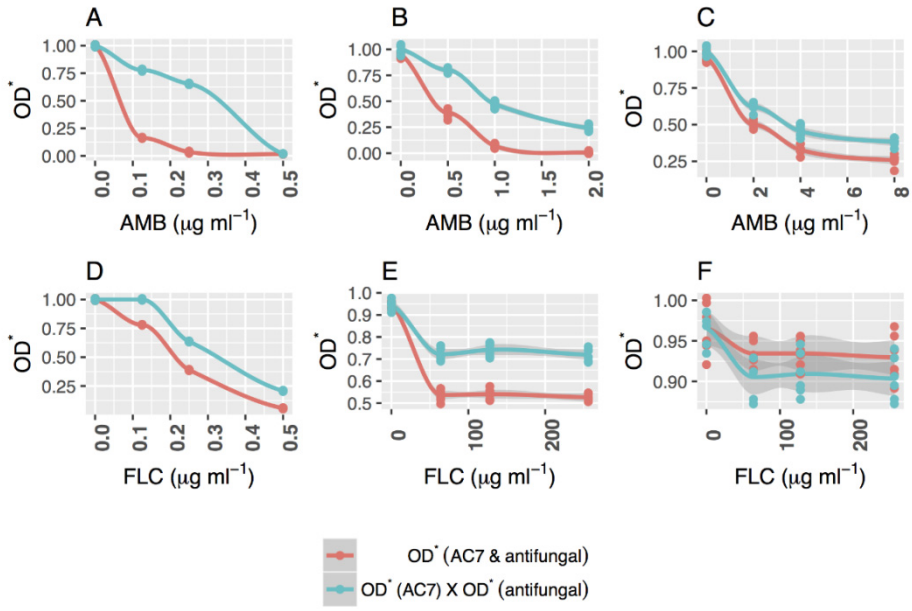

Figure 2. Synergism of antifungal drugs (AMB and FLC) and AC7BS (1 mg ml^{-1}) against *Candida albicans* 40 planktonic (A, D) and sessile cells on intermediate (B, E) and mature (C, F) phases of biofilm formation in co-incubation conditions. Dots (6 for each concentration) represent the experimental data of dose-response curve.

3.2.2. Pre-coating

The antifungal effect of AMB, the anti-adhesive properties of AC7BS and the combination of the two activities (AMB&AC7BS) were evaluated against *C. albicans* 40 biofilm formation in pre-coating conditions. In particular, the activity of AMB (alone or in combination with AC7BS pre-coating) was evaluated in three different experimental settings: AMB added at time 0 to the fungal suspension and at time 1.5 h to the growth medium, at the concentrations of 0.125, 0.25, 0.5 μg ml^{-1} (pre-coating type 1); AMB added only at time 1.5 h to the growth medium, at the concentrations of 0.5, 1, 2 μg ml^{-1} (pre-coating type 2); AMB added at time 24 h to the growth medium, at 2, 4, 8 μg ml^{-1} (pre-coating type 3). These concentrations were again chosen based on the previously calculated MICs and SMICs for AMB on planktonic and sessile *Candida* cells.

In Figure 3, the metabolic activity (OD_{490}) of *C. albicans* 40 in biofilm formation on silicone elastomeric disks pre-coated with or without AC7BS is shown as a function of the concentration of AMB.

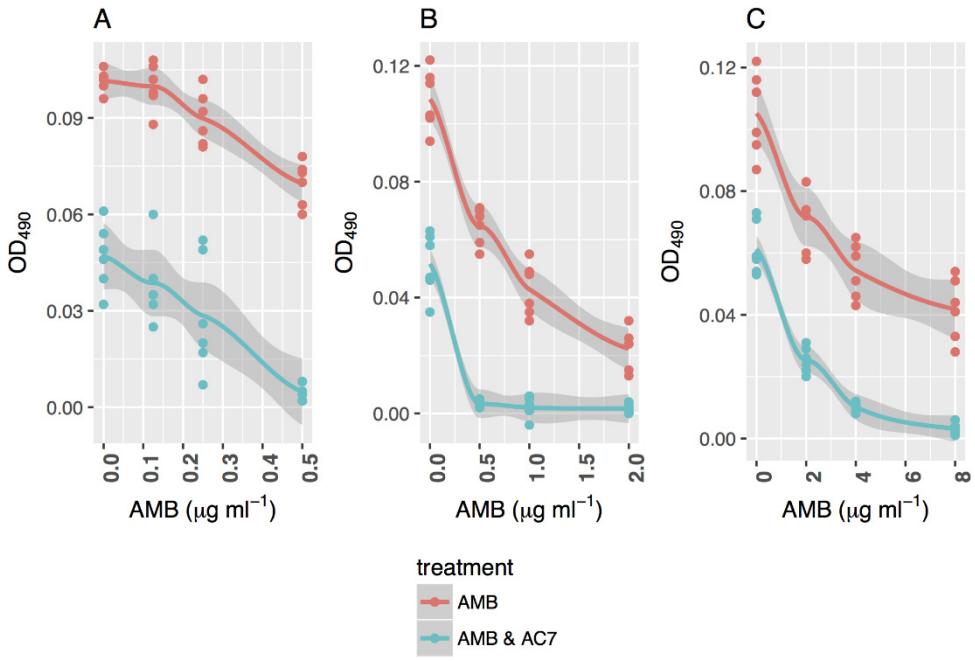

Figure 3. Dose-response (OD) curves of AMB alone or in association with AC7BS (2 mg ml^{-1}) pre-coating on *Candida albicans* 40 biofilm formation on silicone. In pre-coating type 1, AMB was added at time 0 and at time 1.5 h (A); in pre-coating type 2, AMB was added only at time 1.5 h (B) and in pre-coating type 3, AMB was added at time 24 h (C). Dots (6 for each concentration) represent the experimental data of dose-response curve.

In pre-coating type 1, the OD_{490} value of control was 0.102 ± 0.003 and decreased to 0.100 ± 0.007, 0.090 ± 0.008 and 0.070 ± 0.007 when cells were treated with respectively 0.125, 0.25, 0.5 μg ml^{-1}AMB. When cells were treated with AC7BS the value was 0.047 ± 0.010 and decreased to 0.039 ± 0.012, 0.029 ± 0.018 and 0.005 ± 0.003 when AC7BS were associated with the three

concentrations of AMB (Figure 3A).

In pre-coating type 2, the OD_{490} value of control was 0.109 ± 0.011 and decreased to 0.065 ± 0.006, 0.043 ± 0.009 and 0.022 ± 0.007 when cells were treated with respectively 0.5, 1, 2 μg ml^{-1}AMB. When cells were treated with AC7BS the value was 0.052 ± 0.011 and decreased up to 0.02 ± 0.002 when AC7BS was associated with AMB at 2 μg ml^{-1} (Figure 3B).

In pre-coating type 3, the OD_{490} value of control was 0.105 ± 0.014 and decreased to 0.072 ± 0.011, 0.052 ± 0.007 and 0.042 ± 0.010 when cells were treated with respectively 2, 4, 8 μg ml^{-1}AMB. When cells were treated with AC7BS the value was 0.061 ± 0.009 and decreased to 0.025 ± 0.004, 0.010 ± 0.001 and 0.003 ± 0.002 when AC7BS was associated with the three concentrations of AMB (Figure 3C).

Globally, according to ANOVA analysis, *C. albicans* 40 biofilm formation is significantly dependent on the concentration of AMB ($P = 1 \times 10^{-14}$), on the type of treatment ($P = 1 \times 10^{-10}$) and on the pre-coating type ($P = 1 \times 10^{-15}$).

The complete set of percentages of inhibition in pre-coating assays is reported in Table 2.

In pre-coating type 1, with respect to controls, AC7BS significantly reduced *C. albicans* 40 sessile cell of 53.7%. AMB significantly killed cells up to 31.4% at 0.5 μg ml^{-1}, whereas its simultaneous use with AC7BS up to 94.7% (at 0.5 μg ml^{-1}). In addition, in the presence of AC7BS, the $SMIC_{50}$ value was achieved at 0.125 μg ml^{-1}.

In pre-coating type 2, with respect to controls, AC7BS reduced *C. albicans* 40 sessile cells of 52.4% whereas AMB up to 79.4% at 2 μg ml^{-1}. The use of the two molecules together significantly inhibited *C. albicans* 40 up to 98.2% at 2 μg ml^{-1}. In addition, in the presence of AC7BS, the $SMIC_{90}$ value of AMB was reached at 0.5 μg ml^{-1}.

In pre-coating type 3, with respect to controls, AC7BS significantly reduced 24 h-old biofilms of 41.7% whereas AMB of 60.2% at 8 μg ml^{-1}. The joint application of AC7BS and AMB significantly inhibited 24 h-old biofilms up to 97.0% at 8 μg ml^{-1}. Furthermore, a decrease of the $SMIC_{50}$ value, from 4 μg ml^{-1} to 2 μg ml^{-1} was achieved.

Table 2. Inhibition of *Candida albicans* 40 biofilm formation in pre-coating assays.

	AMB (μg ml^{-1})	Percentage of inhibition (%)		
		AC7BS	AMB	AC7BS&AMB
Pre-coating type 1	0.0	53.7		
	0.125		–	61.9
	0.25		11.5	71.9
	0.5		31.4	94.7
Pre-coating type 2	0.0	52.4		
	0.5		40.4	96.9
	1		60.5	98.2
	2		79.4	98.2
Pre-coating type 3	0.0	41.7		
	2		31.9	75.9
	4		50.4	90.3
	8		60.2	97.0

The activity obtained by the association of AC7BS and AMB in the pre-coating assays was graphically expressed in Figure 4.

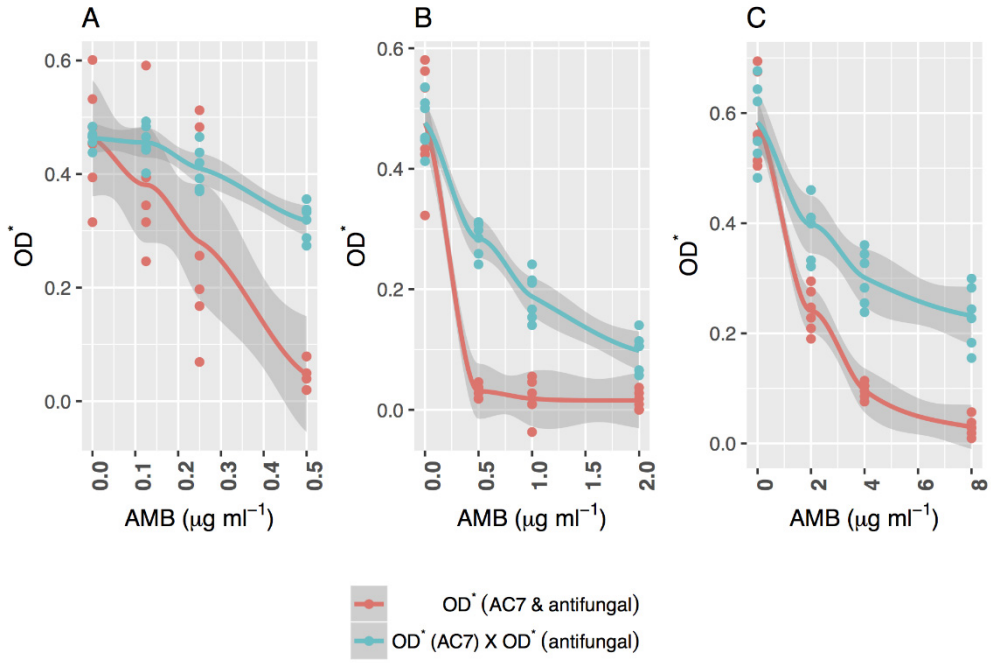

Figure 4. Synergism of AMB and AC7BS (2 mg ml^{-1}) pre-coating against *Candida albicans* 40 biofilm formation on silicone. Pre-coating type 1 (A), pre-coating type 2 (B) and pre-coating type 3 (C). In pre-coating type 1, AMB was added at time 0 and at time 1.5 h (A); in pre-coating type 2, AMB was added only at time 1.5 h (B) and in pre-coating type 3, AMB was added at time 24 h (C). Dots (6 for each concentration) represent the experimental data of dose-response curve.

An interesting synergistic association was reported in all the three type of experiments, confirmed by the higher position of $OD^*_{(AC7BS)} \times OD^*_{(AMB)}$ curve with respect to $OD^*_{(AC7BS\ \&\ AMB)}$ curve.

ANOVA analysis confirms synergy ($P < 1 \times 10^{-3}$) and its dependence on concentration ($P < 1 \times 10^{-7}$).

4. Discussion

Candida species are involved in Candidiasis, the most common opportunistic yeast infection, and *Candida* albicans, the most prevalent, is responsible of approximately 50–90% of cases [20]. *C. albicans* pathogenesis is closely associated to its ability to growth as biofilms, structured cell communities embedded in extracellular matrix, that protect the microorganism from host defences and reduce significantly its susceptibility to antifungal agents [21]. Amphotericin B and fluconazole represent antifungal agents of choice in the treatment of serious *Candida* infections [22]. The interaction of amphotericin B with ergosterol results in pores formation, surface adsorption and ergosterol extraction from plasma membranes leading to membrane damage and rapid fungal cell death [23]. Fluconazole interferes with the ergosterol synthesis preventing the conversion of lanosterol to ergosterol by the inhibition of the fungal cytochrome P450 enzyme 14α-demethylase [24]. Despite

the presence of more effective antifungal agents, active on both planktonic and sessile cells such as AMB and echinocandins, fluconazole is still widely used in the clinic because of its efficacy and low toxicity. Various approaches have been recently proposed to increase the susceptibility of *C. albicans* to fluconazole, such as its combined use with different classes of non-antifungal agents that overcome fungal resistance or enhance antifungal activity [25].

In the presence of biofilm, antifungal drugs are generally less effective or, in some cases, even ineffective. To overcome this serious clinical problem, novel strategies in preventing biofilm development are beginning to explore the combined use of different antimicrobial compounds in order to increase their efficacy. Biosurfactants-secondary metabolites produced by numerous microorganisms-have drawn attention of scientific community thanks to their interesting biological and chemical properties such as the ability to disturb cell membrane integrity and permeability and to affect adhesion of microorganisms [12]. In particular, lipopeptides are involved in the destabilization of membranes lipid packing. They penetrate into the membrane through hydrophobic interactions, influencing in the order of the hydrocarbon chain and varying the membrane thickness and form pores that change membrane permeability and decrease the cooperativity of lipid-lipid interactions [26–30]. Furthermore, lipopeptides are able to reduce the hydrophobicity of surfaces interfering with microbial adhesion and desorption processes [31].

In a previous work by the authors, it has been demonstrated that the lipopeptide biosurfactant AC7BS was characterized by antiadhesive activity against *C. albicans* planktonic and sessile cells [13]. Pre-treatment of silicone elastomeric disks with AC7BS caused the inhibition of fungal adhesion and biofilm formation without altering cell viability of both planktonic and sessile cells. Other studies successfully demonstrated the antiadhesive and anti-biofilm activity of lipopeptides against bacterial and fungal pathogens on polystyrene and silicone materials [32,33].

In the present study, for the first time, the association of AC7BS with two antifungal compounds extensively used in the treatment of invasive fungal infections, amphotericin B (AMB) and fluconazole (FLC), was explored on medical-grade silicone against planktonic and sessile cells of *C. albicans* 40, a clinical isolate from central venous catheter. Moreover, in order to emphasize the clinically oriented approach, the activity of AC7BS and antifungal agents, alone or in association, was assessed in the presence of fetal bovine serum, to mimic medical devices contact with biological fluids during clinical use in internal body areas.

The evaluation of the anti-adhesive and anti-biofilm activity of AC7BS and antifungals was carried out in co-incubation and pre-coating conditions. Co-incubation assays were applied to assess AC7BS biological properties as adjuvant during antifungal treatments, as well as to measure its ability to dislodge pre-formed biofilms. On the other hand, pre-coating assays were used to evaluate the real efficacy and the possible applicability of AC7BS as medical devices coating agent for the prevention of microbial adhesion and biofilm formation, alone or in association to antifungal treatments. Antifungal agents were, generally, tested at three different concentrations: above the MIC, at MIC and at sub-MIC. The first two concentrations were tested with the objective of defining whether the lipopeptide could enhance the killing activity of the antifungal drug. The sub-MIC concentration was tested to assess whether the presence of AC7BS could determine an increase in the antifungal activity, with the consequent decrease of the MIC value. This combined activity could allow the reduction of the therapeutic dose of antifungal agent, thus limiting the occurrence of adverse reactions and the onset of resistances.

In general, when AC7BS was used in association with AMB or FLC, a synergistic activity against

planktonic cells and biofilm formation was observed. The term synergism, meaning working together, is referred to the interaction between two or more molecules when their effect in association is greater than the combination of the effects of the individual compounds.

In the case of planktonic cells, despite the inability of AC7BS to inhibit cells viability, the antifungal activity of AMB and FLC was synergistically increased by the presence of the biosurfactant and a reduction of MICs values was observed for both the antifungal agents.

In anti-biofilm assays, in co-incubation experiments, results demonstrated a significant inhibitory effect of AMB against sessile cells. FLC affected the intermediate phase of biofilm formation but was unable to reduce 24 h-old biofilms. It was also observed that AC7BS alone was not able to inhibit sessile cells but, generally, it increased the killing activity of antifungal agents when used in association. In addition, a reduction of SMICs values was observed for AMB.

It can be hypothesized that the antifungal activity of AMB or FLC is synergistically increased by the interaction of AC7BS with membrane lipids leading to a higher permeability of plasma membranes to antifungal agents.

Another work described a synergistic effect of a lipopeptide in co-incubation with various antibiotics against biofilm of the uropathogenic strain *E. coli* CFT073 on polystyrene [14]. The combined use of V9T14 lipopeptide and six different antibiotics [cefazolin, ciprofloxacin, ceftriaxone, piperacillin, tobramycin and trimethoprim/sulfamethoxazole (SXT) (19:1)] led to a reduction of biofilms in terms of CFU ml^{-1} ranging from 1.0 log10 to 2.1 log10, whereas the association of the biosurfactant with ampicillin led to a complete eradication. Similarly to AC7BS, the presence of V9T14 did not affect cell viability of planktonic and sessile cells but decreased the amount of antibiotics required to obtain the same cell reduction detected with the antibiotic alone.

In pre-coating experiments, the absorption of AC7BS on silicone disks resulted in a significant reduction of biofilm formation confirming the antiadhesive properties of this biosurfactant. The treatment with AMB alone showed a higher inhibitory effect when added after the adhesion phase. A less marked, but still significant, activity was observed when the antifungal agent was added directly to the fungal suspension and on 24-old biofilms. Interestingly, when AC7BS and AMB were used in association, a synergistic activity of the two molecules against *C. albicans* 40 biofilm formation was observed. In particular, the most encouraging results were obtained in pre-coating type 2 experiments, wherein the co-use of AC7BS and AMB at all concentrations decreased biofilm formation of more than 90%. Furthermore, it was also possible to observe a reduction of the concentration of antifungal agent needed to achieve the SMICs values.

In this case, the synergism can be due both to the anti-adhesive activity of AC7BS and to the antifungal effect of AMB, but also to the previously indicated ability of the biosurfactant to decrease the cooperativity of the lipid-lipid interactions in the bilayer membrane, thus facilitating the entry of AMB in the cells.

5. Conclusion

The study demonstrated, for the first time, that the association of lipopeptide AC7 with antifungal agents leads to a synergistic effect in inhibiting *C. albicans* 40 planktonic cells and biofilm formation. Although additional studies are required to determine the molecular basis of these observations, these results suggest that the joint activity of AC7BS and antifungal agents might have potential applicability in prophylactic or therapeutic strategy against *C. albicans* infections related to the use of

medical insertional materials.

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Conflict of Interest

All authors declare no conflicts of interest in this paper.

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