

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

*Original*

Lipopeptides from *Bacillus subtilis* AC7 inhibit adhesion and biofilm formation of *Candida albicans* on silicone / Ceresa, Chiara; Rinaldi, Maurizio; Chiono, Valeria; Carmagnola, Irene; Allegrone, Gianna; Fracchia, Letizia. - In: ANTONIE VAN LEEUWENHOEK. - ISSN 0003-6072. - 109:(2016), pp. 1375-1388. [10.1007/s10482-016-0736-z]

*Availability:*

This version is available at: 11583/2649103 since: 2016-11-25T13:14:15Z

*Publisher:*

Springer Netherlands

*Published*

DOI:10.1007/s10482-016-0736-z

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Schedule Received 27 April 2016  
Revised  
Accepted 12 July 2016

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Abstract *Candida albicans* is the major fungus that colonises medical implants, causing device-associated infections with high mortality. Antagonistic bacterial products with interesting biological properties, such as biosurfactants, have recently been considered for biofilm prevention. This study investigated the activity of lipopeptide biosurfactant produced by *Bacillus subtilis* AC7 (AC7 BS) against adhesion and biofilm formation of *C. albicans* on medical-grade silicone elastomeric disks (SEDs). Chemical analysis, stability, surface activities of AC7 BS crude extract and physicochemical characterisation of the coated silicone disk surfaces were also carried out. AC7 BS showed a good reduction of water surface tension, low critical micelle concentration, good emulsification activity, thermal resistance and pH stability. Co-incubation with 2 mg ml<sup>-1</sup> AC7 BS significantly reduced adhesion and biofilm formation of three *C. albicans* strains on SEDs in a range of 67–69 % and of 56–57 %, respectively. On pre-coated SEDs, fungal adhesion and biofilm formation were reduced by 57–62 % and 46–47 %, respectively. Additionally, AC7 BS did not inhibit viability of *C. albicans* strains in both planktonic and sessile form. Chemical analysis of the crude extract revealed the presence of two families of lipopeptides, principally surfactin and a lower percentage of fengycin. The evaluation of surface wettability indicated that AC7 BS coating of SEDs surface was successful although uneven. AC7 BS significantly prohibits the initial deposition of *C. albicans* and slows biofilm growth, suggesting a potential role of biosurfactant coatings for preventing fungal infection associated with silicone medical devices.

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

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Footnote Information

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

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6 Received: 27 April 2016 / Accepted: 12 July 2016  
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**Keywords** Anti-adhesion · Biofilm · Medical 42  
device · Lipopeptide biosurfactant · Coating · *Candida* 43  
*albicans* 44

**Introduction** 45

In recent years, a significant increase in the incidence 46  
of human fungal infections has been observed. *Can-* 47  
*dida* species are the major problem, especially in 48  
immunocompromised patients (Espinel-Ingroff et al. 49  
2009; van De Veerdonk et al. 2010; Ruhnke et al. 50  
2011), representing the fourth most common cause of 51  
nosocomial bloodstream infections (Wisplinghoff 52  
et al. 2004). Invasive candidiasis presents a high 53

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54 global mortality rate, ranging from 36 to 63 % in  
 55 different patient groups (Guery et al. 2009; van De  
 56 Veerdonk et al. 2010) and represents a significant  
 57 problem in terms of patient management and health-  
 58 care costs in the public health system (Kullberg et al.  
 59 2011).

60 Among the *Candida* species isolated from humans,  
 61 *Candida albicans* is frequently associated with the  
 62 formation of biofilms on a wide variety of medical  
 63 devices (Crump and Collignon 2000; Goldberg et al.  
 64 2000; Karchmer 2000; Maki and Tambyah 2001). *C.*  
 65 *albicans* biofilms consist of structured surface-associ-  
 66 ated cell communities embedded in an extracellular  
 67 matrix that have distinct phenotypes compared to their  
 68 planktonic cell counterparts (Fanning and Mitchell  
 69 2012). The presence of the biofilm plays a key role on  
 70 *C. albicans* pathogenesis, by protecting the microor-  
 71 ganism from host defences and reducing significantly  
 72 its susceptibility to antifungal agents (Hawser and  
 73 Douglas 1994; Lazzell et al. 2009). Furthermore, the  
 74 tenacity with which *C. albicans* infects indwelling  
 75 medical devices necessitates, in almost all the cases,  
 76 their removal (Kojic and Darouiche 2004).

77 For these reasons, the development of new tech-  
 78 nologies to counteract *C. albicans* biofilm growth  
 79 represents a major challenge in clinical practice and  
 80 preventive medicine. Among microbial metabolites,  
 81 biosurfactants have gained importance thanks to their  
 82 interesting biological properties, such as the ability to  
 83 disrupt membranes and to affect the adhesion proper-  
 84 ties of cells/microorganisms (Cameotra and Makkar  
 85 2004; Singh and Cameotra 2004; Seydlová and  
 86 Svobodová 2008; Rodrigues and Teixeira 2010).  
 87 Biosurfactants are amphipathic compounds with both  
 88 hydrophilic and hydrophobic moieties that exhibit  
 89 surface activities at interfaces (Banat et al. 2010).  
 90 Adsorption of biosurfactants to a substratum surface  
 91 modifies its hydrophobicity, interfering with microbial  
 92 adhesion and desorption processes (Rodrigues et al.  
 93 2006a; Biniarz et al. 2015). Pre-coating catheters and  
 94 other medical implantable materials with biosurfac-  
 95 tants could represent a preventive strategy to inhibit  
 96 pathogenic biofilm growth, thus reducing the use of  
 97 pharmaceuticals and antibiotics (Rodrigues et al.  
 98 2006b; Singh et al. 2007; Falagas and Makris 2009).

99 In this study, the ability of the lipopeptide biosur-  
 100 factant AC7 produced by *Bacillus subtilis* (AC7 BS) to  
 101 inhibit adhesion and biofilm formation of three *C.*  
 102 *albicans* strains was evaluated by co-incubation and

pre-coating on silicone elastomer at physiological 103  
 conditions. Lipopeptides activity was determined by 104  
 means of the crystal violet staining and the viable cell 105  
 counting methods. Moreover, AC7 BS was chemically 106  
 characterised and its stability and surface activities 107  
 measured. Surface physicochemical characterisations 108  
 of the AC7 BS coated silicone were also carried out. 109

**Materials and methods** 110

Microorganisms and culture conditions 111

The endophytic biosurfactant-producing strain AC7 112  
 was isolated from the inside of stems of *Robinia* 113  
*pseudoacacia* and was genotypically identified by 114  
 complete 16S rDNA sequence analysis (DSMZ Iden- 115  
 tification Service, Braunschweig, Germany) as *B.* 116  
*subtilis*. For biofilm assays, the strain *C. albicans* 117  
 IHEM 2894 was purchased from The Belgian Co- 118  
 ordinated Collections of Microorganisms (BCCM). *C.* 119  
*albicans* 40 (DSM 29204) and 42 (DSM 29205) are 120  
 two wild strains (courtesy of Hospital “Maggiore della 121  
 Carità”, Novara, Italy), clinically isolated from central 122  
 venous catheter and urinary catheter, respectively, and 123  
 deposited by the Authors in the DSMZ collection. 124  
 Strain *B. subtilis* AC7 was stored at  $-80^{\circ}\text{C}$  in Luria- 125  
 Bertani (LB) broth (Sigma-Aldrich) supplemented 126  
 with 25 % glycerol and grown on LB agar plates for 127  
 24 h at  $28^{\circ}\text{C}$ . *C. albicans* strains were stored at 128  
 $-80^{\circ}\text{C}$  in Sabouraud dextrose broth (Sigma-Aldrich) 129  
 supplemented with 25 % glycerol and grown for 24 h 130  
 at  $37^{\circ}\text{C}$  on Sabouraud Dextrose Agar (SDA) plates. 131

Critical micelle concentration, emulsification 132  
 index and stability study of AC7 BS 133

AC7 BS crude extract was obtained according to the 134  
 method described by Rivardo et al. (2009). Surface 135  
 tension of 0.5, 1.0 and  $2.0\text{ mg ml}^{-1}$  AC7 BS solutions 136  
 in alkaline distilled water was measured by using a 137  
 ring tensiometer (KSV Sigma 703D). Results for 138  
 surface tension measurements were expressed as 139  
 $\text{mN m}^{-1}$  and compared with alkaline distilled water. 140  
 Critical micelle concentration (CMC) was determined 141  
 on serially diluted biosurfactant solutions in alkaline 142  
 distilled water (from  $0.01$  to  $0.5\text{ mg ml}^{-1}$ ). Surface 143  
 tension of each dilution was determined in triplicate. 144  
 The CMC was assessed from the intercept of two 145

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146	straight lines extrapolated from the concentration-	100 µl of sterile PBS were used. <i>C. albicans</i> suspen-	191
147	dependent and concentration-independent sections of	sions at a concentration ranging from 1 to 5 × 10 <sup>5</sup>	192
148	a curve plotted between biosurfactant concentration	colony-forming units (CFU) ml <sup>-1</sup> were prepared in	193
149	and surface tension values.	sterile double-strength Roswell Park Memorial Insti-	194
150	The heat/cold stability of 0.5, 1.0 and 2.0 mg ml <sup>-1</sup>	tute (RPMI) 1640 medium (Sigma-Aldrich) buffered	195
151	AC7 BS solutions was evaluated by measuring the	with 3-( <i>N</i> -morpholino)propanesulfonic acid buffer	196
152	emulsification index at 24 h E <sub>24</sub> (%) (Franzetti et al.	(MOPS) (Sigma-Aldrich) and supplemented with D-	197
153	2012) and surface tension after treatment at 100 °C for	glucose (2 % final concentration), pH 7.0. Subse-	198
154	1 h, at 121 °C for 15 min and at -80 °C for 24 h. To	quently, 100 µl of the <i>Candida</i> suspensions were	199
155	study pH stability, the pH of the AC7 BS solutions	added to each well, to obtain final concentrations of	200
156	(0.5, 1.0, 2.0 mg ml <sup>-1</sup> ) was adjusted to different pH	AC7 BS ranging from 0.03 to 3 mg ml <sup>-1</sup> , and to the	201
157	values (3–11) with 1 M NaOH or 1 M HCl. After-	control wells. Blank wells were prepared by mixing	202
158	wards, the surface tension and the E <sub>24</sub> were measured.	100 µl double-strength RPMI with 100 µl of the	203
159	Assays were carried out in triplicate.	biosurfactant solutions (from 0.06 to 6 mg ml <sup>-1</sup> ) or	204
160	Chemical characterisation of AC7 BS	PBS. The plate was incubated at 37 °C for 24 h in	205
161	The chemical characterisation of the crude extract was	static conditions. Finally, OD <sub>450</sub> was measured for	206
162	performed according to the method described by Pecci	each well using a Ultramark Microplate Imaging	207
163	et al. (2010), with and slight modifications. An aliquot	System (Bio-Rad). The data were normalised with	208
164	of the biosurfactant extract was dissolved in methanol/	respect to the value of the corresponding blank wells.	209
165	acetonitrile (50/50 v/v) to obtain a 1000 µg ml <sup>-1</sup>	Assays were carried out in triplicate.	210
166	stock solution. Freshly prepared working solutions	Medical-grade silicone elastomeric disks	211
167	were made by diluting the stock solution with	preparation	212
168	methanol/water (50/50 v/v) to 10 µg ml <sup>-1</sup> solutions	Medical-grade silicone elastomeric disks (SEDs)	213
169	Mass spectrometry analyses were done on a LCQ	(TECNOEXTR Srl, Italy) used in the study were	214
170	DECA XP Plus (Thermo Finnigan, San Jose, CA,	15 mm in diameter and 1.5 mm in thickness for	215
171	USA) IonTrap mass instrument equipped with an ESI	experiments in 12-well tissue culture plates, and	216
172	source. Samples (10 µg/ml solutions) were injected	10 mm in diameter and 1.5 mm in thickness for	217
173	with a syringe at 5 µl min <sup>-1</sup> flow rate. Source voltage	experiments in 24-well tissue culture plates. Cleaning	218
174	and capillary voltage were at 4.80 kV and 23 V in	and sterilisation of SEDs was carried out according to	219
175	positive mode. The capillary temperature was main-	the method described by Busscher et al. (1997).	220
176	tained at 350 °C and nitrogen was used as nebulising	Briefly, disks were immersed in 200 ml of distilled	221
177	gas at 30 arbitrary units. Data were acquired in	water supplemented with 1.4 % (v/v) of RBS <sup>TM</sup> 50	222
178	positive MS total ion scan mode (mass scan range <i>m/z</i>	solution (Sigma-Aldrich), sonicated for 5 min at	223
179	100–2000) and MS/MS product ion scan mode with	60 kHz using Elma S30H (Elmasonic, VWR Interna-	224
180	normalised collision energy (nce %) optimised for	tional) and rinsed in 1 l of MilliQ water twice. Then,	225
181	each precursor ion selected: <i>m/z</i> 1030, 38 %; 1044 and	disks were submerged in 20 ml of methanol (99 %)	226
182	1058, 39 %; 1478 and 1506, 35 %.	(Sigma-Aldrich), rinsed twice and autoclaved for	227
183	Antifungal susceptibility testing against	15 min at 121 °C.	228
184	<i>Candida albicans</i> planktonic cells	Antifungal susceptibility of <i>Candida albicans</i>	229
185	AC7 BS antifungal activity towards planktonic cells of	biofilms	230
186	<i>C. albicans</i> strains was assessed according to	<i>Candida albicans</i> cells were cultivated for 24 h at	231
187	EUCAST guidelines (Rodriguez-Tudela et al. 2008).	37 °C on SDA plates. Cells were then suspended in	232
188	Briefly, 100 µl of AC7 BS solutions (from 0.06 to	Phosphate Buffered Saline (PBS) solution with 10 %	233
189	6 mg ml <sup>-1</sup> ) were added in a 96-well microtiter plate	Fetal Bovine Serum (FBS) and standardised to	234
190	(Bioster). In control wells (no biosurfactant added),	1 × 10 <sup>7</sup> CFU ml <sup>-1</sup> . One milliliter of this fungal	235

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<sup>-1</sup> 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<sup>-1</sup>) 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<sup>-1</sup>) and 1 µl of 1 mmol l<sup>-1</sup> 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<sub>490</sub> 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.

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

269 *Co-incubation assays*

270 Five hundred microliters of *C. albicans* suspensions  
271 (2 × 10<sup>7</sup> CFU ml<sup>-1</sup>) in PBS + 20 % FBS, were  
272 added to each well of a 24-well plates (Greiner bio-  
273 one) containing a silicone disk together with either  
274 500 µl of 2 × AC7 BS solutions (1, 2, 4 and  
275 6 mg ml<sup>-1</sup>) (test groups) or PBS (control group).  
276 After 1.5 h of incubation (*C. albicans* adhesion  
277 phase), the disks were transferred into a new plate  
278 and placed in either 1 ml of YNBD + 10 % FBS with  
279 0 mg ml<sup>-1</sup> (control group) or 0.5, 1, 2, 3 mg ml<sup>-1</sup>  
280 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<sub>570</sub>  
was measured using a Ultramark microplate imaging  
system (Bio-Rad Laboratories Srl, Segrate MI, Italy).  
Assays were carried out in triplicate and the experi-  
ments 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<sup>-1</sup> (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<sup>7</sup>  
CFU ml<sup>-1</sup>. 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<sup>-1</sup> 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 detach-  
ment. 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<sub>10</sub> CFU/  
disk ± standard deviations.

327 Surface physicochemical characterisation

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

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

346 The surface chemical properties of SEDs were  
 347 analysed by infrared spectroscopy in an IR Perkin-  
 348 Elmer Frontier spectrophotometer equipped with an  
 349 attenuated total reflectance (ATR-FTIR) device using  
 350 a Germanium crystal. Spectra were recorded with a  
 351 resolution of 4 cm<sup>-1</sup> and averaged over 36 scans.

352 Statistical analysis of data

353 Statistical analysis and graphs were elaborated by  
 354 means of the statistical program R, 3.1.2 (R Develop-  
 355 ment Core Team, <http://www.R-project.org>). Two-  
 356 way ANOVA was used to compare optical densities of  
 357 planktonic cells and pre-formed biofilm at different  
 358 AC7 BS concentrations for the three *C. albicans*  
 359 strains. Tukey's Honest Significant Difference (HSD)  
 360 method was used as ANOVA post hoc test. The Welch  
 361 Two Sample *t* test was performed to investigate the  
 362 effect of AC7 BS on the three *Candida* stains adhesion  
 363 and biofilm formation in pre-coating assays, carried  
 364 out by means of the viable cell counting method.  
 365 Results were considered to be statistically significant  
 366 when *P* < 0.05. To estimate log<sub>10</sub> CFU/disk from  
 367 colony counts, the R package dupiR was used (Co-  
 368 moglio et al. 2013). This package allows estimation,  
 369 from a set of counts, the population size and its  
 370 uncertainty using a Bayesian approach under minimal  
 371 information on the distributions; this is particularly  
 372 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<sup>-1</sup> reduced the surface  
 tension of alkaline distilled water from 72.4 to 31.4  
 mN m<sup>-1</sup> (Fig. 1). Serial dilutions of this solution  
 showed a gradual increase of surface tension up to  
 38.2 mN m<sup>-1</sup> at the concentration of 62.5 µg ml<sup>-1</sup>.  
 Then, surface tension rapidly increased to  
 54.9 mN m<sup>-1</sup> at the concentration of 7.8 µg ml<sup>-1</sup>.  
 The CMC value for AC7 BS was 31.9 µg ml<sup>-1</sup>.

Studies on the pH stability of AC7 BS, carried out at  
 0.5, 1.0 and 2.0 mg ml<sup>-1</sup>, 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 concentra-  
 tions tested. In particular, the highest surface activity  
 of AC7 BS solutions at 0.5, 1.0 and 2.0 mg ml<sup>-1</sup> was  
 found at pH 6.0 with values of 28.45, 28.53 and  
 29.26 mN m<sup>-1</sup>, respectively. At pH 11.0 the values  
 were 30.76, 30.91 and 30.92 mN m<sup>-1</sup>. At pH ≤ 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<sup>-1</sup>. The mean emulsification  
 index at 24 h (E<sub>24</sub>) 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

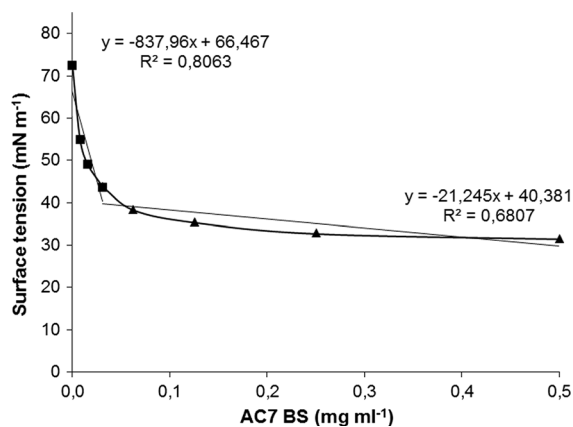


Fig. 1 A plot of surface tension as a function of concentration of AC7 BS after purification. Standard deviation was ranging between ±0.3 mN m<sup>-1</sup>

Author Proof

**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 <sup>-1</sup>			1 mg ml <sup>-1</sup>			2 mg ml <sup>-1</sup>		
	Surface tension (mN m <sup>-1</sup> )		$E_{24h}$ (%)	Surface tension (mN m <sup>-1</sup> )		$E_{24h}$ (%)	Surface tension (mN m <sup>-1</sup> )		$E_{24h}$ (%)
	Mean	SD <sup>a</sup>		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<sup>-1</sup>

<sup>a</sup> SD standard deviation

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

406 The studies on the effect of heat/cold treatment on  
407 AC7 BS solutions at 0.5, 1.0 and 2.0 mg ml<sup>-1</sup>  
408 demonstrated no evident changes in surface tension  
409 and  $E_{24}$ . In particular, the treatment at 100 °C for 1 h,  
410 at 121 °C for 15 min and at -80 °C for 24 h did not  
411 alter the ability of AC7 BS solutions to decrease water  
412 surface tension and its emulsification property; more-  
413 over, the emulsions remained indefinitely stable.

#### 414 Chemical characterisation of AC7 BS

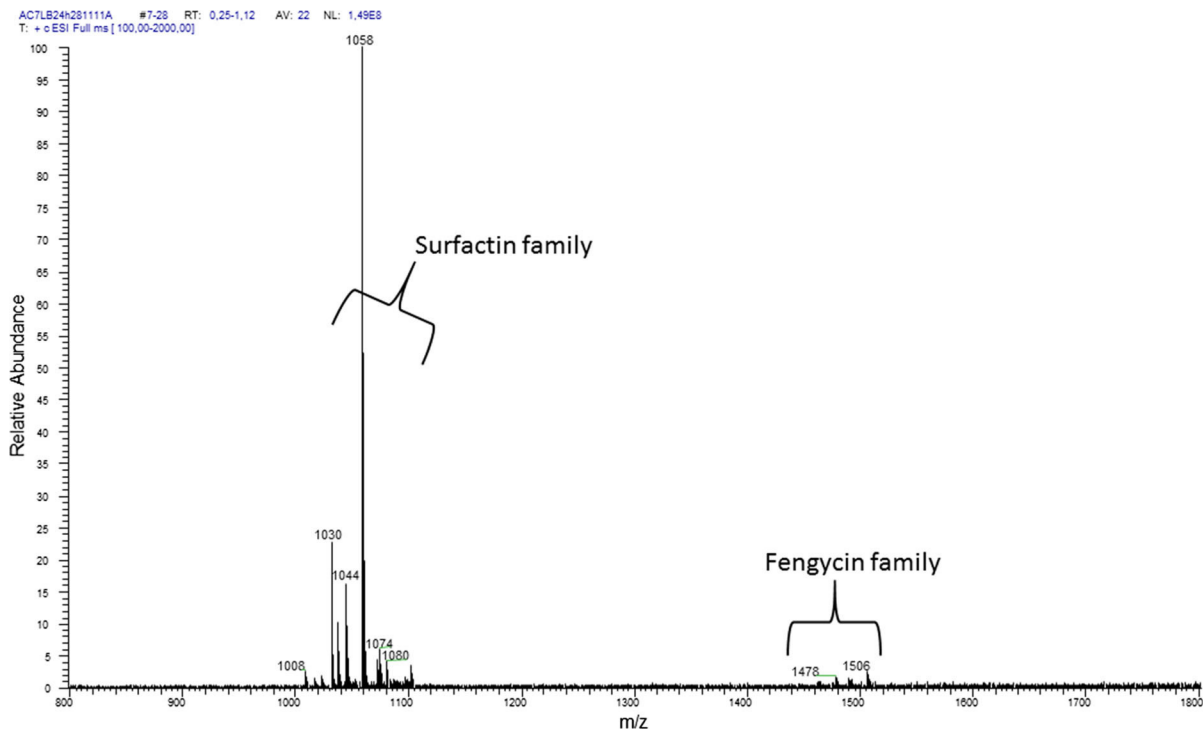
415 The positive ESI-MS analysis of the crude extract  
416 showed the presence of homologues of two lipopep-  
417 tide families, surfactin and fengycin, respectively  
418 (Fig. 2). The surfactin family member was composed  
419 mainly of C13, C14 and C15 surfactin homologues,  
420 whose structures were confirmed by the product ion  
421 spectra of the sodiated molecules [M + Na]<sup>+</sup> at  $m/z$   
422 1030, 1044 and 1058. The fengycin family member  
423 was composed of two main fengycin isoforms corre-  
424 sponding to C17 fengycin A and C17 fengycin B,  
425 whose structures were confirmed by the product ion  
426 spectra of the protonated molecules [M + H]<sup>+</sup> at  $m/z$   
427 1478 and 1506, respectively. The relative amount of  
428 the two families in the crude extract was about 98 %  
429 surfactin and 2 % fengycin.

Antifungal susceptibility testing against *Candida* 430  
*albicans* planktonic cells and biofilms 431

432 AC7 BS susceptibility testing was carried out on *C.* 432  
*albicans* strains 40, 42 and IHEM 2894 planktonic 433  
cells and pre-formed biofilms by means of EUCAST 434  
and XTT methods, respectively. Optical densities at 435  
450 nm and at 490 nm versus the biosurfactant 436  
concentration are shown in Fig. 3. Two-way ANOVA 437  
analysis indicated that both OD<sub>450</sub> and OD<sub>490</sub> were not 438  
significantly associated with biosurfactant concentra- 439  
tions, showing that no antifungal activity against 440  
planktonic cells or biofilms was detected for any of the 441  
strains. For biofilm formation, OD<sub>490</sub> was significantly 442  
different among the three strains ( $P = 0.006371$ ). 443  
Tukey's HSD post hoc test showed that OD<sub>490</sub> was 444  
significantly higher for *C. albicans* IHEM 2894 445  
suggesting that this strain is a stronger biofilm 446  
producer. 447

Anti-adhesion and anti-biofilm activity of AC7 BS 448  
against *Candida albicans* strains 449

450 The anti-adhesion and anti-biofilm activity of AC7 BS 450  
concentrations ranging from 0.5 to 3 mg ml<sup>-1</sup> in co- 451  
incubation and in pre-coating conditions were evalu- 452  
ated by the CV method, which stains total biofilm 453  
biomass (cells and extracellular matrix). Figure 4 454  
shows how uncoated and AC7 BS coated SEDs 455  
appeared after treatment with CV. On uncoated SEDs, 456



**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

457 CV is uniformly distributed on the surface whereas on  
 458 pre-coated SEDs, the violet colour intensity and  
 459 coverage area decrease with the increase of AC7 BS  
 460 concentration with a maximum reduction observed at  
 461  $2 \text{ mg ml}^{-1}$ , both at 1.5 and 24 h incubation. To  
 462 quantify the CV staining, the disks were submerged in  
 463 acetic acid, and the absorbance of the eluted stain  
 464 measured. Figure 5 shows the  $OD_{570}$  as a function of  
 465 the biosurfactant concentration.

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

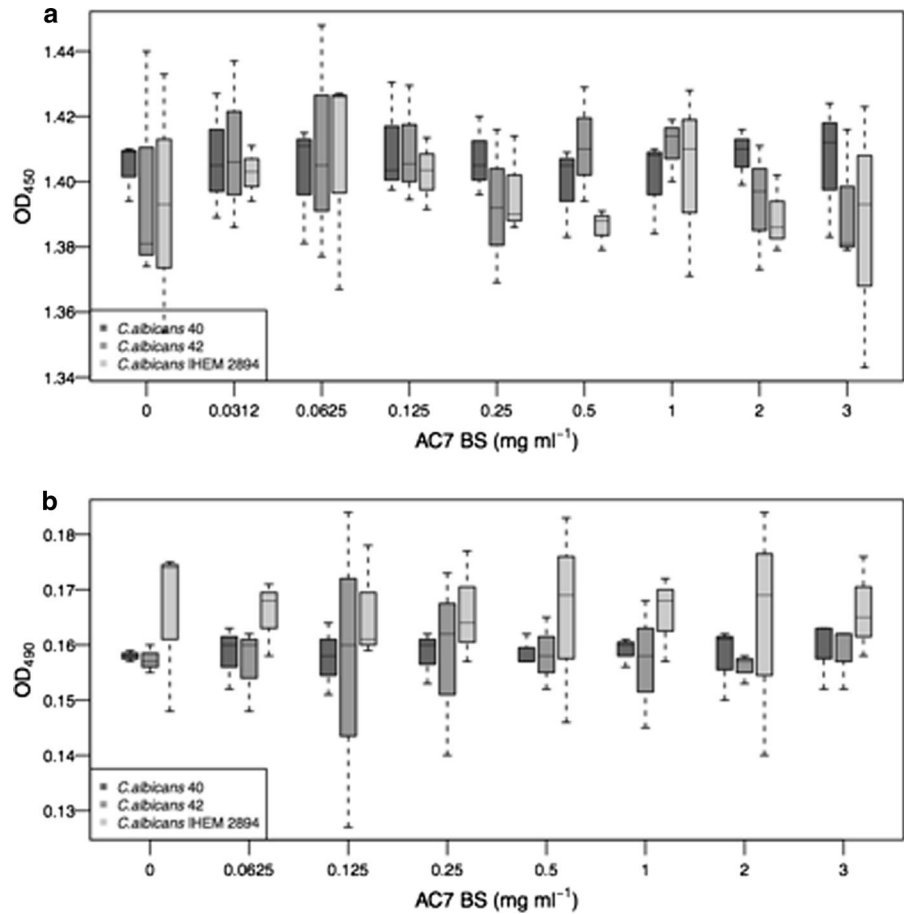
475 Percentages of reduction of  $OD_{570}$  are reported in  
 476 Table 2. In particular, the reduction at  $2 \text{ mg ml}^{-1}$   
 477 ranged (among the three strains) between 67 and 69 %  
 478 at 1.5 h, and between 56 and 57 % at 24 h. Two-way

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 concentra-  
 tion 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 concentra-  
 tion of  $2 \text{ mg ml}^{-1}$  both at 1.5 and 24 h incubation  
 (Fig. 5b, d). As previously observed, at the highest  
 concentration ( $3 \text{ mg ml}^{-1}$ ), adhesion and biofilm  
 formation slightly increased. Percentages of reduction  
 of  $OD_{570}$  are reported in Table 2. In particular,  
 at  $2 \text{ mg ml}^{-1}$  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<sub>450</sub> and OD<sub>490</sub>, respectively. For each condition (concentration and strain) minimum, maximum median and interquartile range are illustrated using a box plot



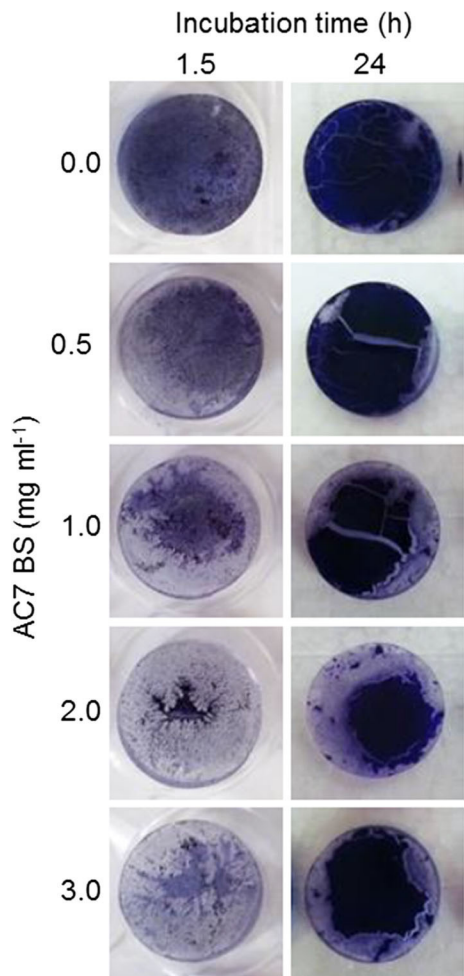
501 on the strain while at 24 h biofilm formation was  
 502 significantly dependent on concentration (but not on  
 503 the strain). In particular, Tukey's HSD test revealed a  
 504 significant reduction (both at time 1.5 h and at time  
 505 24 h) with a *P* value adjusted for multiple comparison  
 506 of  $<10^{-9}$ .

507 The effect of SEDs pre-coating with an AC7 BS  
 508 concentration of 2 mg ml<sup>-1</sup> was further investigated  
 509 by the viable cell counting method. Table 3 sum-  
 510 marises the results obtained for the three *C. albicans*  
 511 strains expressed as means and standard deviations for  
 512 log<sub>10</sub> CFU/disk. In addition, the results of the Welch  
 513 Two Sample t-test comparing AC7 BS treated and  
 514 control samples are reported as *P* values and 95 %  
 515 confidence intervals for the differences. The final  
 516 column of Table 3 indicates the percentages of  
 517 inhibition calculated as  $(1-10^{\mu}) \times 100$ , where  $\mu$  is  
 518 the difference in log<sub>10</sub> CFU/disk of AC7 BS treated  
 519 and control samples. Fungal adhesion and biofilm  
 520 formation on treated disks was significantly lower (at

both incubation times) than on untreated disks. The  
 521 difference was more evident at time 1.5 h. It should be  
 522 noted that, at time 1.5 h, fungal counts were very low  
 523 compared to 24 h as *C. albicans* stains are in the initial  
 524 phase of adhesion. With respect to controls, the  
 525 adhesion of the three fungal stains to SEDs treated  
 526 with 2 mg ml<sup>-1</sup> AC7 BS was significantly reduced in  
 527 a range of 57.7–62.0 % at 1.5 h and biofilm formation  
 528 was significantly inhibited in a range of 45.9–47.6 %  
 529 after 24 h of incubation (*P* values in Table 3). Two-  
 530 ways ANOVA confirmed that *C. albicans* viable  
 531 counts were significantly dependent on the disk  
 532 treatment (untreated or AC7 BS pre-coated) and on  
 533 incubation time (*P* < 10<sup>-15</sup>).  
 534

535 Surface physicochemical characterisation

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



**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)

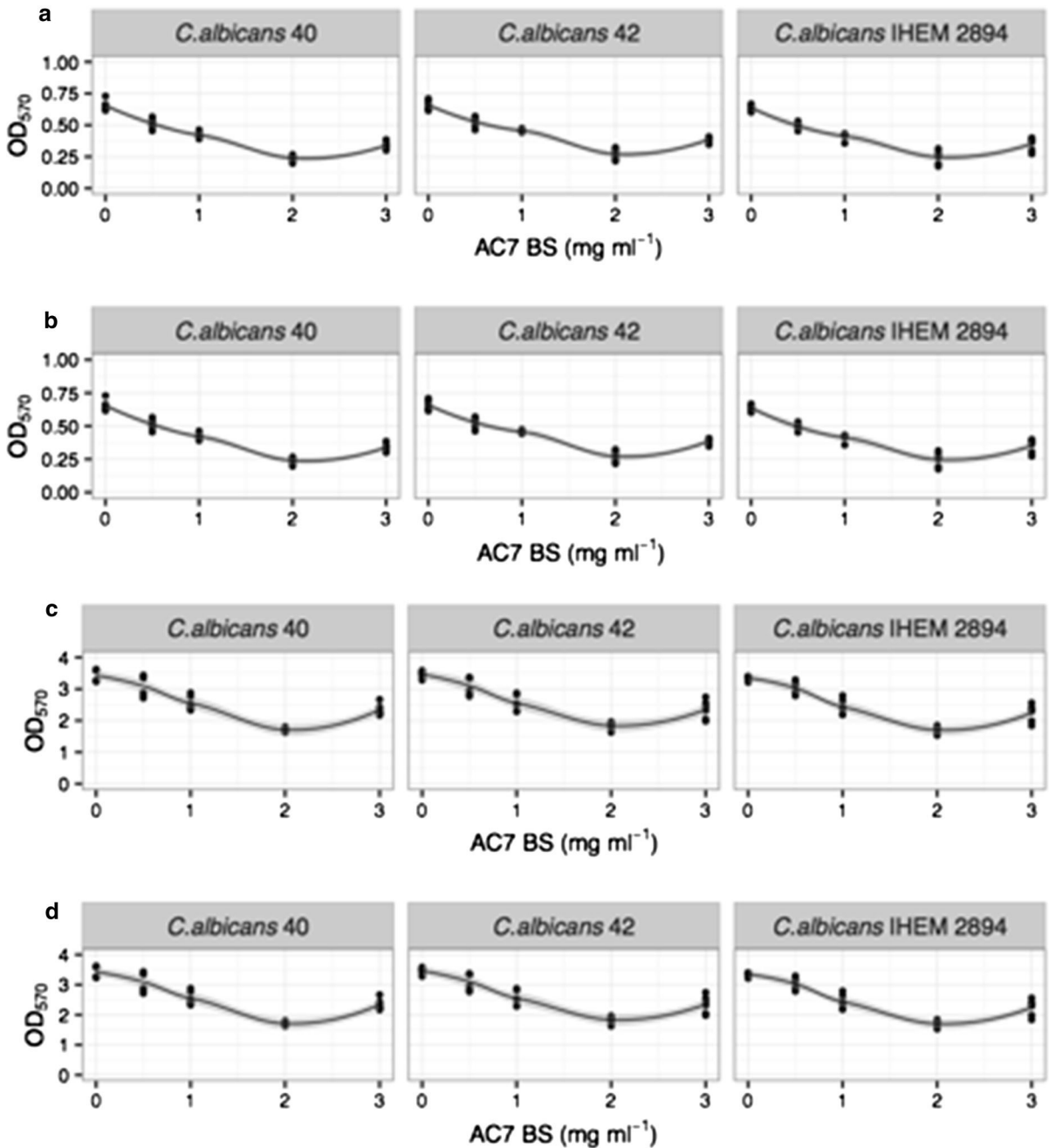
539 (control SEDs of the simulated pre-coating assay),  
 540 were hydrophobic ( $110.6^\circ \pm 3.0^\circ$  and  $112.0^\circ \pm 0.6^\circ$ ,  
 541 respectively). AC7 BS pre-coated SEDs, as well as  
 542 AC7 BS pre-coated SEDs further incubated for 1.5 h  
 543 in YNBD + 10 % FBS (treated SEDs of the simulated  
 544 pre-coating assay), were hydrophobic, although they  
 545 showed a reduced average static contact angle com-  
 546 pared to their respective control samples ( $94.4^\circ \pm$   
 547  $10.0^\circ$  and  $103.8^\circ \pm 15.0^\circ$ , respectively). The enhanced  
 548 surface wettability was a consequence of AC7 BS  
 549 surface adsorption. The wide standard deviation of the  
 550 contact angle value of AC7 BS treated samples  
 551 compared to the respective control samples suggested  
 552 that surface coating by physical adsorption was  
 553 uneven.

The FTIR-ATR spectra of each analysed SED 554  
 showed the characteristic bands at  $830\text{--}1110\text{ cm}^{-1}$  555  
 due to Si–O–Si stretching, at  $1259.4\text{ cm}^{-1}$  due to  $\text{CH}_3$  556  
 symmetric stretching of  $\text{Si}\backslash\text{CH}_3$ , at  $1412.4\text{ cm}^{-1}$  due 557  
 to  $\text{CH}_3$  asymmetric stretching of Si-CH<sub>3</sub>, and at 558  
 $2962.8\text{ cm}^{-1}$  due to C–H stretching. AC7 BS physical 559  
 absorption onto silicone disks did not alter the FTIR- 560  
 ATR spectra suggesting that the surface amount of 561  
 AC7 BS was below the detection limit of the FTIR- 562  
 ATR technique. 563

## Discussion 564

*Candida albicans* is one of the most important 565  
 nosocomial pathogens frequently involved in 566  
 implanted device-associated infection (Horn et al. 567  
 2009) and represents a serious public health problem 568  
 with important medical and economic consequences 569  
 (Almirante et al. 2005; Lai et al. 2012). *C. albicans* 570  
 biofilm formation is a complex, multicellular process 571  
 in which adhesion of cells to materials or host cells is a 572  
 primary prerequisite (Ramage et al. 2005). There is, 573  
 therefore, a need for biomaterials with antimicrobial- 574  
 coated surfaces for the inhibition of the microbial 575  
 adhesion and the eradication of biofilms. The main 576  
 drawbacks of antimicrobial coatings arise from time 577  
 limited effectiveness and potential toxicity towards 578  
 human cells (de Sainte 2009; Hegstad et al. 2010). In 579  
 this context, biosurfactants have recently emerged as a 580  
 new generation of anti-adhesive and antimicrobial 581  
 agents with enhanced biocompatibility and potential 582  
 commercial application in pharmaceutical and 583  
 biomedical fields (Cameotra and Makkar 2004; Frac- 584  
 chia et al. 2015). Among biosurfactants, lipopeptides 585  
 form the most widely reported class with antimicro- 586  
 bial/antiadhesive activities due to their ability to 587  
 disrupt phospholipid membranes and to affect cell- 588  
 to-surface interactions by decreasing hydrophobicity 589  
 and, thus, interfering with cell deposition processes 590  
 and microbial adhesion (Rodrigues et al. 2006b). 591

Previous research about the activity of biosurfac- 592  
 tants from endophytes and from a *Lactobacillus brevis* 593  
 isolate against *C. albicans* biofilm formation reported 594  
 significant reductions in biofilm cell number and 595  
 biomass on polystyrene, denture resin and silicone 596  
 elastomer (Fracchia et al. 2010; Cochis et al. 2012; 597  
 Ceresa et al. 2015). In the present study, the activity of 598  
 AC7 BS was investigated on two clinically relevant 599



**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<sub>570</sub>. 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 %)

600 wild strains of *C. albicans* and on a culture collection  
601 strain. The experiments were carried out on industrially  
602 produced medical-grade silicone disks, during the

603 initial phases of biofilm formation and a number of  
604 different complementary methods (fungal biomass  
605 staining, viable cell counting, and surface

Author Proof

**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 <sup>-1</sup> )							
		0.5	1	2	3	0.5	1	2	3
Co-incubation	<i>C. albicans</i> 40	35	63	69	51	26	47	57	49
	<i>C. albicans</i> 42	32	63	67	53	23	40	55	50
	<i>C. albicans</i> IHEM 2894	32	62	68	51	27	45	57	55
Pre-coating	<i>C. albicans</i> 40	22	36	63	48	10	26	50	32
	<i>C. albicans</i> 42	20	31	59	41	10	26	47	32
	<i>C. albicans</i> 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 <sup>a</sup>	Mean	SD			μ <sup>b</sup>	Percentage of inhibition (%) <sup>c</sup>
1.5	<i>C. albicans</i> 40	6.66	0.0489	6.24	0.0534	(0.371, 0.471)	3.76 × 10 <sup>-12</sup>	-0.42	62.0
	<i>C. albicans</i> 42	6.66	0.0693	6.29	0.1130	(0.278, 0.469)	1.07 × 10 <sup>-6</sup>	-0.37	57.7
	<i>C. albicans</i> IHEM 2894	6.67	0.0595	5.26	0.0757	(0.333, 0.470)	2.19 × 10 <sup>-9</sup>	-0.4	60.3
24	<i>C. albicans</i> 40	7.63	0.0455	7.35	0.0918	(0.188, 0.384)	1.99 × 10 <sup>-4</sup>	-0.29	48.3
	<i>C. albicans</i> 42	7.63	0.0408	7.36	0.0601	(0.206, 0.328)	1.36 × 10 <sup>-6</sup>	-0.27	45.9
	<i>C. albicans</i> IHEM 2894	7.63	0.0508	7.35	0.0384	(0.222, 0.339)	1.45 × 10 <sup>-6</sup>	-0.28	47.6

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

<sup>a</sup> SD standard deviation

<sup>b</sup> μ = (log<sub>10</sub> CFU/disk<sub>AC7 BS</sub> - log<sub>10</sub> CFU/disk<sub>Control</sub>)

<sup>c</sup> Percentage of inhibition = (1 - 10<sup>μ</sup>) × 100

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

608 Chemical analysis of the AC7 BS crude extract  
609 revealed the presence of surfactin and fengycin,  
610 similar to other lipopeptide biosurfactants (Joshi  
611 et al. 2008; Rivardo et al. 2009; Kim et al. 2010;  
612 Pecci et al. 2010). Surface tension, CMC and emul-  
613 sification capacity of AC7 BS were comparable to  
614 those observed for other lipopeptide biosurfactants  
615 (Lee et al. 2006; Nitschke and Pastore 2006). More-  
616 over, AC7 BS showed a high stability to heat/cold  
617 treatments and over a wide range of pH. In particular,  
618 surface tension remained stable between pH 6 and 11,  
619 with the maximum activity observed at pH 6. Similar

620 results were described by Kim et al. (1997) for the  
621 lipopeptide biosurfactant from *B. subtilis* C9; by  
622 Ghojavand et al. (2008) for the biosurfactant produced  
623 by *B. subtilis* PTCC 1696; and by Rivardo et al. (2009)  
624 for the lipopeptides V9T14 and V19T21. On the  
625 contrary, surface tension and emulsification activity of  
626 AC7 BS were affected by low pH values due to  
627 biosurfactant precipitation in acidic conditions, as  
628 reported for other biosurfactants (Rivardo et al. 2009;  
629 Amani et al. 2010; Kanna et al. 2014; Elazzazy et al.  
630 2015).

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

634 experiments and after its absorption on the silicone  
635 surface, in order to imitate a functional coating. The  
636 assays were performed in combination with a pro-  
637 teinaceous solution, i.e. FBS, to mimic blood contact  
638 upon the silicone surface during clinical use. More-  
639 over, FBS is known to promote the morphogenic  
640 switching between yeast and filamentous states, an  
641 important step for biofilm formation by *C. albicans*  
642 (Chandra et al. 2008), that plays a significant role in  
643 fungal infection (Thompson et al. 2011; Mayer et al.  
644 2013). These stringent conditions were intended to  
645 evaluate the activity of the biosurfactant in the  
646 presence of a solution that stimulates the production  
647 of biofilm.

648 AC7 BS treatments resulted in a significant reduc-  
649 tion of the total adherent cells and biofilm biomass  
650 compared to controls for all three *C. albicans* strains,  
651 as evaluated by the CV method. The anti-adhesive and  
652 anti-biofilm activity of AC7 BS was concentration-  
653 dependent, with a maximum activity observed at about  
654 2 mg ml<sup>-1</sup>, both in the co-incubation and pre-coating  
655 conditions. The effect of SEDs pre-coating with this  
656 concentration of biosurfactant was, thus, further  
657 investigated by the viable cell counting method. Cell  
658 adhesion and biofilm formation were significantly  
659 altered by AC7 BS treatment in terms of difference in  
660 the number of log<sub>10</sub> CFU/disk. Notably, in pre-coating  
661 assays, the percentages of reduction were almost  
662 similar for the CV and viable cell counting biofilm  
663 quantification assays (about 60 % reduction of adhe-  
664 sion and 50 % reduction of biofilm formation for all  
665 the three stains). In general, the highest performance  
666 of AC7 BS was observed during the *C. albicans*  
667 adhesion phase, whereas during the biofilm formation  
668 phase, the inhibition was lower but still significant.

669 Research into the activity of lipopeptides against *C.*  
670 *albicans* biofilm on silicone is scarce, as most  
671 experiments have been conducted on polystyrene.  
672 Janek et al. (2012) visually demonstrated that the  
673 pretreatment of silicone urethral catheters with pseud-  
674 ofactin II, a cyclic lipopeptide, and the inclusion of the  
675 biosurfactant in the growth medium caused an  
676 efficient reduction of *C. albicans* biofilm growth.  
677 The same authors demonstrated that the pre-treatment  
678 of polystyrene with pseudofactin II strongly inhibited  
679 *C. albicans* adhesion (>90 %), whereas the post-  
680 adhesion treatment dislodged biofilms grown on  
681 untreated surfaces to a lower extent (29–39 %).  
682 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-depen-  
dent fungal growth inhibition and fungicidal activity.  
Moreover, when added to the growth media, biosur-  
factant 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 success-  
ful, as the average value of the static contact angle  
decreased compared to control silicone disks. How-  
ever, 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 percent-  
ages 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<sup>-1</sup>, suggesting that  
AC7 BS inhibited pathogen adhesion without affect-  
ing 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 antimicro-  
bial activity.

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

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

734 **Acknowledgments** This work was partially funded by  
735 Regione Piemonte Grant POR-FESR Asse I—AGROBIOC  
736 Project.

737 **Compliance with ethical standards**

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

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