Lipopeptides from Bacillus subtilis AC7 inhibit adhesion and biofilm formation of Candida albicans on silicone

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Journal Name	Antonie van Leeuwenhoek	
Corresponding Author	Family Name	**Fracchia**
	Particle	
	Given Name	**Letizia**
	Suffix	
	Division	Department of Pharmaceutical Sciences
	Organization	Università del Piemonte Orientale “A. Avogadro”
	Address	Largo Donegani 2, 28100, Novara, Italy
	Email	letizia.fracchia@uniupo.it
	ORCID	http://orcid.org/0000-0003-3505-1919
Author	Family Name	**Ceresa**
	Particle	
	Given Name	**Chiara**
	Suffix	
	Division	Department of Pharmaceutical Sciences
	Organization	Università del Piemonte Orientale “A. Avogadro”
	Address	Largo Donegani 2, 28100, Novara, Italy
	Email	
	ORCID	
Author	Family Name	**Rinaldi**
	Particle	
	Given Name	**Maurizio**
	Suffix	
	Division	Department of Pharmaceutical Sciences
	Organization	Università del Piemonte Orientale “A. Avogadro”
	Address	Largo Donegani 2, 28100, Novara, Italy
	Email	
	ORCID	
Author	Family Name	**Chiono**
	Particle	
	Given Name	**Valeria**
	Suffix	
	Division	Department of Mechanical and Aerospace Engineering
	Organization	Politecnico di Torino

	Address	Corso Duca degli Abruzzi 24, 10129, Turin, Italy
	Email	
	ORCID	
Author	Family Name	**Carmagnola**
	Particle	
	Given Name	**Irene**
	Suffix	
	Division	Department of Mechanical and Aerospace Engineering
	Organization	Politecnico di Torino
	Address	Corso Duca degli Abruzzi 24, 10129, Turin, Italy
	Email	
	ORCID	
Author	Family Name	**Allegrone**
	Particle	
	Given Name	**Gianna**
	Suffix	
	Division	Department of Pharmaceutical Sciences
	Organization	Università del Piemonte Orientale “A. Avogadro”
	Address	Largo Donegani 2, 28100, Novara, Italy
	Email	
	ORCID	

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Keywords (separated by '-')	Anti-adhesion - Biofilm - Medical device - Lipopeptide biosurfactant - Coating - *Candida albicans*
Footnote Information	

Antonie van Leeuwenhoek
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ORIGINAL PAPER

Lipopeptides from *Bacillus subtilis* AC7 inhibit adhesion and biofilm formation of *Candida albicans* on silicone

Chiara Ceresa · Maurizio Rinaldi · Valeria Chiono · Irene Carmagnola · Gianna Allegrone · Letizia Fracchia

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Keywords Anti-adhesion · Biofilm · Medical device · Lipopeptide biosurfactant · Coating · *Candida albicans*

A1 C. Ceresa · M. Rinaldi · G. Allegrone · L. Fracchia (✉)
A2 Department of Pharmaceutical Sciences, Università del
A3 Piemonte Orientale "A. Avogadro", Largo Donegani 2,
A4 28100 Novara, Italy
A5 e-mail: letizia.fracchia@uniupo.it

A6 V. Chiono · I. Carmagnola
A7 Department of Mechanical and Aerospace Engineering,
A8 Politecnico di Torino, Corso Duca degli Abruzzi 24,
A9 10129 Turin, Italy

Introduction

In recent years, a significant increase in the incidence of human fungal infections has been observed. *Candida* species are the major problem, especially in immunocompromised patients (Espinel-Ingroff et al. 2009; van De Veerdonk et al. 2010; Ruhnke et al. 2011), representing the fourth most common cause of nosocomial bloodstream infections (Wisplinghoff et al. 2004). Invasive candidiasis presents a high

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global mortality rate, ranging from 36 to 63 % in different patient groups (Guery et al. 2009; van De Veerdonk et al. 2010) and represents a significant problem in terms of patient management and healthcare costs in the public health system (Kullberg et al. 2011).

Among the *Candida* species isolated from humans, *Candida albicans* is frequently associated with the formation of biofilms on a wide variety of medical devices (Crump and Collignon 2000; Goldberg et al. 2000; Karchmer 2000; Maki and Tambyah 2001). *C. albicans* biofilms consist of structured surface-associated cell communities embedded in an extracellular matrix that have distinct phenotypes compared to their planktonic cell counterparts (Fanning and Mitchell 2012). The presence of the biofilm plays a key role on *C. albicans* pathogenesis, by protecting the microorganism from host defences and reducing significantly its susceptibility to antifungal agents (Hawser and Douglas 1994; Lazzell et al. 2009). Furthermore, the tenacity with which *C. albicans* infects indwelling medical devices necessitates, in almost all the cases, their removal (Kojic and Darouiche 2004).

For these reasons, the development of new technologies to counteract *C. albicans* biofilm growth represents a major challenge in clinical practice and preventive medicine. Among microbial metabolites, biosurfactants have gained importance thanks to their interesting biological properties, such as the ability to disrupt membranes and to affect the adhesion properties of cells/microorganisms (Cameotra and Makkar 2004; Singh and Cameotra 2004; Seydlová and Svobodová 2008; Rodrigues and Teixeira 2010). Biosurfactants are amphipathic compounds with both hydrophilic and hydrophobic moieties that exhibit surface activities at interfaces (Banat et al. 2010). Adsorption of biosurfactants to a substratum surface modifies its hydrophobicity, interfering with microbial adhesion and desorption processes (Rodrigues et al. 2006a; Biniarz et al. 2015). Pre-coating catheters and other medical implantable materials with biosurfactants could represent a preventive strategy to inhibit pathogenic biofilm growth, thus reducing the use of pharmaceuticals and antibiotics (Rodrigues et al. 2006b; Singh et al. 2007; Falagas and Makris 2009).

In this study, the ability of the lipopeptide biosurfactant AC7 produced by *Bacillus subtilis* (AC7 BS) to inhibit adhesion and biofilm formation of three *C. albicans* strains was evaluated by co-incubation and pre-coating on silicone elastomer at physiological conditions. Lipopeptides activity was determined by means of the crystal violet staining and the viable cell counting methods. Moreover, AC7 BS was chemically characterised and its stability and surface activities measured. Surface physicochemical characterisations of the AC7 BS coated silicone were also carried out.

Materials and methods

Microorganisms and culture conditions

The endophytic biosurfactant-producing strain AC7 was isolated from the inside of stems of *Robinia pseudoacacia* and was genotypically identified by complete 16S rDNA sequence analysis (DSMZ Identification Service, Braunschweig, Germany) as *B. subtilis*. For biofilm assays, the strain *C. albicans* IHEM 2894 was purchased from The Belgian Coordinated Collections of Microorganisms (BCCM). *C. albicans* 40 (DSM 29204) and 42 (DSM 29205) are two wild strains (courtesy of Hospital "Maggiore della Carità", Novara, Italy), clinically isolated from central venous catheter and urinary catheter, respectively, and deposited by the Authors in the DSMZ collection. Strain *B. subtilis* AC7 was stored at −80 °C in Luria–Bertani (LB) broth (Sigma-Aldrich) supplemented with 25 % glycerol and grown on LB agar plates for 24 h at 28 °C. *C. albicans* strains were stored at −80 °C in Sabouraud dextrose broth (Sigma-Aldrich) supplemented with 25 % glycerol and grown for 24 h at 37 °C on Sabouraud Dextrose Agar (SDA) plates.

Critical micelle concentration, emulsification index and stability study of AC7 BS

AC7 BS crude extract was obtained according to the method described by Rivardo et al. (2009). Surface tension of 0.5, 1.0 and 2.0 mg ml^{-1} AC7 BS solutions in alkaline distilled water was measured by using a ring tensiometer (KSV Sigma 703D). Results for surface tension measurements were expressed as mN m^{-1} and compared with alkaline distilled water. Critical micelle concentration (CMC) was determined on serially diluted biosurfactant solutions in alkaline distilled water (from 0.01 to 0.5 mg ml^{-1}). Surface tension of each dilution was determined in triplicate. The CMC was assessed from the intercept of two

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straight lines extrapolated from the concentration-dependent and concentration-independent sections of a curve plotted between biosurfactant concentration and surface tension values.

The heat/cold stability of 0.5, 1.0 and 2.0 mg ml^{-1} AC7 BS solutions was evaluated by measuring the emulsification index at 24 h E_{24} (%) (Franzetti et al. 2012) and surface tension after treatment at 100 °C for 1 h, at 121 °C for 15 min and at −80 °C for 24 h. To study pH stability, the pH of the AC7 BS solutions (0.5, 1.0, 2.0 mg ml^{-1}) was adjusted to different pH values (3–11) with 1 M NaOH or 1 M HCl. Afterwards, the surface tension and the E_{24} were measured. Assays were carried out in triplicate.

Chemical characterisation of AC7 BS

The chemical characterisation of the crude extract was performed according to the method described by Pecci et al. (2010), with and slight modifications. An aliquot of the biosurfactant extract was dissolved in methanol/acetonitrile (50/50 v/v) to obtain a 1000 µg ml^{-1} stock solution. Freshly prepared working solutions were made by diluting the stock solution with methanol/water (50/50 v/v) to 10 µg ml^{-1} solutions Mass spectrometry analyses were done on a LCQ DECA XP Plus (Thermo Finnigan, San Jose, CA, USA) IonTrap mass instrument equipped with an ESI source. Samples (10 µg/ml solutions) were injected with a syringe at 5 µl min^{-1} flow rate. Source voltage and capillary voltage were at 4.80 kV and 23 V in positive mode. The capillary temperature was maintained at 350 °C and nitrogen was used as nebulising gas at 30 arbitrary units. Data were acquired in positive MS total ion scan mode (mass scan range *m/z* 100–2000) and MS/MS product ion scan mode with normalised collision energy (nce %) optimised for each precursor ion selected: *m/z* 1030, 38 %; 1044 and 1058, 39 %; 1478 and 1506, 35 %.

Antifungal susceptibility testing against *Candida albicans* planktonic cells

AC7 BS antifungal activity towards planktonic cells of *C. albicans* strains was assessed according to EUCAST guidelines (Rodriguez-Tudela et al. 2008). Briefly, 100 µl of AC7 BS solutions (from 0.06 to 6 mg ml^{-1}) were added in a 96-well microtiter plate (Bioster). In control wells (no biosurfactant added), 100 µl of sterile PBS were used. *C. albicans* suspensions at a concentration ranging from 1 to 5 × 10^5 colony-forming units (CFU) ml^{-1} were 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. Subsequently, 100 µl of the *Candida* suspensions were added to each well, to obtain final concentrations of AC7 BS ranging from 0.03 to 3 mg ml^{-1}, and to the control wells. Blank wells were prepared by mixing 100 µl double-strength RPMI with 100 µl of the biosurfactant solutions (from 0.06 to 6 mg ml^{-1}) or PBS. The plate was incubated at 37 °C for 24 h in static conditions. Finally, OD_{450} was measured for each well using a Ultramark Microplate Imaging System (Bio-Rad). The data were normalised with respect to the value of the corresponding blank wells. Assays were carried out in triplicate.

Medical-grade silicone elastomeric disks preparation

Medical-grade silicone elastomeric disks (SEDs) (TECNOEXTR Srl, Italy) used in the study were 15 mm in diameter and 1.5 mm in thickness for experiments in 12-well tissue culture plates, and 10 mm in diameter and 1.5 mm in thickness for experiments in 24-well tissue culture plates. Cleaning and sterilisation of SEDs was carried out according to the method described by Busscher et al. (1997). Briefly, disks were immersed in 200 ml of distilled water supplemented with 1.4 % (v/v) of RBS™ 50 solution (Sigma-Aldrich), sonicated for 5 min at 60 kHz using Elma S30H (Elmasonic, VWR International) and rinsed in 1 l of MilliQ water twice. Then, disks were submerged in 20 ml of methanol (99 %) (Sigma-Aldrich), rinsed twice and autoclaved for 15 min at 121 °C.

Antifungal susceptibility of *Candida albicans* biofilms

Candida albicans cells were cultivated for 24 h at 37 °C on SDA plates. Cells were then suspended in Phosphate Buffered Saline (PBS) solution with 10 % Fetal Bovine Serum (FBS) and standardised to 1 × 10^7 CFU ml^{-1}. One milliliter of this fungal

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suspension was used to submerge silicone disks previously inserted in a 24-well plate (Greiner bio-one). After incubation in static conditions at 37 °C for 1.5 h (adhesion phase), disks were transferred into a new 24-well plate and submerged in 1 ml of Yeast Nitrogen Base with 50 mmol l^{-1} Dextrose (YNBD) medium with 10 % of FBS and incubated at 37 °C for 24 h at 90 rpm (biofilm growth phase). Mature biofilms were then treated with different concentrations of AC7 BS in YNBD + 10 % FBS (ranging from 0.06 to 3 mg ml^{-1}) and incubated for an additional 24 h at 37 °C in static conditions. As controls, mature biofilms were submerged in 1 ml YNBD + 10 % FBS. The antifungal activity of AC7 BS was evaluated by the {2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide} (XTT) (Sigma-Aldrich) colorimetric assay. Disks were transferred into new plates and submerged in 1 ml of PBS containing 12.5 µl of XTT solution (1 mg ml^{-1}) and 1 µl of 1 mmol l^{-1} menadione solution (Sigma-Aldrich). Blank disks (disks without biofilm), submerged in the XTT/menadione mixture were also prepared. Plates were covered with an aluminum foil and incubated at 37 °C for 5 h at 90 rpm. Finally, 150 µl were collected from each well and transferred to a 96-well plate for OD_{490} measurement using an Ultramark microplate imaging system (Bio-Rad Laboratories Srl, Segrate MI, Italy). The data were normalised with respect to the value of blanks (background). All assays were carried out in triplicate.

Anti-adhesion and anti-biofilm assays against *C. albicans* strains

Co-incubation assays

Five hundred microliters of *C. albicans* suspensions (2×10^7 CFU ml^{-1}) in PBS + 20 % FBS, were added to each well of a 24-well plates (Greiner bio-one) containing a silicone disk together with either 500 µl of 2 × AC7 BS solutions (1, 2, 4 and 6 mg ml^{-1}) (test groups) or PBS (control group). After 1.5 h of incubation (*C. albicans* adhesion phase), the disks were transferred into a new plate and placed in either 1 ml of YNBD + 10 % FBS with 0 mg ml^{-1} (control group) or 0.5, 1, 2, 3 mg ml^{-1} AC7 BS (test groups) and incubated for 24 h at 37 °C at 90 rpm. The reduction of adherent cells and biofilm biomass was evaluated after 1.5 and 24 h by crystal violet (CV) staining. The supernatants were discarded and the disks were washed three times with PBS for removal of non-adherent cells. Afterwards, disks were dried at 37 °C for 2 h and submerged into 1 ml of a 0.2 % CV solution for 10 min. The CV solution was removed by washing with distilled water and the disks air-dried. Finally, bound CV was released by adding 2 ml of 33 % acetic acid (Sigma-Aldrich) and OD_{570} was measured using a Ultramark microplate imaging system (Bio-Rad Laboratories Srl, Segrate MI, Italy). Assays were carried out in triplicate and the experiments were repeated two times.

Pre-coating assays

SEDs were dipped in 2 ml of AC7 BS solution at concentrations ranging from 0.5 to 3 mg ml^{-1} (test groups) or in PBS only (control group) and incubated at 37 °C for 24 h at 140 rpm. Disks were then placed in 12-well plates containing two milliliters of *C. albicans* suspensions, standardised to 1×10^7 CFU ml^{-1}. After 1.5 h of incubation (adhesion phase), the disks were transferred into 2 ml YNBD + 10 % FBS and incubated at 37 °C with gentle shaking for 24 h. The reduction of adherent cells and biofilm biomass were evaluated after 1.5 and 24 h with CV staining method as indicated previously.

Furthermore, the anti-adhesion and anti-biofilm activity of silicone disks pre-coated with AC7 BS at a concentration of 2 mg ml^{-1} was evaluated by means of the viable-cell counting method. Silicone disks and *C. albicans* suspensions were prepared as described previously. After 1.5 h and 24 h of incubation, the supernatants were discarded and the disks were washed three times with PBS to remove non-adherent cells. Then, the disks were inserted into 50 ml tubes containing 10 ml PBS and subjected to four cycles of sonication (30 s) and stirring (30 s) for cells detachment. The disrupted biofilm cells were serially diluted in PBS and 1 ml of each dilution was incorporated into melted SDA using the pour-plate method.

Agar plates were incubated at 37 °C for 24 h and colonies were then enumerated. Assays were carried out in triplicate and experiments were repeated two times. Results were expressed as mean $\log_{10}$ CFU/disk ± standard deviations.

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Surface physicochemical characterisation

For the physicochemical characterisation, a set of six AC7 BS pre-coated and six control (PBS treated) SEDs was prepared as described previously. In order to simulate a pre-coating assay, three of the AC7 BS pre-coated and three of the control SEDs were subjected to the same procedures as described in the previous section (up to the three washing steps with PBS after 1.5 h of incubation) with the only difference that sterile YNBD + 10 % FBS was added to the disks instead of the *C. albicans* suspensions.

The wettability of SEDs was evaluated by water contact angle measurements using a CAM 200 KSV Instrument (Biolin Scientific), equipped with Tetha software. Static water contact angle was measured using the sessile drop l Milli-Q water drops) at room temperature. The static contact angle method was calculated as the average value from five measurements.

The surface chemical properties of SEDs were analysed by infrared spectroscopy in an IR Perkin-Elmer Frontier spectrophotometer equipped with an attenuated total reflectance (ATR-FTIR) device using a Germanium crystal. Spectra were recorded with a resolution of 4 cm^{-1} and averaged over 36 scans.

Statistical analysis of data

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 used to compare optical densities of planktonic cells and pre-formed biofilm at different AC7 BS concentrations for the three *C. albicans* strains. Tukey's Honest Significant Difference (HSD) method was used as ANOVA post hoc test. The Welch Two Sample *t* test was performed to investigate the effect of AC7 BS on the three *Candida* stains adhesion and biofilm formation in pre-coating assays, carried out by means of the viable cell counting method. Results were considered to be statistically significant when $P < 0.05$. To estimate $\log_{10}$ CFU/disk from colony counts, the R package dupiR was used (Comoglio et al. 2013). This package allows estimation, from a set of counts, the population size and its uncertainty using a Bayesian approach under minimal information on the distributions; this is particularly helpful in situations where one faces with low counts.

Results

Critical micellar concentration and stability study of AC7 BS

Critical micellar concentration (CMC) was evaluated for the AC7 BS crude extract. An AC7 BS solution at a concentration of 0.5 mg ml^{-1} reduced the surface tension of alkaline distilled water from 72.4 to 31.4 mN m^{-1} (Fig. 1). Serial dilutions of this solution showed a gradual increase of surface tension up to 38.2 mN m^{-1} at the concentration of 62.5 μg ml^{-1}. Then, surface tension rapidly increased to 54.9 mN m^{-1} at the concentration of 7.8 μg ml^{-1}. The CMC value for AC7 BS was 31.9 μg ml^{-1}.

Studies on the pH stability of AC7 BS, carried out at 0.5, 1.0 and 2.0 mg ml^{-1}, demonstrated that it was stable over a wide pH range (Table 1). At pH ranging from 6 to 11, the surface tension was preserved without large deviations at all of the three concentrations tested. In particular, the highest surface activity of AC7 BS solutions at 0.5, 1.0 and 2.0 mg ml^{-1} was found at pH 6.0 with values of 28.45, 28.53 and 29.26 mN m^{-1}, respectively. At pH 11.0 the values were 30.76, 30.91 and 30.92 mN m^{-1}. At pH $\leq$ 5, the surface tension of the three AC7BS solutions increased and reached, at pH 3, values of 49.01, 40.64 and 37.20 mN m^{-1}. The mean emulsification index at 24 h (E_{24}) of AC7 BS solutions was not altered at pH from 7 to 11 (about 60 % at all three concentrations tested), but it was absent when the pH

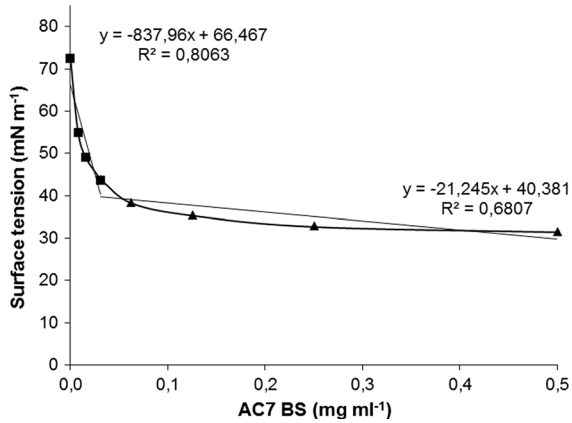

Fig. 1 A plot of surface tension as a function of concentration of AC7 BS after purification. Standard deviation was ranging between ±0.3 mN m^{-1}

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Table 1 Surface tension and emulsification index at 24 h (E_{24}) of AC7 BS solutions as a function of pH

pH	0.5 mg ml^{-1}			1 mg ml^{-1}			2 mg ml^{-1}		
	Surface tension (mN m^{-1})		E_{24h} (%)	Surface tension (mN m^{-1})		E_{24h} (%)	Surface tension (mN m^{-1})		E_{24h} (%)
	Mean	SD[a]		Mean	SD		Mean	SD	
3.0	49.01	0.35	0	40.64	0.17	0	37.20	0.13	0
4.0	49.40	0.29	0	36.54	0.25	0	35.42	0.08	0
5.0	37.53	0.39	0	31.32	0.17	0	30.74	0.28	0
6.0	28.45	0.10	0	28.53	0.36	0	29.26	0.43	0
7.0	30.50	0.17	60.5	30.49	0.22	60	30.36	0.17	60
8.0	30.95	0.18	60.5	30.45	0.17	60	30.64	0.06	60
9.0	30.96	0.14	60.5	30.47	0.30	60	30.84	0.44	60
10.0	31.13	0.22	60.5	30.82	0.27	60	30.62	0.31	60
11.0	30.76	0.32	60.5	30.91	0.30	60	30.92	0.14	60

Surface tension of alkaline distilled water was 72.4 mN m^{-1}

[a] *SD* standard deviation

was reduced. At pH 7 (the condition used in the anti-adhesion and anti-biofilm assays reported here), AC7 BS solutions showed an E_{24} of about 60 % and a mean surface tension value of 30.45 mN m^{-1}.

The studies on the effect of heat/cold treatment on AC7 BS solutions at 0.5, 1.0 and 2.0 mg ml^{-1} demonstrated no evident changes in surface tension and E_{24}. In particular, the treatment at 100 °C for 1 h, at 121 °C for 15 min and at −80 °C for 24 h did not alter the ability of AC7 BS solutions to decrease water surface tension and its emulsification property; moreover, the emulsions remained indefinitely stable.

Chemical characterisation of AC7 BS

The positive ESI–MS analysis of the crude extract showed the presence of homologues of two lipopeptide families, surfactin and fengycin, respectively (Fig. 2). The surfactin family member was composed mainly of C13, C14 and C15 surfactin homologues, whose structures were confirmed by the product ion spectra of the sodiated molecules $[M + Na]^+$ at *m/z* 1030, 1044 and 1058. The fengycin family member was composed of two main fengycin isoforms corresponding to C17 fengycin A and C17 fengycin B, whose structures were confirmed by the product ion spectra of the protonated molecules $[M + H]^+$ at *m/z* 1478 and 1506, respectively. The relative amount of the two families in the crude extract was about 98 % surfactin and 2 % fengycin.

Antifungal susceptibility testing against *Candida albicans* planktonic cells and biofilms

AC7 BS susceptibility testing was carried out on *C. albicans* strains 40, 42 and IHEM 2894 planktonic cells and pre-formed biofilms by means of EUCAST and XTT methods, respectively. Optical densities at 450 nm and at 490 nm versus the biosurfactant concentration are shown in Fig. 3. Two-way ANOVA analysis indicated that both OD_{450} and OD_{490} were not significantly associated with biosurfactant concentrations, showing that no antifungal activity against planktonic cells or biofilms was detected for any of the strains. For biofilm formation, OD_{490} was significantly different among the three strains ($P = 0.006371$). Tukey's HSD post hoc test showed that OD_{490} was significantly higher for *C. albicans* IHEM 2894 suggesting that this strain is a stronger biofilm producer.

Anti-adhesion and anti-biofilm activity of AC7 BS against *Candida albicans* strains

The anti-adhesion and anti-biofilm activity of AC7 BS concentrations ranging from 0.5 to 3 mg ml^{-1} in co-incubation and in pre-coating conditions were evaluated by the CV method, which stains total biofilm biomass (cells and extracellular matrix). Figure 4 shows how uncoated and AC7 BS coated SEDs appeared after treatment with CV. On uncoated SEDs,

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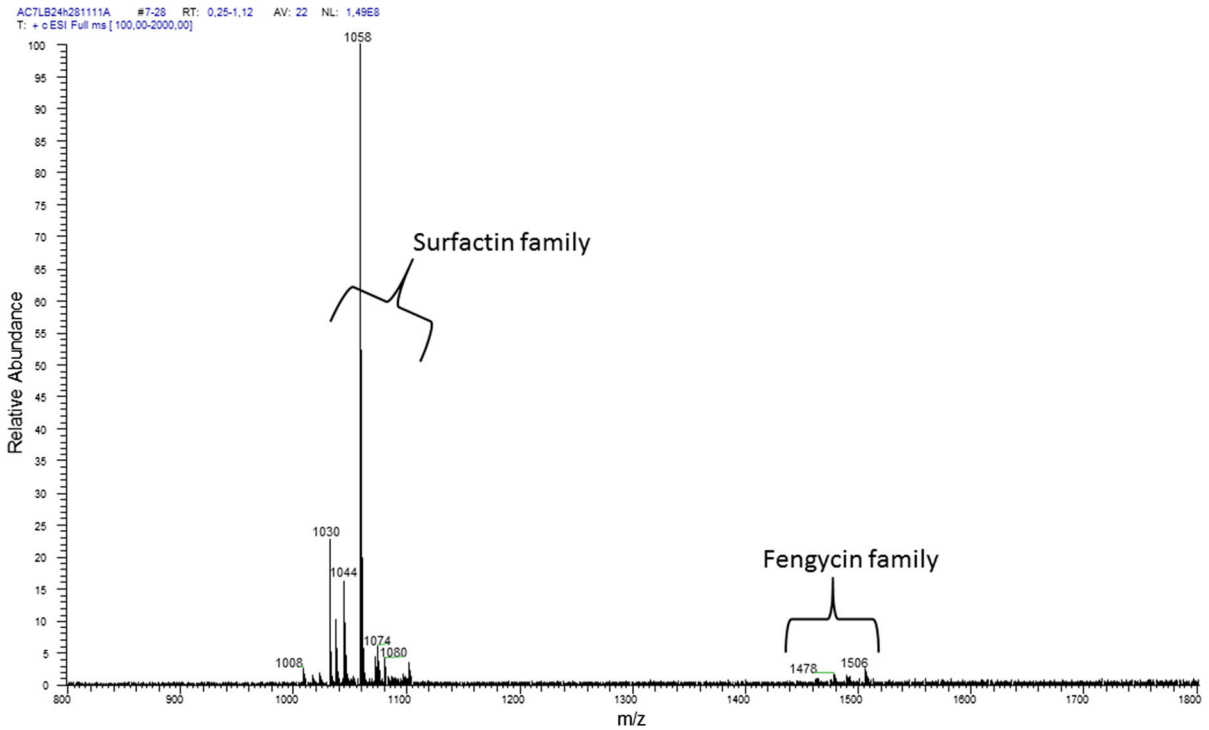

Fig. 2 (+) ESI–MS analysis (direct infusion) of lipopeptides produced by *B. subtilis* AC7. Two clusters of peaks revealed two sets of homologue molecules. The first set evidenced four main signals corresponding to the $[M + Na]^+$ of surfactin family. The second set evidenced two main signals corresponding to the protonated molecules of fengycin family

CV is uniformly distributed on the surface whereas on pre-coated SEDs, the violet colour intensity and coverage area decrease with the increase of AC7 BS concentration with a maximum reduction observed at 2 mg ml^{-1}, both at 1.5 and 24 h incubation. To quantify the CV staining, the disks were submerged in acetic acid, and the absorbance of the eluted stain measured. Figure 5 shows the OD_{570} as a function of the biosurfactant concentration.

In the co-incubation experiment, the adhesion and biofilm formation of the three *C. albicans* strains to SEDs (as measured by OD_{570}) were progressively reduced as a function of biosurfactant concentration, with a minimum reached at a concentration range between 1 and 2 mg ml^{-1} at time 1.5 h, and at concentration of 2 mg ml^{-1} at time 24 h (Fig. 5a, c). At the highest concentration (3 mg ml^{-1}), *C. albicans* adhesion and biofilm formation slightly increased.

Percentages of reduction of OD_{570} are reported in Table 2. In particular, the reduction at 2 mg ml^{-1} ranged (among the three strains) between 67 and 69 % at 1.5 h, and between 56 and 57 % at 24 h. Two-way ANOVA showed that at time 1.5 h adhesion was significantly dependent on biosurfactant concentration (but not on the strain) while at time 24 h biofilm formation was significantly dependent on concentration and on the strain. In particular, Tukey's HSD test revealed a significant reduction (both at time 1.5 h and at time 24 h) with a P value adjusted for multiple comparison of $<10^{-9}$.

In the pre-coating assay, the adhesion and biofilm formation of the three *C. albicans* strains to SEDs were progressively reduced as a function of biosurfactant concentration, with a minimum reached at the concentration of 2 mg ml^{-1} both at 1.5 and 24 h incubation (Fig. 5b, d). As previously observed, at the highest concentration (3 mg ml^{-1}), adhesion and biofilm formation slightly increased. Percentages of reduction of OD_{570} are reported in Table 2. In particular, at 2 mg ml^{-1} the mean reduction ranged (among the three strains) between 59 and 63 % at 1.5 h, and between 47 and 50 % at 24 h. Two-way ANOVA showed that at 1.5 h adhesion was significantly dependent on AC7 BS concentration and also

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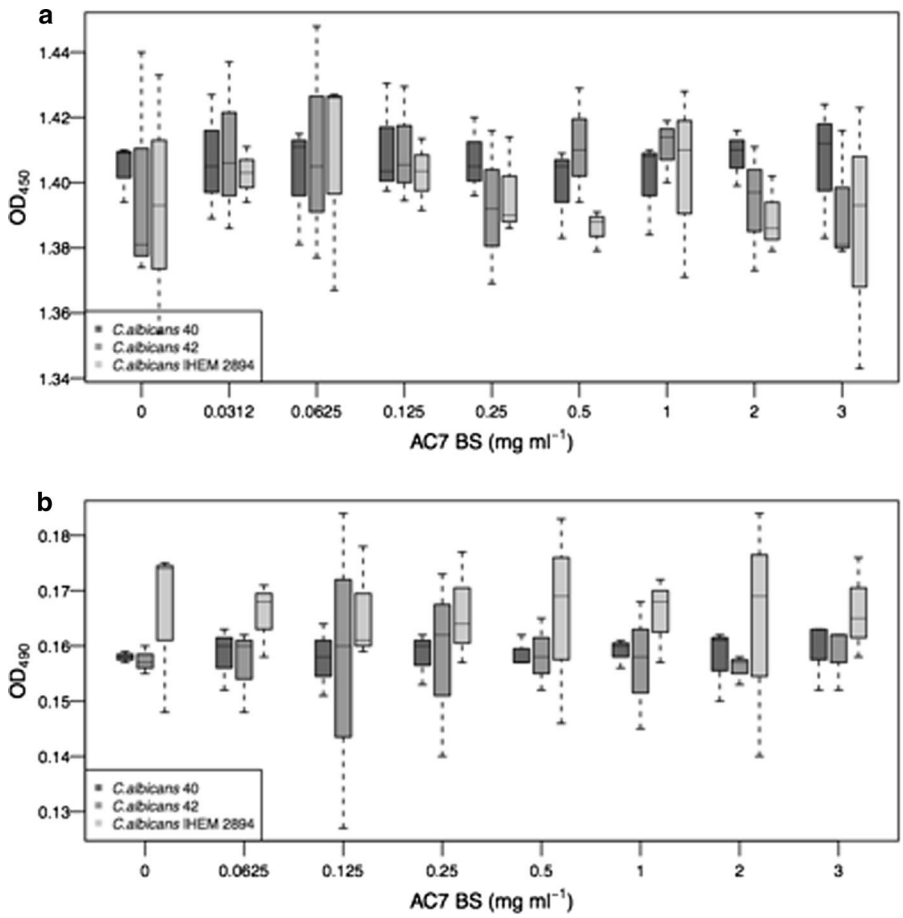

Fig. 3 Antifungal susceptibility testing against *C. albicans* planktonic cells and biofilms. AC7 BS activity at 24 h on **a** *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 planktonic cells and **b** pre-formed biofilm, measured by OD_{450} and OD_{490}, respectively. For each condition (concentration and strain) minimum, maximum median and interquartile range are illustrated using a box plot

on the strain while at 24 h biofilm formation was significantly dependent on concentration (but not on the strain). In particular, Tukey's HSD test revealed a significant reduction (both at time 1.5 h and at time 24 h) with a *P* value adjusted for multiple comparison of $<10^{-9}$.

The effect of SEDs pre-coating with an AC7 BS concentration of 2 mg ml^{-1} was further investigated by the viable cell counting method. Table 3 summarises the results obtained for the three *C. albicans* strains expressed as means and standard deviations for $\log_{10}$ CFU/disk. In addition, the results of the Welch Two Sample t-test comparing AC7 BS treated and control samples are reported as *P* values and 95 % confidence intervals for the differences. The final column of Table 3 indicates the percentages of inhibition calculated as $(1–10^{\mu}) \times 100$, where μ is the difference in $\log_{10}$ CFU/disk of AC7 BS treated and control samples. Fungal adhesion and biofilm formation on treated disks was significantly lower (at both incubation times) than on untreated disks. The difference was more evident at time 1.5 h. It should be noted that, at time 1.5 h, fungal counts were very low compared to 24 h as *C. albicans* stains are in the initial phase of adhesion. With respect to controls, the adhesion of the three fungal stains to SEDs treated with 2 mg ml^{-1} AC7 BS was significantly reduced in a range of 57.7–62.0 % at 1.5 h and biofilm formation was significantly inhibited in a range of 45.9–47.6 % after 24 h of incubation (*P* values in Table 3). Two-ways ANOVA confirmed that *C. albicans* viable counts were significantly dependent on the disk treatment (untreated or AC7 BS pre-coated) and on incubation time ($P < 10^{-15}$).

Surface physicochemical characterisation

The static contact angle measurements showed that the PBS-treated SEDs, as well as the PBS-treated SEDs further incubated for 1.5 h in YNBD + 10 % FBS

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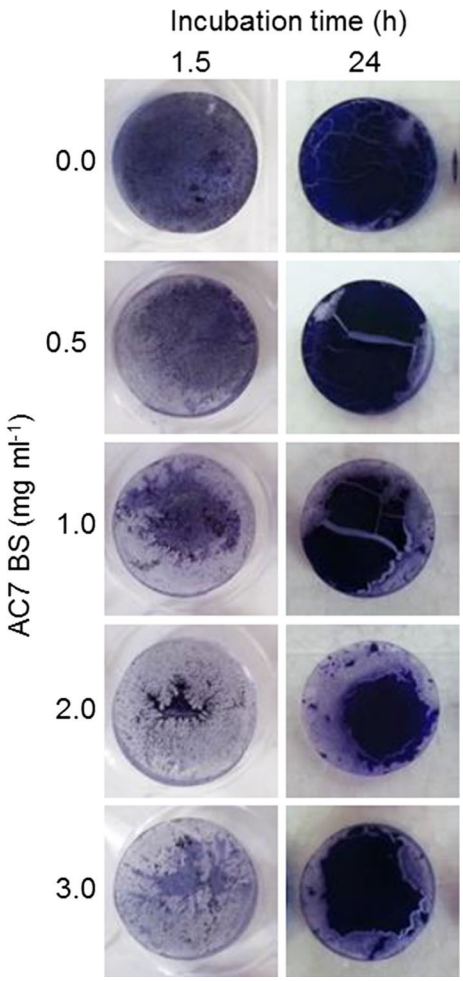

Fig. 4 CV stained SEDs, pre-coated with different concentrations of AC7 BS, after *C. albicans* IHEM 2894 adhesion (1.5 h) and biofilm formation (24 h)

(control SEDs of the simulated pre-coating assay), were hydrophobic (110.6° ± 3.0° and 112.0° ± 0.6°, respectively). AC7 BS pre-coated SEDs, as well as AC7 BS pre-coated SEDs further incubated for 1.5 h in YNBD + 10 % FBS (treated SEDs of the simulated pre-coating assay), were hydrophobic, although they showed a reduced average static contact angle compared to their respective control samples (94.4° ± 10.0° and 103.8° ± 15.0°, respectively). The enhanced surface wettability was a consequence of AC7 BS surface adsorption. The wide standard deviation of the contact angle value of AC7 BS treated samples compared to the respective control samples suggested that surface coating by physical adsorption was uneven.

The FTIR-ATR spectra of each analysed SED showed the characteristic bands at 830–1110 cm^{-1} due to Si–O–Si stretching, at 1259.4 cm^{-1} due to CH_3 symmetric stretching of Si\CH_3, at 1412.4 cm^{-1} due to CH_3 asymmetric stretching of Si-CH_3, and at 2962.8 cm^{-1} due to C–H stretching. AC7 BS physical absorption onto silicone disks did not alter the FTIR-ATR spectra suggesting that the surface amount of AC7 BS was below the detection limit of the FTIR-ATR technique.

Discussion

Candida albicans is one of the most important nosocomial pathogens frequently involved in implanted device-associated infection (Horn et al. 2009) and represents a serious public health problem with important medical and economic consequences (Almirante et al. 2005; Lai et al. 2012). *C. albicans* biofilm formation is a complex, multicellular process in which adhesion of cells to materials or host cells is a primary prerequisite (Ramage et al. 2005). There is, therefore, a need for biomaterials with antimicrobial-coated surfaces for the inhibition of the microbial adhesion and the eradication of biofilms. The main drawbacks of antimicrobial coatings arise from time limited effectiveness and potential toxicity towards human cells (de Sainte 2009; Hegstad et al. 2010). In this context, biosurfactants have recently emerged as a new generation of anti-adhesive and antimicrobial agents with enhanced biocompatibility and potential commercial application in pharmaceutical and biomedical fields (Cameotra and Makkar 2004; Fracchia et al. 2015). Among biosurfactants, lipopeptides form the most widely reported class with antimicrobial/antiadhesive activities due to their ability to disrupt phospholipid membranes and to affect cell-to-surface interactions by decreasing hydrophobicity and, thus, interfering with cell deposition processes and microbial adhesion (Rodrigues et al. 2006b).

Previous research about the activity of biosurfactants from endophytes and from a *Lactobacillus brevis* isolate against *C. albicans* biofilm formation reported significant reductions in biofilm cell number and biomass on polystyrene, denture resin and silicone elastomer (Fracchia et al. 2010; Cochis et al. 2012; Ceresa et al. 2015). In the present study, the activity of AC7 BS was investigated on two clinically relevant

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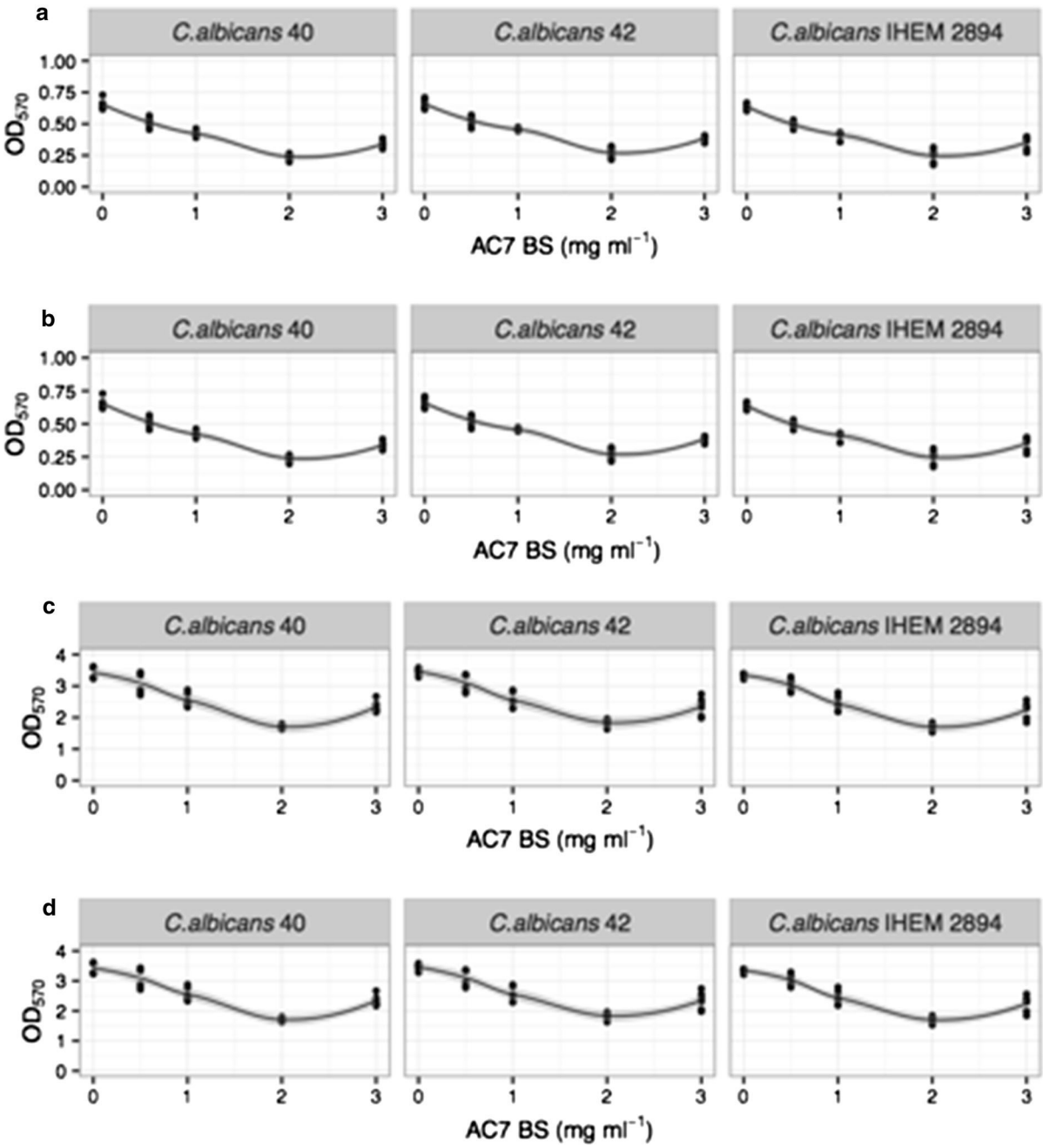

Fig. 5 AC7 BS activity against *C. albicans* adhesion and biofilm formation evaluated by the CV method. Inhibition of *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 adhesion and biofilm formation on silicone disks by different concentrations of AC7 BS at 1.5 h, in co-incubation (**a**) and in pre-coating assays (**b**) and at 24 h, in co-incubation (**c**) and in pre-coating assays (**d**). The inhibition of adhesion and biofilm formation is evaluated by means of the CV method and measured by OD_{570}. The different scales on the y-axes reflect the progression in biofilm formation with time. Each scatterplot includes a Loess curve (*local regression curve*) and a Loess confidence region (95 %)

wild strains of *C. albicans* and on a culture collection strain. The experiments were carried out on industrially produced medical-grade silicone disks, during the initial phases of biofilm formation and a number of different complementary methods (fungal biomass staining, viable cell counting, and surface

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Table 2 Percentages of inhibition of the *Candida albicans* strains adhesion (1.5 h) and biofilm formation (24 h)

Experimental condition	Strain	Time (h)							
		1.5				24			
		AC7 BS concentration (mg ml^{-1})							
		0.5	1	2	3	0.5	1	2	3
Co-incubation	*C. albicans* 40	35	63	69	51	26	47	57	49
	C. albicans 42	32	63	67	53	23	40	55	50
	C. albicans IHEM 2894	32	62	68	51	27	45	57	55
Pre-coating	*C. albicans* 40	22	36	63	48	10	26	50	32
	C. albicans 42	20	31	59	41	10	26	47	32
	C. albicans IHEM 2894	22	35	61	45	10	27	49	33

Table 3 AC7 BS inhibition of *Candida albicans* adhesion and biofilm formation in pre-coating assays

Time (h)	Strain	Control (C)		AC7 BS		95 % confidence interval (C-AC7 BS)	*P* value	Inhibition measures	
		Mean	SD[a]	Mean	SD			μ[b]	Percentage of inhibition (%)[c]
1.5	*C. albicans* 40	6.66	0.0489	6.24	0.0534	(0.371, 0.471)	3.76×10^{-12}	−0.42	62.0
	C. albicans 42	6.66	0.0693	6.29	0.1130	(0.278, 0.469)	1.07×10^{-6}	−0.37	57.7
	C. albicans IHEM 2894	6.67	0.0595	5.26	0.0757	(0.333, 0.470)	2.19×10^{-9}	−0.4	60.3
24	*C. albicans* 40	7.63	0.0455	7.35	0.0918	(0.188, 0.384)	1.99×10^{-4}	−0.29	48.3
	C. albicans 42	7.63	0.0408	7.36	0.0601	(0.206, 0.328)	1.36×10^{-6}	−0.27	45.9
	C. albicans IHEM 2894	7.63	0.0508	7.35	0.0384	(0.222, 0.339)	1.45×10^{-6}	−0.28	47.6

The table represents the mean *C. albicans* concentrations expressed as Log_{10} CFU/disk (as calculated by the R package dupiR) recovered on silicone disks, 95 % confidence interval, *P* values and inhibition measures

[a] SD standard deviation

[b] $\mu = (\log_{10} \text{CFU/disk}_{\text{AC7 BS}} - \log_{10} \text{CFU/disk}_{\text{Control}})$

[c] Percentage of inhibition $= (1 - 10^{\mu}) \times 100$

physicochemical characterisation) were used, to address the underlying mechanisms.

Chemical analysis of the AC7 BS crude extract revealed the presence of surfactin and fengycin, similar to other lipopeptide biosurfactants (Joshi et al. 2008; Rivardo et al. 2009; Kim et al. 2010; Pecci et al. 2010). Surface tension, CMC and emulsification capacity of AC7 BS were comparable to those observed for other lipopeptide biosurfactants (Lee et al. 2006; Nitschke and Pastore 2006). Moreover, AC7 BS showed a high stability to heat/cold treatments and over a wide range of pH. In particular, surface tension remained stable between pH 6 and 11, with the maximum activity observed at pH 6. Similar results were described by Kim et al. (1997) for the lipopeptide biosurfactant from *B. subtilis* C9; by Ghojavand et al. (2008) for the biosurfactant produced by *B. subtilis* PTCC 1696; and by Rivardo et al. (2009) for the lipopeptides V9T14 and V19T21. On the contrary, surface tension and emulsification activity of AC7 BS were affected by low pH values due to biosurfactant precipitation in acidic conditions, as reported for other biosurfactants (Rivardo et al. 2009; Amani et al. 2010; Kanna et al. 2014; Elazzazy et al. 2015).

The efficacy of AC7BS to inhibit *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 biofilm formation on SEDs was evaluated in co-incubation

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experiments and after its absorption on the silicone surface, in order to imitate a functional coating. The assays were performed in combination with a proteinaceous solution, i.e. FBS, to mimic blood contact upon the silicone surface during clinical use. Moreover, FBS is known to promote the morphogenic switching between yeast and filamentous states, an important step for biofilm formation by *C. albicans* (Chandra et al. 2008), that plays a significant role in fungal infection (Thompson et al. 2011; Mayer et al. 2013). These stringent conditions were intended to evaluate the activity of the biosurfactant in the presence of a solution that stimulates the production of biofilm.

AC7 BS treatments resulted in a significant reduction of the total adherent cells and biofilm biomass compared to controls for all three *C. albicans* strains, as evaluated by the CV method. The anti-adhesive and anti-biofilm activity of AC7 BS was concentration-dependent, with a maximum activity observed at about 2 mg ml^{-1}, both in the co-incubation and pre-coating conditions. The effect of SEDs pre-coating with this concentration of biosurfactant was, thus, further investigated by the viable cell counting method. Cell adhesion and biofilm formation were significantly altered by AC7 BS treatment in terms of difference in the number of $\log_{10}$ CFU/disk. Notably, in pre-coating assays, the percentages of reduction were almost similar for the CV and viable cell counting biofilm quantification assays (about 60 % reduction of adhesion and 50 % reduction of biofilm formation for all the three stains). In general, the highest performance of AC7 BS was observed during the *C. albicans* adhesion phase, whereas during the biofilm formation phase, the inhibition was lower but still significant.

Research into the activity of lipopeptides against *C. albicans* biofilm on silicone is scarce, as most experiments have been conducted on polystyrene. Janek et al. (2012) visually demonstrated that the pretreatment of silicone urethral catheters with pseudofactin II, a cyclic lipopeptide, and the inclusion of the biosurfactant in the growth medium caused an efficient reduction of *C. albicans* biofilm growth. The same authors demonstrated that the pre-treatment of polystyrene with pseudofactin II strongly inhibited *C. albicans* adhesion (>90 %), whereas the post-adhesion treatment dislodged biofilms grown on untreated surfaces to a lower extent (29–39 %). Rautela et al. (2014) evaluated the influence of lipopeptides from *Bacillus amyloliquefaciens* strain AR2 on *C. albicans* biofilm grown in polystyrene plates. Biosurfactant exhibited concentration-dependent fungal growth inhibition and fungicidal activity. Moreover, when added to the growth media, biosurfactant inhibited *C. albicans* biofilm formation in a range of 46–100 % (depending on the concentration and on *Candida* strains) and, less efficiently, dislodged preformed biofilm from polystyrene plates. Very recently, Biniarz et al. (2015) demonstrated that the lipopeptide biosurfactants pseudofactin and surfactin were able to limit fungal adhesion to polystyrene both in co-incubation and in pre-coating conditions.

Surface wettability measurements showed that AC7 BS coating by physical adsorption was successful, as the average value of the static contact angle decreased compared to control silicone disks. However, the wide standard deviation of the average contact angle of AC7 BS coated disks suggested that the biosurfactant was unevenly distributed on the sample surface. This may explain why lower percentages of inhibition of *C. albicans* adhesion and biofilm formation were observed in pre-coating assays rather than in co-incubation. Alternative coating methods are being investigated include plasma pre-treatment of the silicone disk surfaces (Ferreira et al. 2013) and AC7 BS physical adsorption and/or chemical grafting.

Finally, no antifungal activity towards *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 planktonic cells and pre-formed biofilms was observed at concentrations up to 3 mg ml^{-1}, suggesting that AC7 BS inhibited pathogen adhesion without affecting cell growth. Similarly, biosurfactants from *L. brevis* CV8LAC (Fracchia et al. 2010) and from *Bacillus licheniformis* V9T14 (Rivardo et al. 2011) were reported to have anti-biofilm but not antimicrobial activity.

Our results indicate that AC7 BS can be used as a coating agent to reduce efficiently *C. albicans* adhesion and biofilm formation on medical device materials. To our knowledge, this is the first time that the ability of lipopeptides to limit microbial adhesion on silicone has been demonstrated at physiological conditions and in the presence of FBS. However, further investigations are in progress to develop coating methodologies that will allow an even and stable distribution of the biosurfactant on the surface. In conclusion, we suggest that biosurfactant AC7, thanks to its anti-adhesive properties, could represent a

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potential candidate to effectively limit colonisation of medical devices and prevent *C. albicans* infections.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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