Università degli Studi del Piemonte Orientale “Amedeo Avogadro”

Dipartimento di Scienze del Farmaco

Dottorato di Ricerca in Biotecnologie Farmaceutiche ed Alimentari

XXVII ciclo a.a. 2011-2014

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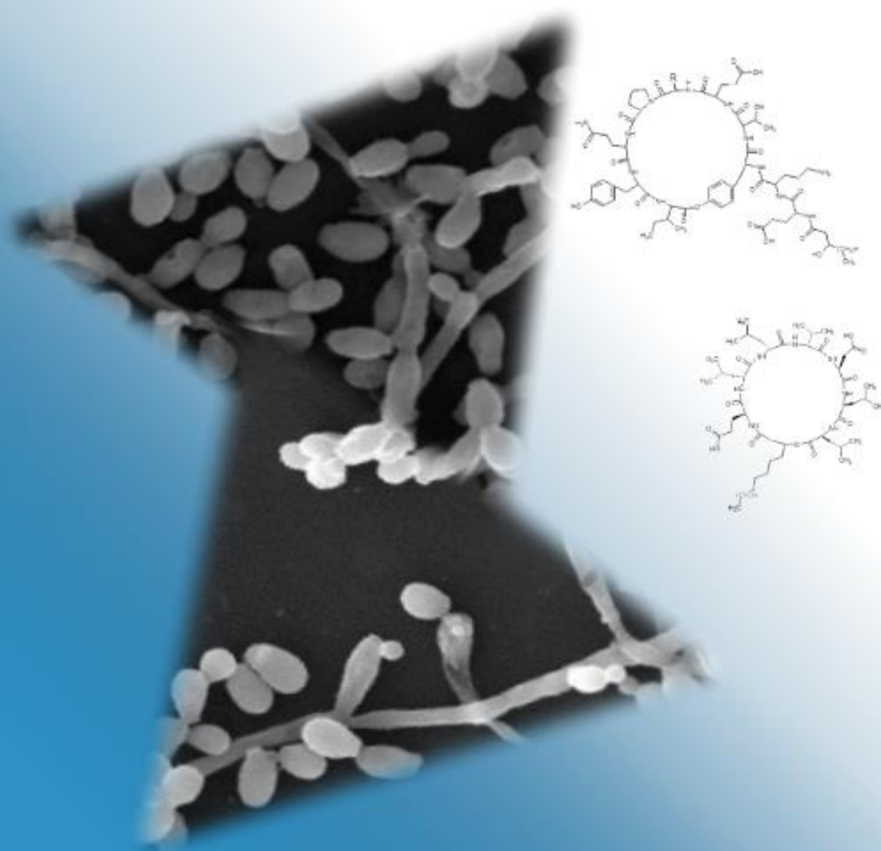

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Activity of bacterial biosurfactants against *Candida albicans* adhesion and biofilm formation on medical-grade silicone

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1. Introduction

Biosurfactants (BSs) comprise a wide range of structurally different organic compounds produced by numerous prokaryotic and eukaryotic microorganisms. These molecules, generally localized on microbial cell surfaces or excreted extracellularly, are characterized by the presence of both hydrophilic and hydrophobic moieties within the same structure with allow them to exhibit surface activities [1]. Even if, in literature the terms 'biosurfactants' and 'bioemulsifiers' are often used interchangeably, molecules that reduce surface and interfacial tension at gas-liquid-solid interfaces are indicated as biosurfactants while those that are able to reduce the interfacial tension between immiscible liquids or at the solid-liquid interfaces forming stable emulsion are called bioemulsifiers [2].
The hydrophobic moiety of these compounds aggregates at the surface that represents the hydrophobic phase while the hydrophilic moiety is oriented towards the solution or hydrophilic phase. These structural orientation on the surfaces and interfases confers to these compounds a range of properties, such as the ability to lower surface and interfacial tension of liquids and the formation of micelles and microemulsions between different phases [3,4]. In heterogeneous systems, they tend to aggregate at the phase boundaries or interfaces, and form a molecular interfacial film that affects the properties of the original surface.

In the last twenty years a large amount of research activity has been dedicated to biosurfactants as potential replacement for synthetic surfactants in many environmental and industrial applications such as bioremediation, enhanced oil recovery, paint, textile, detergent, cosmetic, food, agrochemical fields and several commercial products have already been issued [5].

More recently, numerous investigations have lead to the discovery of many interesting chemical and biological properties of biosurfactants and several pharmaceutical and medical applications have been envisaged [1,2,6]. In particular, their ability to destabilize membranes by disturbing their integrity and permeability

leading to metabolite leakage and cell lysis [7-11], as well as their propensity to partition at the interfaces, modifying surface properties and thus affecting microorganisms adhesion, are important functions for antimicrobial and antibiofilm applications. Furthermore, some experimental results suggest that they are non-toxic or less toxic than synthetic surfactants [12-14], a valuable characteristic for biomedical applications.

2. Classification

BSs are mainly classified according to their chemical composition, molecular weight, and mode of action. They are divided into two groups: low molecular weight molecules (biosurfactants) that efficiently reduce surface and interfacial tension and high molecular weight polymers (bioemulsifiers) that stabilize emulsion without lower the surface tension [15,16]. Their hydrophobic moiety may be composed of an acid, peptide cations, or anions, mono-, di- or polysaccharides, and the hydrophobic moiety may include unsaturated or saturated hydrocarbon chains or fatty acids [1].

The best studied biosurfactants are lipopeptides such as surfactin and fengicin. and glycolipids such as rhamnolipids, trehalolipids, sophorolipids and mannosylerythritol lipids.

2.1. Lipopeptides

Lipopeptides are small molecules that are formed by cyclic or short linear peptides linked with a lipid tail or other lipophilic molecules [17,18]. There are different families and each family is constituted of several variants, which can differ in their fatty acid chain and their peptide moiety [19-22]. Lipopeptides are synthesized by many species of *Bacillus* and other species such as *Actinoplanes*,

Lyngbya, *Pseudomonas*, *Streptomyces*, *Tolypothrix* and in the fungi *Aspergillus nidulans* [23]. A large collection of these molecules (polymyxins, polypeptins, and octapeptins) can be classified as cyclic cationic lipopeptides. These molecules are cyclized at the C-terminus by an ester or amide bond and the lipid tail is incorporated through acylation of the N-terminal amino acid. The overall cationic charge derives from the incorporation of multiple residues of the non-proteogenic amino acid 2,4-diaminobutyric acid. Cyclic non-cationic lipopeptides include iturin, surfactin and fengycin. Surfactins (A, B and C) consist in a loop of seven amino acids, with the chiral sequence LLDLLDL, linked to a β-hydroxy fatty acid chain via a lactone bond [24]. At position 7, surfactin A presents L-Leu, surfactin B presents L-Val and surfactin C presents L-Ile [25]. Furthermore, surfactin is a mixture of isoforms characterized by a different acyl chain length (C13-C15) which confers selective properties to the biosurfactant [26,27]. Iturins (iturin A, C, A_L, mycosubtilin, bacillomycin L, D, F and Lc) are heptapeptides with the chiral sequence LDDLLDL and are cyclized by an amide bond between the N-terminal β-amino fatty acid and the C-terminus. Furthermore, acyl chain length range from 14 to 17 C atoms, resulting in a mixture of isomers. Fengycin is a mixture of two homologues differing for their amino acid sequence. Its structure consists of a β-hydroxy fatty acid linked to the N-terminus of a decapeptide, including four D-amino acids residues and the rare amino acid ornithine. The C-terminal residue of the peptide moiety is linked to the tyrosine residue at position 3, forming the branching point of the acyl peptide and the eight-membered cyclic lactone. The chiral sequence is LDDDLDLLLL. Fengycin A and B present at position 6 the amino acid D-Ala and D-Val respectively. A series of isoforms is present in fengycin family by varying the length of β-hydroxy fatty acid tail, linked at position 1, from 14 to 18 C atoms in both fengycin A and B.

2.2. Glicolipids

Glycolipids are commonly mono disaccharides molecules in combination with long chain aliphatic acids or hydroxyaliphatic acids. Rhamnolipids, mainly produced by *Pseudomonas* and *Burkholderia* genus, consist of one or two rhamnose sugar moieties linked to one or two β-hydroxy fatty acid chains [28]. Investigations have revealed a large diversity of congeners and homologs produced by this strain following different culture conditions and by other bacterial species [29].

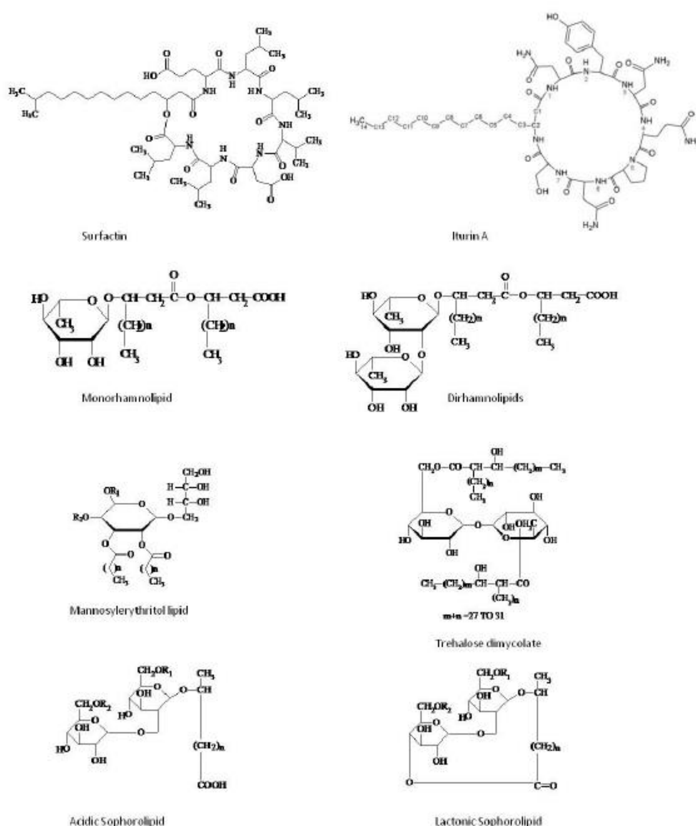

Figure 1. Chemical structures of the main low molecular weight microbial surface active compounds [2].

Trehalolipids production is associated with most species of *Mycobacterium*, *Rhodococcus* and *Corynebacterium* [30]. These molecules are composed by a trehalose, linked by an ester bond to a-branched b-hydroxy fatty acids [31]. The most reported trehalose lipid is trehalose 6,6'-dimycolate, which is a α -branched chain mycolic acid esterified to the C6 position of each glucose [31]. Sophorolipids are most commonly produced by *Candida bombicola* and *Candida apicola*, along with *Rhodotorula bogoriensi, Wicherhamiella domercqiae, Pichia anomala* [32,33]. They can be classified in two major groups: the acidic sophorolipids comprising of a disaccharide, sophorose, linked to the sub-terminal or terminal carbon of the fatty acid chain and the lactonic sophorolipids where the carboxylic acid portion of the fatty acid is joined to carbon 4'' of the disaccharide unit [34]. Mannosylerythritol lipids (MELs) are generally produced by *Pseudozyma* yeasts species, *P. rugulosa*, *P. aphidis* and *P. Antarctica* [35]. These molecules contain a 4-O-b-D-mannopyranosyl-D-erythritol connected to two medium length chains of fatty acyl esters [36]. Based on the degree of acetylation MELs have been classified as MEL-A when diacetylated, MEL-B and MEL-C when monoacetylated and MEL-D when no acetylated [37].

3. Biosurfactants as biological control agents

The urgent need for new antimicrobial compounds remains a major concern nowadays because of the newly emerged pathogens and conventional others which have become almost insensitive to existing antibiotics [38,39]. Microbial metabolites are recognized as a major source of compounds endowed with potent biological activities and, among these, some biosurfactants have been described as alternatives to synthetic medicines and antimicrobial agents [6,40,41]. Moreover, thanks to their ability to modulate the interaction of cells with surfaces, biosurfactants are able to interfere with microbial adhesion and biofilm formation, an important and mostly hazardous occurrence on medical devices, especially as

bacteria within such biofilms usually become highly resistant to antibiotics and adverse environmental challenges [42,43]. From this point of view, it could be useful to increase the efficacy of known antibiotics and biocides with alternative strategies aimed at decreasing bacterial adhesion and reducing the biofilm populations on medical device surfaces.

3.1.Mechanisms of action

Understanding the functional mechanisms of biosurfactants is of great help for the disclosure of interesting applications. Among biosurfactants, lipopeptides and glicolipids have the most potent antimicrobial activity and represent an important source for the identification of new antibiotics.

The antimicrobial activity of lipopeptides is due to their ability to self-associate and form a pore-bearing channel or micellular aggregate inside a lipid membrane [44,45]. Thanks to these properties, lipopeptides usually cause membrane disruption, increased membrane permeability, metabolite leakage and cell lysis. Furthermore, changes in membrane structure or disruption of protein conformations alter important membrane functions such as transport and energy generation [23,46]. It has been observed that pore formation in membranes occurs after lipopeptide oligomer binding, some of which are Ca^{2+} dependent multimers [47]. These pores may cause transmembrane ion influxes, including Na^{+} and K^{+}, leading to membrane disruption and cell death [47-49]. The bactericidal activity of lipopeptides increases with the presence of a lipid tail length of 10-12 carbons atoms whereas an enhanced antifungal activity is exhibited in lipopeptides with a lipid tail length of 14 or 16 carbon atoms [23]. In addition, due to the difficulty of the target cells to reorganize their membranes, the development of resistant strains is extremely reduced [48].

Surfactin, known as one of the most powerful biosurfactants, destabilize membranes disturbing their integrity and permeability [50]. Infact, surfactin creates

changes in physical membrane structure or disrupts protein conformations which alter important membrane functions such as transport and energy generation [7-11,51,52]. A key step for membrane destabilization and leakage is the dimerization of surfactin into the bilayer [44]. In vitro, the incorporation of surfactin into the membrane gives rise to dehydration of the phospholipid polar head groups and the perturbation of lipid packing which strongly compromise the bilayer stability, leading to the disturbance of the membrane barrier properties [44]. The degree of phospholipid bilayer perturbation depends on the concentration of surfactin. At low concentrations surfactin penetrates into the cell membrane, where it is completely miscible with the phospholipids and forms mixed micelles. At moderate concentrations this lipopeptide creates domains segregated within the phospholipid bilayer that may contribute to the formation ion-conducting pores in the cell membrane; at high concentrations, surfactin operates as a detergent, leading to membrane disruption and permeabilization [53,54].

Mechanisms of action and activity of other lipopeptides have been reviewed by Cochrane and Vederas [55]. Polymyxins primarily exert their strong bactericidal effect against Gram-negative bacteria through the binding of the lipid A component of lipopolysaccharide (LPS) and disruption of the outer membrane, followed by permeabilization and disruption of the inner membrane [56,57]. Octapeptins A and B display broad-spectrum activity against both Gram-positive and Gram-negative bacteria and have also antimicrobial activity against some filamentous fungi, protozoa and yeasts due to their ability to disrupt the cytoplasmic membrane [58,59]. The iturin family exerts fungicidal action through the interaction with sterol components in the fungal membrane, leading to an increase in K^+ permeability [60,61]. Concerning the fengycin family, even if the complete mode of action is not known, current studies suggest it operates through a membrane disruption mechanism [45,62,63].

Concerning glycolipids mode of action, Sotirova et al. [64] demonstrated that the exposure of *P. aeruginosa* to rhamnolipids causes a multi-component response

of the bacterial cells characterized by a reduction of total cellular LPS content, an increase in cell hydrophobicity and changes in membrane proteins and surface morphology. At the same manner, the antimicrobial activity of sophorolipids involves mechanisms that cause destabilization and alteration of the permeability of the cellular membrane [65]. Furthermore, Ortiz et al. [7,8] have recently studied the interactions of bacterial biosurfactants trehalose lipids with phosphatidylserine and phosphatidylethanolamine membranes. The results demonstrated that trehalose lipids, when incorporated into the bilayers, increased hydrocarbon chain conformational disorder and decreased the hydration of the interfacial region of the bilayer, leading to structural perturbations that might affect the function of the membranes.

The ability to reduce microbial cells adhesion to surfaces, thus limiting biofilm formation, is another well-known property of biosurfactants. The initial deposition rates and numbers of microorganisms adhering to a surface are determined by a complex interplay of hydrophobicity (interfacial free energies), electrostatic interactions, the presence of specific receptor sites on the microbial cell surfaces and possible biosurfactants produced [66]. In particular, biofilm formation on solid surfaces is generally directly proportional to the hydrophobicity of the surface, as long as the suspended medium is a simple buffer [67]. Microbial adhesion on hydrophobic substrates (e.g. silicone rubber) is presumably related to the removal of interfacial water between microorganism and surface interacting surfaces, which facilitates close approach [67]. These Authors suggested that biosurfactants reduce hydrophobic interactions, resulting in a decrease in the hydrophobicity of the surface, which interferes with the microbial adhesion to the surface and consequently, alters biofilm development.

3.2.Antibacterial and antifungal activity of biosurfactants

Lipopeptides form the most commonly reported class of biosurfactants with antimicrobial activity. Antimicrobial lipopeptides are surfactin, fengycin, iturin, bacillomycins and mycosubtilins produced by *B. subtilis*, lichenysin, pumilacidin and polymyxin B produced by *B. licheniformis*, *Bacillus pumilus* and *Bacillus polymyxa,* respectively, and cyclic lipopeptides such as daptomycin, from *Streptomyces roseosporus* and viscosin, from *Pseudomonas* [55]. Glycolipids, as well, have been reported to exhibit antimicrobial activities, in particular, rhamnolipids from *P. aeruginosa*, sophorolipids from *C. bombicola*, mannosylerythritol lipids (MEL-A and MEL-B) from *Candida antarctica* [68].

Ghribi et al. [69] investigated the antimicrobial activity of a biosurfactant produced by the strain *B. subtilis* SPB1 against bacteria and fungi. The biosurfactant exhibited a broad spectrum of action, including antimicrobial activity against microorganisms with multidrug-resistant profiles. The compound showed higher activity against Gram-positive cocci than against Gram-negative bacilli and its activity was particularly significant against *Enterococcus faecalis*. Ding et al. [70] isolated two lipopeptide antibiotics, pelgipeptins C and D from the strain *Paenibacillus elgii* B69, active against a number of Gram-positive and Gram-negative bacteria and against pathogenic fungus strains of *Candida*. In particular, pelgipeptin D exhibited rapid and effective bactericidal action against a methicillin resistant strain of *S. aureus* and, according to acute toxicity test, the intraperitoneal LD50 value of pelgipeptin D was slightly higher than that of the structurally related antimicrobial agent polymyxin B. Tabbene et al. [71] studied three anti-*Candida albicans* compounds derived from the strain *Bacillus subtilis* B38, designated a1, a2 and a3, and identified as analogues of bacillomycin D-like lipopeptides. The compound a3 displayed the strongest fungicidal activity and was even more active than amphotericin B against the pathogenic strain *C. albicans* sp. 311 isolated from finger nail. More recently, a lipopeptide produced by *B. licheniformis* M104 was

investigated as antimicrobial agent against Gram-positive bacteria (*B subtilis, B. thuringiensis, B. cereus, Staphylococcus aureus, L. monocytogenes*), Gram-negative bacteria (*P. aeruginosa, E. coli, S. typhimurium, P. vulgaris, K. pneumonia*) and *C. albicans* [72]. All the tested microorganisms, with the exception of *L. monocytogenes* and *K. pneumonia*, were affected by the biosurfactant and *S. aureus* was the most susceptible. The antimicrobial effect the lipopeptide was time and concentration-dependent. The maximum inhibitory activity was observed at a concentration of 48 µg ml^{-1} after 12 h of treatment. The lipopeptide 6-2 produced by *Bacillus amyloliquefaciens* was found to have an interesting antifungal activity against *Candida albicans, Metschnikowia bicuspidate, Candida tropicalis, Yarrowia lipolytica, and Saccharomyces cerevisiae* [73]. The authors evaluated how lipopeptide 6-2 was able to kill *C. albicans* cells by scanning electronic microscope, revealing the presence of invaginations of the cell wall, the disruption of the whole cells and the disappearance of integrity of the cell wall. In addition, it was shown that the plasma membrane of the yeast cells was damaged by the treatment with lipopeptide 6-2 as well as the biosurfactant was responsible of the lysis of the *C. albicans* protoplast [73]. Very recently, Sharma et al. [74] purified and characterized a novel lipopeptide from *Streptomyces amritsarensis* sp. nov. The antimicrobial activity of biosurfactant was evaluated on a broad spectrum of bacteria and fungi. The MIC values of purified lipopeptide against *B. subtilis* (MTCC 619), *S. epidermidis* (MTCC 435), *M. smegmatis* (MTCC 6) and Methicillin Resistant *Staphylococcus aureus* (MRSA) were found to be 10, 15, 25 and 45 µg ml^{-1}, respectively. No activity against any of the tested Gram-negative bacteria and against fungi was observed. The results concerning biosurfactant heat stability demonstrated that a treatment at 100 °C or 121 °C for 15 minutes reduced the antimicrobial action of 13.7% and 18.2% respectively. In addition, the lipopeptide demonstrated to be non-cytotoxic and non-mutagenic, which is an important prerequisite for the development of a drug. Liang et al. [75] analyzed the antimicrobial effect of a

biosurfactant obtained by cultivating the strain *Paenibacillus macerans* TKU029 in a medium with 2% (w/v) squid pen powder as carbon/nitrogen source. The purified TKU029 biosurfactant showed significant antimicrobial activity, which remained active after high-temperature treatment (121 °C) and at different pH (pH 4-10). TKU029 biosurfactant displayed a significant inhibitory effect on *E. coli* BCRC13086 and *S. aureus* BCRC10780 at concentrations of 2 and 1.5 mg m^{-1} respectively. TKU029 BS also showed a good antifungal activity against *F. oxysporum* BCRC32121 and *A. fumigatus* BCRC30099.

Samadi et al. [76] evaluated some biological activities of mono and di-rhamnolipids produced by *Pseudomonas aeruginosa* MN1 isolated from oil contaminated soil. The mono-rhamnolipid containing fraction was a more potent antibacterial agent than the di-rhamnolipid fraction, in particular, against Gram-positive bacteria that were inhibited at 25 μg ml^{-1} concentration. Moreover, the rhamnolipids remarkably enhanced the activity of oxacillin against Methicillin-resistant *Staphylococcus aureus* strains and lowered the minimum inhibitory concentrations of oxacillin to the range of 3.12-6.25 μg ml^{-1}. Abdel-Megeed et al [29] analysed the antimicrobial activity of a glycolipid produced by a strain of *Rhodococcus erythropolis* isolated from contaminated sites. It exhibited high inhibitory activity against *Escherichia coli, Pseudomonas aeruginosa, Aspergillus niger* and *Aspergillus flavus*. Investigation of the glycolipid effects by scanning electronic microscope, showed that bacteria were totally deformed and exhibited severe destruction. In other works Luna et al. [77] and Rufino et al. [78] demonstrated antimicrobial activity of two biosurfactants derived respectively from *Candida sphaerica* UCP0995 and *Candida lipolytica* UCP 0988 against Gram-positive strains such as *Streptococcus agalactiae, Streptococcus mutans, Streptococcus sanguis, Streptococcus oralis, Staphylococcus epidermidi* and against *Candida albicans*. In the study conducted by Lotfabad et al. [79] the antibacterial ability of rhamnolipids produced produced by two *P. aeruginosa* strains, isolated from oil excavation areas in south of Iran, was elucidated. MR01

and MASH1 biosurfactants did not affect Gram-negative bacteria growth. On the other hand, they exhibited a strain dependent inhibitory effect against Gram-positive bacteria. The MIC values of MR01 biosurfactant for *S. epidermidis* ATCC 12228, *B. cereus* PTCC1247, *E. faecalis* ATCC 29212, and the clinical isolated *E. faecalis* were higher than the values obtained for MASH1 biosurfactant, whereas, MR01 biosurfactant presented lower MIC values for *S. aureus* ATCC 29213 and for the clinical isolated *M. luteus* in comparison to MASH1 biosurfactant. Rhamnolipids were also examined to evaluate their antimicrobial potential against *L. monocytogenes* and their combined effect with nisin against two wild-type isolates of *L. monocytogenes* with different susceptibility to these biosurfactants [80]. Rhamnolipids alone inhibited the 90.6% of the tested cultures and were characterized by MIC values ranging from 78.1 μg ml^{-1} to 2500 μg ml^{-1}. The combination of nisin and rhamnolipids was bactericidal at lower concentration than for the individual antimicrobials, revealing a strong synergistic effect against *L. monocytogenes* isolates. Interestingly, Joshi-Navare and Prabhune [81] have paid attention to sophorolipids (SL) ad their synergistic effect with antibiotics. Tetracycline alone, at the concentration of 15 μg ml^{-1}, was not able to totally kill *S. aureus* cells after 6 h of exposure but, when combined with sophorolipids (300 μg ml^{-1}), a total inhibition of the strain was achieved after 4 hour and an increase of the inhibition of 22% in comparison to SL alone was observed after 2 hours of exposure. Similarly, Cefaclor achieved almost total inhibition of *E. coli* after 6 h exposure, SL alone was unable to completely inhibit bacterial growth, but when administered in combination, they resulted in faster killing of the bacterium. Scanning electron microscopy revealed that the cells treated with mixtures of SL and antibiotics were characterized by cell membrane damage and pore formation, leading to leakage of the cytoplasmic contents and accumulation of cell debris. A glycolipid biosurfactant from *Halomonas sp* BS4, containing 1, 2-Ethanediamine N, N, N’, N’- tetra and (Z)-9-octadecenamide, showed antibacterial activity against

S. aureus, K. pneumonia, S. pyrogenes and *S. typhi* and antifungal activity against *Aspergillus niger, Fusarium sp, Aspergillus flavus* and *T. rubrum* [82].

3.3.Biosurfactants as anti-adhesives and role in biofilms

The continuous increase in the use of medical devices is associated with a significant risk of infectious complications, including systemic infections, septic thrombophlebitis, endocarditis, metastatic infections and sepsis. These microbial infections are due to the formation of biofilms, complex biological structures adhering to the medical device consisting of a sessile and multicellular community encapsulated in a hydrated matrix of polysaccharides and proteins. Once a mature biofilm is developed, the bacteria growing in the biofilm become highly resistant to both antimicrobial agents [83] and host immune response. The Gram-positive bacteria *Staphylococcus epidermidis, S. aureus, Enteroccocus faecalis*, constitute more than 50% of the species isolated from patients with medical device-associated infections. *Candida* spp., *Pseudomonas aeruginosa* and uropathogenic *Escherichia coli* are the remaining causal agents. Catheter-associated infections (CAIs), in particular, have become one of the most common sources of healthcare-associated infections [84,85]. Similarly, orthopaedic metallic prostheses are associated with a significant risk of infection [86,87].

Current biofilm preventive strategies are essentially aimed at coating medical surfaces with antimicrobial agents, a process not always successful [88]. Surface modification strategies based on plasma, UV and corona discharge treatment of typical catheter materials, such as silicone and polyurethanes, have been developed with the aim to increase material hydrophilicity, thus decreasing microbial adhesion and biofilm formation [89,90]. Such modifications have a temporary effect on silicone, due to the rapid rearrangement of macromolecular chains, leading to surface hydrophobicity recovery [91]. Surface coatings releasing biocides (e.g. nitric oxide, antibiotics or silver) have been developed on metallic

and polymer biomaterials, as short term antimicrobial strategies [92]. To obtain surfaces with permanent antimicrobial properties, polymeric and metal surfaces have been frequently modified via direct covalent coupling of antifouling (e.g. polyethylene glycole (PEG) [93]), cationic (e.g. polycarbonate copolymers [94], chitosan [95], quaternary ammonium salts [96] and cationic peptides [97]) or zwitterionic molecules (e.g. poly(sulfobetaine methacrylate) [98]). Main drawbacks of antimicrobial coatings arise from time limited effectiveness (as in the case of PEG-based coatings, which are susceptible to oxidative degradation [99]), development of microorganism resistance and potential toxicity towards human cells (as in the case of quaternary ammonium salts coatings [100]).

In this context, biosurfactants have recently emerged as a potential new generation of antiadhesive agents with enhanced biocompatibility. Biosurfactants, have demonstrated the ability to interfere with biofilm formation, modulating microbial interaction with interfaces by altering the physical and chemical condition of the environment where biofilms are developing [101-107].

Rivardo et al. [108], observed that a lipopeptide biosurfactant produced by the strain *B. subtilis* V9T14 in association with antibiotics synergistically increased the efficacy of antibiotics against biofilm formation of the pathogenic *E. coli* CFT073 and, in some combinations, led to the total eradication its biofilm. An international patent on this application has also been issued [109]; the biosurfactant composition can be used in combination with biocides, as an adjuvant, to aid in preventing formation and/or eradicating bacterial growth as planktonic cells or as a biofilm on biotic and abiotic surfaces. Janek et al. [110] investigated the role and applications of pseudofactin II, cyclic lipopeptide biosurfactant secreted by *Pseudomonas fluorescens* BD5, as an antiadhesive compound for medicinal and therapeutic applications. Pseudofactin II lowered the adhesion of *Escherichia coli*, *Enterococcus faecalis, Enterococcus hirae, Staphylococcus epidermidis, Proteus mirabilis* and *Candida albicans* to glass, polystyrene and silicone. In particular, pre-treatment of a polystyrene surface with 0.5 mg ml^{-1} pseudofactin II inhibited

bacterial adhesion by 36-90% and that of *C. albicans* by 92-99%. The same concentration of pseudofactin II dislodged 26-70% of pre-existing biofilms grown on previously untreated surfaces. Pseudofactin II also caused a marked inhibition of the initial adhesion of *E. faecalis*, *E. coli*, *E. hirae* and *C. albicans* strains to silicone urethral catheters. The highest concentration tested (0.5 mg ml^{-1}) caused a total growth inhibition of *S. epidermidis*, partial (18-37%) inhibition of other bacteria and 8-9% inhibition of *C. albicans* growth. In other work, a lipopeptide biosurfactants from *Paenibacillus polymyxa* was able to inhibit single and mixed species biofilms [111]. This biosurfactant complex, mainly composed of fusaricidin B and polymyxin D1, reduced the biofilm biomass of *Bacillus subtilis, Micrococcus luteus, Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus bovis*, and inhibited a self-assembling marine biofilm in co-incubation assays by 99.3% and disrupted the established marine biofilm thickness by 72.4%. Biofilm inhibition and antimicrobial activity of a lipopeptide biosurfactant produced by a soil strain of *Bacillus cereus* resistant to the heavy metals iron, lead and zinc was described by Sriram et al. [112]. It inhibited the biofilm formation in pathogenic strains of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. The highest biofilm inhibition (57%) was observed against *S. epidermidis* at a concentration of 15 mg ml^{-1}. In another work, marine bacterial culture supernatants of *Bacillus pumilus* and *B. indicus* significantly inhibited the initial attachment process and biofilm formation and dispersal of mature biofilms of *Vibrio* spp. strains [113]. The bacterial supernatants also reduced the surface hydrophobicity of *Vibrio* spp., which is one of the important requirements for biofilm development. Zeraik and Nitschke [114] assessed the effect of different temperatures on the anti-adhesive activity of surfactin and rhamnolipids biosurfactants on polystyrene surfaces, regarding the attachment of *Staphylococcus aureus*, *Listeria monocytogenes*, and *Micrococcus luteus*. Surfactin inhibited bacterial adhesion at all tested conditions, and its activity increased with the decrease in temperature, giving a 63-66% adhesion reduction in the bacterial strains

at 4 °C. Rhamnolipids promoted a slight decrease in the attachment of *S. aureus* but were not as effective. Prevention of *Candida albicans* biofilm formation on silicone disks and on acrylic resins for denture prostheses by lipopeptide biosurfactants produced by *Bacillus* sp. were reported by Cochis et al. [14]. Precoating with biosurfactants caused a greater reduction in biofilm cell number and viability than chlorhexidine. The antiadhesion activity of the biosurfactants was observed at low concentrations (78.12 μg ml^{-1} and 156.12 μg ml^{-1}) which were noncytotoxic. In another work, the lipopeptide biosurfactant produced by *Bacillus tequilensis* CH (CHBS) was able to inhibit biofilm formation of pathogenic bacteria on both hydrophilic and hydrophobic surfaces [115]. *E. coli* and *Streptococcus mutans* biofilms were grown with different concentrations of biosurfactant on glass pieces or polyvinyl chloride surfaces. Biofilms of *E. coli* and *S. mutans* were observed on the surfaces co-incubated with 0 and 25 μg ml^{-1} CHBS, whereas there was a complete absence of biofilm on the surfaces incubated with 50 and 75 μg ml^{-1} CHBS. Interestingly, CHBS did not inhibit the growth of *E. coli* and *S. mutans* planktonic cells under all tested concentrations, demonstrating that CHBS was not a bactericidal agent but only contrasted bacterial adhesion to different surfaces [115].

The anti-biofilm potential of a glycolipid surfactant produced by a tropical marine strain of *Serratia marcescens* was analyzed by Dusane et al. [116]. Pre-coating of microtiter plate wells with the surfactants effectively reduced the development of *Y. lipolytica* biofilms. Moreover, rhamnolipid treatment disrupted pre-formed biofilms both in microtiter plates and on glass slides in a more effective manner than chemical surfactants. Confocal laser scanning microscopy confirmed the effective removal of biofilms from glass surfaces.

Rhamnolipids and other plant biosurfactants have also recently been reported to have some role in the inhibition of complex biofilms and as adjuvants to enhance some antibiotics microbial inhibitors [43]. In another study, a glycolipid biosurfactant from *Pseudomonas aeruginosa* DSVP20 was evaluated for its ability

to disrupt *C. albicans* biofilm. The treatment with the di-rhamnolipid RL-2 at concentrations ranging from 0.04 to 5.0 mg ml^{-1} significantly reduced *C. albicans* adhesion on polystyrene surfaces (PS) in a dose-dependent manner. Data showed a reduction of the number of adherent cells, after 2 h of treatment, of about 50 % with 0.16 mg ml^{-1} RL-2, that gradually increased up to a completely inhibition of adherence at a concentration of 5 mg ml^{-1}. Moreover, *C. albicans* biofilm on PS surface was disrupted up to 70 % and 90 % with RL-2 treatment at concentrations of 2.5 and 5.0 mg ml^{-1}, respectively [117]. Very recently, Pradhan et al. [118] have studied a new glycolipid obtained from *Lysinibacillus fusiformis* S9. This biosurfactant showed a remarkable antibiofilm activity against pathogenic bacteria such as *E. coli* and *S. mutans*, without affecting microbial cell viability. In particular, the biosurfactant was able to completely contain the biofilms formation at a concentration of 40 µg ml^{-1}.

Anti-adhesive activities of two BSs named Rufisan and Lunasan, respectively produced by *Candida lipolytica* UCP0988 and *Candida sphaerica* UCP0995, against Gram-positive, Gram-negative and *Candida albicans* pathogenic bacteria were also described by Rufino et al. [78] and Luna et al. [77]. More recently, Padmapriya and Suganthi [119] have partially purified two biosurfactant produced by *C. tropicalis* and *C. albicans* and tested their anti-adhesive activity on different types of urinary and clinical pathogens. The results showed a reduction of adherent cells on the surface of urinary catheter pre-coated with biosurfactants and a higher activity of the biosurfactant synthesized by *C. tropicalis* in comparison with the biosurfactant synthesized by *C. albicans*.

Anti-adhesive activity against two *C. albicans* pathogenic biofilm-producing strains was described by Fracchia et al. [120] with a biosurfactant produced by the strain *Lactobacillus* sp. CV8LAC, isolated from fresh cabbage. The biosurfactant significantly inhibited the adhesion of fungal pathogens to polystyrene of about 80%. No inhibition of both *C. albicans* planktonic cells was observed, thus indicating that the biosurfactant displayed specific anti-biofilm formation but not

antimicrobial activity. The effect of the *Lactobacillus acidophilus* DSM 20079 biosurfactant on adherence and on the expression level of the genes gtfB and gtfC in *Streptococcus mutans* biofilm cells were analyzed by Tahmourespour et al. [121]. The *L. acidophilus* biosurfactant was able to interfere with the adhesion and biofilm formation of *S. mutans* to glass slide and could also make streptococcal chains shorter. Moreover, several properties of *S. mutans* cells (surface properties, biofilm formation, adhesion ability and gene expression) were changed after treatment with *L. acidophilus* biosurfactant. *Lactobacillus* biosurfactants have also been patented as inhibitors of adherence and colonization of bacterial pathogens on medical devices, in particular for preventing urogenital infection in mammals [122]. The antiadhesive activity of a lipopeptide biosurfactant secreted by the probiotic strain *Propionibacterium freudenreichii* was analyzed by Hajfarajollah et al. [123]. The biosurfactant showed a significant anti-adhesive action against a wide range of pathogenic bacteria and fungi (*E. coli. S. aureus, P. aeruginosa, B. cereus*). The highest adhesion reduction was obtained for *P. aeruginosa* (67.1 %) at the concentration of 40 mg ml^{-1}, whereas a lower activity was observed for *S. aureus* (32.3 %), *B. cereus* (39.1 %) and *E. coli* (47.7 %), at the same concentration.

3.4. Industrial application in the pharmaceutical and biomedical fields

In spite of the high number of publication describing the antimicrobial activity of biosurfactants and of patents addressed to their usage for health improvement, real applications in the biomedical and pharmaceutical industry remain quite limited [5]. Some lipopeptides have reached a commercial antibiotic status, like daptomycin [124], and the echinocandins caspofungin [125], micafungin [126] and anidulafungin [127]. Daptomycin (Cubicin®, Cubist Pharmaceuticals), a branched

cyclic lipopeptide isolated from cultures of *Streptomyces roseosporus* [124], was approved in 2003 for the non-topical treatment of skin structure infections caused by Gram-positive pathogens, including methicillin-resistant *Staphylococcus aureus* (MRSA) and in 2006 for the treatment of bacteremia and endocarditis caused by *S. aureus* strains and MRSA. Daptomycin displays strong antibacterial activity against other important pathogens, such as vancomycin resistant *Enterococci* (VRE), glycopeptide-intermediate-susceptible *S. aureus* (GISA), coagulase-negative *Staphylococci* (CNS), and penicillin-resistant *Streptococcus pneumoniae* [128]. The echinocandins caspofungin, micafungin and anidulafungin are low-toxic synthetically modified lipopeptides, originally derived from the fermentation broths of various fungi [129]. Echinocandins can inhibit fungal cell wall formation [130,131]. In particular, they show a fungicidal activity against most isolates of *Candida* spp., *Aspergillus* spp. and *Pneumocystis carinii* [132]. Caspofungin was the first licensed echinocandin, approved since 2001 for the treatment in adults - and since 2008 in pediatric patients - of oesophageal and invasive candidiasis, invasive aspergillosis in patients refractory or intolerant to standard therapy and for empirical therapy of suspected fungal infections in neutropenic patients [125]. Micafungin is used in immune compromised children to combat invasive fungal infections by *Candida* and *Aspergillus* species [126] whereas anidulafungin in the treatment of candidemia and other forms of candidiasis [127]. Lipopeptides with antimicrobial activity suitable for the treatment and prevention of microbial infections were also described in several preparations [133-137] showing potential for pharmaceutical applications. For example, the lipopeptides viscosin and analogues have been patented as therapeutic compounds that inhibit the growth of *Mycobacterium tuberculosis*, *Herpes simplex* virus 2 and/or *Trypanosoma cruzi* [138].

4. *Candida* species medical device-associated infections

Candida species are commensal fungi, belonging to the normal microbiota of mucosal oral cavity, gastrointestinal tract and vagina, that can be isolated from approximately 70% of the healthy population [139]. In healthy individuals their growth is localized by the action of immune system and the presence of other commensal microorganism. However, they can become opportunistic pathogens in critically ill or immunocompromised patients causing disabling and lethal infections [140,141].

Candida spp. are considered important pathogens due to their versatility and ability to survive in various anatomical sites. Their pathogenicity is associated with a number of virulence factor, the most important of which are the ability to evade host defenses, adhere to host tissue and medical devices, form biofilm, and product tissue-damaging hydrolytic enzymes [142]. Moreover, virulence includes host recognition, the production of degradative enzymes and the ability to transit from yeast to filamentous cells [143].

Invasive candidiasis presents a high global mortality rate, ranging from 36% to 63% in different patient groups [144,145], and is a significant problem in terms of patient management and healthcare costs in the public health system [146]. In particular, *Candida albicans* causes more than 50% of the cases of candidaemia in Europe [147]. Recent studies revealed that the majority of disease produced by *Candida* spp. is associated with biofilm formation [148]. Cells grow forming a multicellular community, both in tissues and on prostheses, catheters and other surfaces rather than living in their planktonic free form [149].

Transplantation procedures, the use of chronic indwelling devices, immunosuppression and prolonged intensive care unit stays are specific factor risks that contribute to increase the prevalence of these fungal diseases [150]. *Candida* spp. causes 10% of the overall infections of intravenous catheters and cardiac

devices prosthetic valves as well as 21% of the total cases of urinary catheters infection with a mortality rate of 20-40% [151].

4.1. Central venous catheters

The increased use of central venous catheters has been related to a steady rise of nosocomial bloodstream infections with significant increases in hospital costs, duration of hospitalization, and patient morbidity [152]. As shown by Scanning Electron Microscopy (SEM) and Transmission Electron Microscopy (TEM), biofilms can be found on the outside or the inner lumen of virtually all central venous catheter [153]. Infections may arise at any time during hospitalization. Frequently, contamination occurs in the infusion fluid itself, or in the catheter hub, but, more often, organisms are introduced from the patient's skin or from the hands of nursing staff. In some cases, the distal tip of the catheter is contaminated at the time of insertion but it is also possible that organisms can migrate down the catheter wound. Alternatively, *Candida* spp. can contaminate the catheter tip endogenously, if they are able to penetrate the intestinal mucosa and invade the bloodstream [154].
Management of *Candida* catheter-related infections should include catheter removal plus treatment with antifungal therapy for at least 14 days after the last positive blood culture and when signs and symptoms of infection have resolved [155].

4.2. Urinary catheters

Candida spp. are the microbial pathogens that are most frequently isolated from the urine samples of patients in surgical intensive care units (ICUs), with about 10–15% of nosocomial urinary tract infections being caused by this yeast [156]

Candida infections of the urinary tract are strongly associated with the presence of a urinary catheter [151]. Catheterization can cause infection by introducing organisms during the catheterization process or by allowing migration of organisms into the bladder along the surface of the catheter from the external periurethral surfaces [155]. Distinction between *Candida* colonization of the urinary tract and infection is often problematic, and symptomatic candiduria occurs in a large proportion of catheterized ICU patients [156]. In these cases, candiduria, when treated, can be eradicated by antifungal therapy or catheter removal. Bladder irrigation with amphotericin B and oral fluconazole were equally efficient but recurrences were common with both approaches [151].

5. *Candida albicans* biofilm

C. albicans is the fourth and third leading cause of hospital-acquired bloodstream and urinary tract infections, respectively [157] and represents the fungus most frequently associated with the formation of biofilms on a wide variety of medical devices [152,158-160].

Biofilms are structured microbial communities attached to a surface and surrounded by a self-produced extracellular, often slimy, matrix [161]. Cells in biofilm display altered phenotypes from those associated with planktonic counterpart. Biofilms help fungi to maintain their role as pathogen protecting cells from host defenses, withstanding competitive pressure from other organisms, and giving cells a markedly enhanced resistance to antimicrobial agents [162]. Drugs concentrations necessary for a 50% reduction of metabolic activity is 5-8 times higher in biofilms compared to planktonic cells, as well as minimum inhibitory concentrations (MICs) are increased 30- to 20,000-fold [163]. Consequently, biofilm related infections are extremely difficult, if not impossible, to eradicate, leading to surgical removal and later replacement of the infected device [164].

In general, *C. albicans* biofilm formation *in vitro* consists of four stages: (1) adherence of yeast cells to a surface, (2) growth of the attached yeast cells into a thin layer of cells, (3) maturation of the biofilm with the development of pseudohyphae and hyphae, excretion of matrix material and (4) dispersal of yeast cells from the biofilm leading to colonization of other location within the host [150,165].

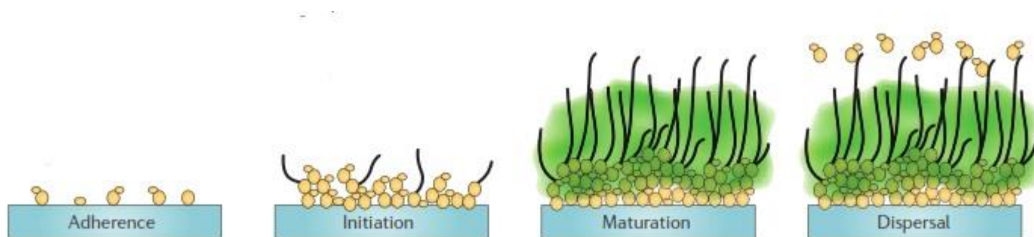

Figure 2. Stages of *Candida albicans* biofilm formation. Biofilm development can be viewed as a series of sequential steps: 1) the adherence phase, 2) the initiation phase, 3) the maturation phase and 4) the dispersal phase (modified from Finkel and Mitchell [166])

Candida biofilms formed in *in vivo* models seem to follow the same sequence [167]. However, maturation is faster and thickness is greater in these biofilms compared to those grown in *in vitro* systems. The thickness of a biofilm grown *in vitro* can range from 25 µm to 450 µm [150,168,169], whereas it usually exceeds 100 µm in *in vivo* models [167].

The first factor that influences the fungal colonization of human tissues is the adhesion to host surfaces or biomaterials. This process is mediated by non-specific factors (hydrophobicity and electrostatic forces) and promoted by specific adhesins present on the surface of fungal cells that recognize and bind to amino acids and sugars on the surface of other cells or promote adherence to abiotic surfaces [170]. One of the most clearly defined biofilm adhesins that mediate surface binding is Eap1 [171]. This evidence is demonstrated by three observations: the expression of Eap1 in a non-adherent *Saccharomyces cerevisiae* strain confers adherence to

polystyrene; a *C. albicans* Eap1$^{-/-}$ deletion mutant is defective in adherence to polystyrene; and a *C. albicans* Eap1$^{-/-}$ deletion mutant is defective in biofilm formation, as assayed both in vitro and in an vivo catheter model [172]. The closely related cell wall proteins Als1 and Als3 also assume an important role in biofilm surface attachment [173]. It has been demonstrated that their expression in *S. cerevisiae* promotes binding to several different proteins [174] while their lacking give a *C. albicans* mutant defective in biofilm formation *in vitro* and *in vivo* (Nobile et al. 2008). Furthermore, catheter surfaces inoculated with a double mutant was found virtually devoid of cells after incubation *in vivo* [175]. Als1 expression is detectable in cells grown as either yeast or hyphal cell types, whereas Als3 is expressed primarily or exclusively in hyphae. This finding suggest that the initial adherence step that leads to biofilm formation in vivo can be carried out by either yeast-form cells or hyphae [176].

The capacity of *C. albicans* to switch from yeast to hyphae is a crucial step in the formation of biofilms. Genetic analyses indicate that both yeast cells and hyphae are crucial for biofilm formation, which suggests that each cell type has a unique role in the process [166]. This morphological transition is induced by many environmental factors, such as serum, a temperature of 37°C, and neutral pH and repressed by the quorum sensing molecule E,E-farnesol [177]. Biofilm formation is prevented by this molecule, if provided during adherence, because it inhibits hyphal growth and the expression of necessary morphology-specific genes. In particular, farnesol inhibits the yeast-to-mycelium conversion of *C. albicans* [178]. The limited biofilms that form in the presence of farnesol comprise mainly yeast and pseudohyphal cells, rather than hyphae. Furthermore, farnesol also accumulates in supernatants of mature biofilms, where it stimulates the production of yeast cells and promotes biofilm dispersal [179].

The matrix is one of the most distinctive features of a microbial biofilm. It forms a three-dimensional, gel-like, highly hydrated and locally charged environment in which the micro-organisms are largely immobilized [180]. Matrix potentially

serves several special functions in the growing biofilm, such as defending against phagocytic cells, providing a scaffold to maintain biofilm integrity, limiting active drug diffusion, or a combination of all these. In *C. albicans* biofilms, matrix consists of carbohydrate, together with small amounts of proteins, hexosamine, phosphorus and uronic acid [181]. β-1,3 glucan is one of the main extracellular carbohydrate constituent and its increased production is associated with biofilm cells rather than planktonic counterparts. Furthermore, another important elements of matrix is represented by extracellular DNA. A recent study, reported that the detection of extracellular DNA through the addition of DNase to a mature biofilm partially disrupts the biofilm. At the contrary, the addition of extracellular DNA at the beginning of biofilm development results in mature biofilms with increased biofilm biomass, confirming that extracellular DNA in the matrix contributes to the structure and stability of a mature biofilm [179,181].

Mature *C. albicans* biofilms, mostly present after 24–48 h, have a highly heterogeneous architecture in terms of distribution of fungal cells and extracellular material. In addition, matrix-enclosed microcolonies are separated by water channels, which provide a mechanism for nutrient circulation within the biofilm [182]. Commonly, it is formed by a basal layer composed of several thicknesses cells in the yeast form adhering to the surface and, above this, a thick heterogeneous outer layer of filamentous cells in the hyphal form and a extensive exopolymeric matrix [183].

In the last phase, cells are released from biofilm and can disseminate into host tissues and initiate the formation of new biofilms in another sides. The majority of dispersed cells are yeast cells. This finding suggests that the transition from yeast cells to hyphae that occurs during the initial phase of biofilm formation is reversed for this step. In addition, dispersed cells have a distinct phenotype when compared with planktonic cells displaying elevated adherence, filamentation capacity, and increased pathogenicity in a disseminated infection model [165].

Hawser and Douglas were the first to create a model to study for *C. albicans* biofilm development in 1994 [184]. Since then, different model systems have been developed both *in vitro* and *in vivo* by several research groups [148,185,186].

Quantification of biofilms is evaluated by a colorimetric assay that depends on the reduction of a tetrazolium salt [168,187], by [^{3}H] leucine incorporation [184], or dry weight measurements [185]. The overall morphology and architecture of *Candida* biofilms is generally examined with fluorescence microscopy, scanning electron microscopy (SEM) or confocal scanning laser microscopy (CLSM) techniques. SEM is able to visualize detailed surface topography and morphology, whereas CLSM is used to give an image of the three-dimensional structure of biofilms and the emergence of extracellular matrix during biofilm formation [185].

Biofilm development is influenced by nature of the device surface, presence of a conditioning films, and liquid flow [150,184]. Hawser and Douglas [184] evaluated the ability of *C. albicans* to form biofilm on various catheter materials. The most extensive biofilm was observed for latex urinary catheters, followed by PVC and polyurethane. In contrast, 100% silicone was capable of significantly less biofilm formation. Serum, as a conditioning film, is important in the early adhesion events of biofilm formation, providing receptor binding sites for planktonic *C. albicans* [188], and in the interaction with *Candida* cells to initiate and promote biofilm formation and maturation [189]. The amount of matrix depends on incubation conditions. Gentle shaking produce a flow of liquid over the surface of the cells leading to an increase of the amount of the matrix, not observed in static condition. Similarly, matrix production is increased when conventional flow systems or perfused biofilm fermenter are used [154].

5.1.Standard antifungal classes

Current therapies against *Candida* infections may be grouped into four classes of antifungal compounds: polyenes, azoles, echinocandins and nucleoside analogues.

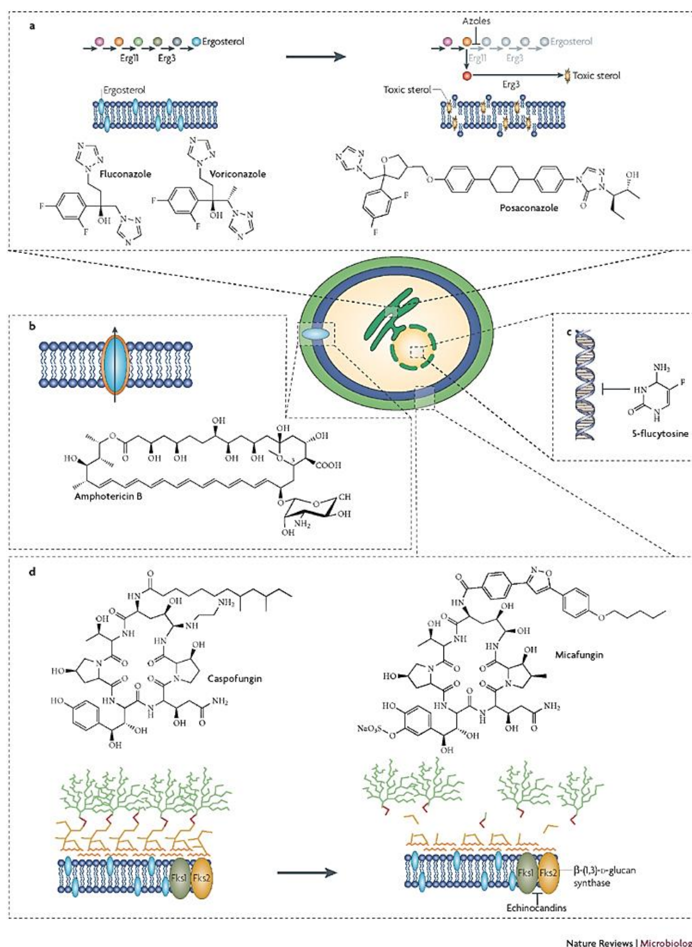

Figure 3. Antifungal drugs and their targets. The main classes of antifungal drugs that are in clinical use and how they exert their effects on the fungal cell.

Polyene antifungal agents bind sterols in the fungal cell membrane and cause electrolyte leakage via formation of transmembrane channels [190]. A second proposed mechanism of action involves a cascade of oxidation reactions and

interactions with lipoproteins that impair membrane permeability through the release of free radical. Acquired resistance to amphotericin B is relatively rare and biofilms are approximately eight-times more resistant to amphotericin B than their planktonic counterparts [191]. The specific mechanisms of resistance to polyenes are not known, but might involve alterations in the cell membrane composition [192].

Azoles target ergosterol biosynthesis via blockage of the fungal cytochrome P450-dependent enzyme lanosterol 14α-demethylase encoded by the ERG11 gene. Although acquired resistance is not common, cells in the biofilm environment are up to 1000-fold more azole resistant than their planktonic counterparts, making azoles an ineffective option [193]. The most frequently observed resistance mechanisms include alteration of the target enzyme either by overexpression or as a result of point mutations in the gene that encodes it and upregulation of membrane-bound efflux pumps [194].

Pyrimidine analogs arrest fungal DNA and RNA synthesis following their incorporation in a growing RNA/DNA strand. The group is solely composed of flucytosine. It is brought into the cell via a cytosine permease and metabolized, by a cytosine deaminase into a toxic version of uridine triphosphate. Flucytosine is also converted into a metabolite that inhibits the thymidylate synthetase, leading to decrease the availability of nucleotides for DNA synthesis [195]. The most common causes of drug resistance are mutations in the cytosine permease gene FCY2, or in the cytosine deaminase gene FCY1. *Candida* strains that are heterozygous for these mutations show partial resistance and can quickly acquire further mutations to gain full resistance upon drug exposure [196].

Echinocandins block the enzyme β-1,3-glucan synthase and thereby inhibit incorporation of β-1,3-glucans in the cell wall disturbing its integrity [132]. Echinocandins are the most recent advances in antifungal drug development. These fungicidal compounds are semi-synthetic amphiphilic lipopeptides composed of a

cyclic hexapeptide core linked to a variably configured lipid side chain [197]. Compared to planktonic cells, biofilms are approximately 2–20-fold more resistant [191]. The low resistance to echinocandins has been linked to acquired or intrinsic FKS1 point mutations in *C. albicans* [198,199]

Concerning biofilms, of the classes mentioned above, only echinocandins and the polyene amphotericin B lipid formulations have been shown to be effectively active both *in vitro* [200,201] and *in vivo* [202,203].

5.2.*Candida* biofilm resistance mechanisms

In the last years, the incidence of fungal infections has increased significantly. This is due to an increase in antimicrobial resistance and to the restricted number of antifungal drugs. The ability of *Candida albicans* to form drug resistant biofilms is an important factor in their contribution to human disease [163]. Recent investigations have started to elucidate the mechanisms behind the profound resistance associated with the biofilm. This resistance appears to be multifactorial, involving mechanisms of planktonic antifungal resistance (e.g. upregulation of drug efflux pumps, upregulation of target gene expression) as well as mechanisms specific to the biofilm lifestyle (e.g. presence of matrix, persister cells) [204]. The following section describes some of the main factors that play a role in fungal biofilm resistance.

- *Efflux pumps*. Upregulation of drug efflux pumps has been described as a causative factor in biofilm drug resistance for several biofilm-forming microorganisms [205]. In *C. albicans*, two groups of efflux pumps have been shown to contribute to drug resistance: the ATP binding cassette (ABC) transporters encoded by the CDR-genes and the major facilitator (MF) superfamily encoded by the MDR genes [206]. It has been observed that the overexpression of efflux pumps is involved in azole resistance [207-209], but not in resistance to echinocardins and Amphotericin B

[210,211]. It has been demonstrated that transcription of both MDR1 and CDR1 was more abundant in 24 h *C. albicans* biofilms than planktonic cultures of the same age [193,208]. Furthermore several studies showed that CDR1, CDR2 and MDR1 single and double mutants are susceptible to azoles when grown planktonically but preserve their resistance when grown in a biofilm structure, suggesting that the presence of these genes is not necessary for resistance in biofilms [193,208,212]. In addition, these findings supported the hypothesis that up-regolation of efflux pumps contributed to resistance during the early biofilm developmental phase, but their role in mature biofilms appeared to be minimal.

- *Cell density.* To examine the role of high cell density on biofilm drug-resistance, Perumal et al. [212] compared the susceptibility of planktonic *C. albicans* cells with intact and disrupted biofilms. It was demonstrated that both type of cells exhibited azoles, amphotericin B and caspofungin sensitivity at low cell numbers, but became resistant at a high cell density, indicating that the increased resistance was indeed associated with the biofilm architecture. Similar conclusions were also obtained by Seneviratne et al. [213] for the azole ketoconazole and the pyrimidine analog 5-flucytosine, suggesting that even if the high cell density influences *C. albicans* antifungal resistance, this is not a feature that can be applied only to biofilms, since a similar trend has been detected also in planktonic cells.
- *Persister cells.* The presence of persister cells in *Candida* biofilms was first shown by LaFleur et al. [214]. It has been hypothesized that the inability of antibiotics to kill persister cells is a consequence of the dormant state in which persister cells are present, since antibiotics need an active target to perform their function [215,216]. Furthermore, a study conducted by Khot et al. [217] demonstrated that these cells showed differential regulation of genes involved in both ergosterol (ERG1 and ERG25) and β-1,6 glucan

(SKN1 and KRE1) pathways, suggesting the possibility that the transition to a persister cell involves changes in both cell membrane and cell wall.

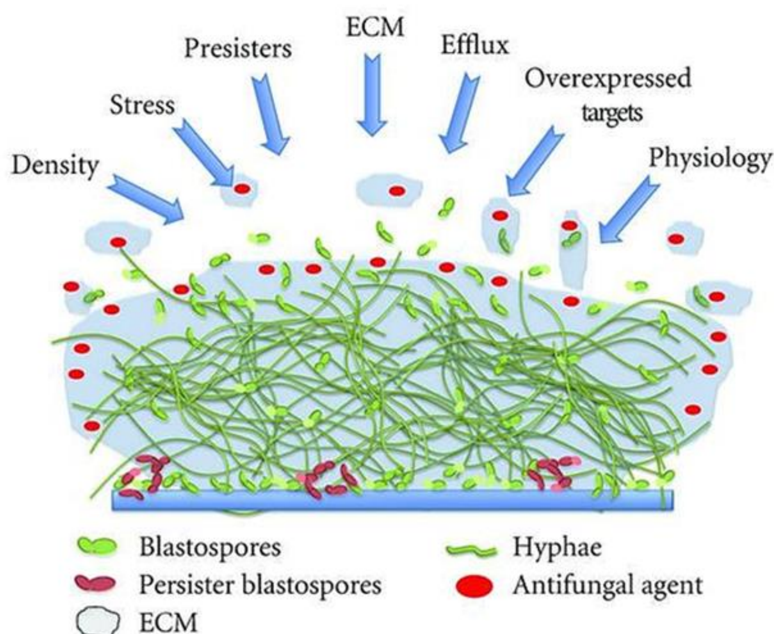

Figure 4. Schematic overview of fungal biofilm resistance mechanisms. The figure illustrates the density and complexity of the *C. albicans* biofilm, with different morphotypes present surrounded by extracellular matrix (ECM). The arrows represent the different factors that drive antifungal resistance within the biofilm, including density, stress, persisters, ECM, efflux, overexpressed targets, and the general physiology of the biofilm [218].

❖ *Stress*. In the last years, it has been shown that drug resistance in *Candida* biofilms can be also promoted by the activation of several stress responses. One of the most important is the cell-wall integrity pathway. In particular, it was observed that the lacking of the mitogen-activated protein kinase (MAPK) mkc1p, which is activated by contact stress, causes the development of deficient biofilms with reduced filamentation and susceptible to MICs 100-fold lower than the sessile wild type and both planktonic strains [219]. Calcineurin, a Ca^{2+} calmodulin-activated serine/ threonine-specific protein phosphatase plays many critical stress roles in the

fungal cell and it has also implicated in mediating resistance to the azoles. *C. albicans* sessile cells are up to 1,000-fold more resistant to fluconazole than planktonic counterparts. Inhibiting calcineurin pharmacologically or impairing calcineurin function, genetically increased the azoles activity against *C. albicans* indicating that inhibitors could be used in combinations as novel therapeutic interventions to treat or prevent biofilms [220]. Another stress response pathway contributing to *Candida* biofilm resistance involves a heat shock protein HSP90. Recent studies demonstrated that genetic depletion of Hsp90 reduced C. *albicans* biofilm growth and maturation and interestingly impaired dispersal of biofilm cells and support azoles susceptibility [221,222]. Furthermore, a marked decrease in matrix glucan levels was observed, providing that Hsp90 might regulate biofilm azole resistance acting as a regulator of the matrix sequestration pathway [222].

❖ *Extracellular matrix.* Biofilm matrix material may also impair drug delivery, either via steric hindrance or by actively binding or sequestrating antifungals. Comparing biofilms grown under continuous flow with statically grown biofilms, Al-Fattani and Douglas [181] were able to link *Candida* biofilm resistance to the production of an extracellular matrix [181]. Nett et al. [157] observed that planktonic cells surrounded by purified matrix material mimicked the biofilm drug-resistant phenotype, confirming the idea that matrix may prevent the reaching of antifungals to their intracellular target. This resistance appears to correlate with production of a matrix carbohydrate, β-1,3 glucan. Its contribution was clarified when it was shown that biofilm cell-walls bound 4- to 5 fold more azole than planktonic counterparts, thereby decreasing its potential to control biofilm-associated cells [223]. Further studies have shown that β-1,3 glucans are also responsible for sequestering echinocandins, pyrimidines,

and polyenes [224]. Since this discovery, the involvement of different genes in this process has been elucidated. One of these genes (FKS1), encoding *C. albicans* glucan synthase, that was found to be involved in the production of extracellular matrix glucan and in the biofilm drug-resistant phenotype, both *in vitro* and in an animal model [225]. Genes SMI1 and RLM1, which control cell-wall glucan content in response to stress, were shown to be essential for *C. albicans* matrix and cell-wall β -1,3-glucan content [226]. Recently, it was discovered that extracellular DNA is also an important component of biofilm matrix material that affects drug resistance. Treatment with DNAse decreased *C. albicans* biofilm biomass [227] and enhanced the activity of AmB and caspofungin but, surprisingly, had no impact on the activity on fluconazole [228].

- *Physiology*. The general physiological state of sessile cells may also influence the susceptibility profiles of biofilms [229-231]. Nevertheless, factors including pH, temperature, oxygen availability, and other environmental conditions may alter the biofilm architecture, and possibly antifungal sensitivity [232,233], suggesting that it is more likely that more complex factors are involved in biofilm resistence.

- *Overexpressed targets*. The fungistatic nature of the azoles towards *C. albicans* induces surviving population to evolve drug resistance by the alteration of the target 1,4-α-demethylase enzyme encoded by ERG11 through the development of point mutations or overexpression [234-236]. Alteration of ergosterols in biofilm membranes may explain their resistance to polyene-derived antifungal agents. Transcriptional analysis of biofilm population, 10 times more resistant to amphotericin B, for genes from the beta-1,6-glucan pathways indicated a possible association between the high level of resistance and upregulation of CaSKN1 and CaKRE1 in the biofilm cells compared to planktonic counterparts [237]. Furthermore, it has been

shown that transcriptional responses in young and mature biofilms after exposure to high doses of antifungals differed depending to the type of drug tested [238]. Exposure of both young and mature biofilms to fluconazole induced upregulation of genes encoding enzymes involved in ergosterol biosynthesis (CaERG1,CaERG3,CaERG11, and CaERG25), whereas treatment with amphotericin B resulted in an overexpression of predominantly CaSKN1, with a modest upregulation of CaKRE1.

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Outline of the thesis

The search for novel antimicrobial agents to combat the emerging pathogens and to elude increased resistance shown by others against existing antimicrobial drugs has drawn attention to natural products. Microbial metabolites are recognized as a major source of compounds endowed with potent biological activities and, among these, some biosurfactants have been described as alternatives to synthetic medicines and antimicrobial agents. Moreover, thanks to their ability to modulate the interaction of cells with surfaces, biosurfactants are able to interfere with microbial adhesion and biofilm formation, an important and mostly hazardous occurrence on medical devices, especially as microrganisms within such biofilms usually become highly resistant to drugs and adverse environmental challenges.

Candida albicans is the major human fungal pathogen in clinical setting causing various clinical manifestation, from superficial to high severe widespread invasive infections.

In this work, the biological activity of two biosurfactants (BS), CV8LAC BS and AC7 BS, was evaluated against *C. albicans* biofilms grown on medical-grade silicone elastomer disks (SEDs), at physiological conditions, in the presence of a proteinaceous solution, i.e. fetal bovine serum, to mimic blood contact during clinical use in internal body. In addition, bisurfactant AC7 activity has been assessed in combination with farnesol, an important quorum sensing molecule involved in *C. albicans* biofilm development.

Biosurfactants CV8LAC, produced by *Lactobacillus brevis,* and AC7, from *Bacillus subtilis,* are able to reduce significantly *C. albicans* cell adhesion and biofilm formation on medical grade silicone disks. In particular, these biosurfactants show the highest inhibitory activity during the adhesion phase, that persists, although at lower levels, at further incubation times, where a still significant reduction of biofilm growth is observed.

Furthermore, both compounds do not inhibit *C. albicans* planktonic cells or pre-formed biofilms, thus suggesting an anti-adhesive but not antifungal activity. In addition, when lipopeptide biosurfactant AC7 is used together with farnesol against *C. albicans* adhesion, growing biofilm and pre-formed biofilm, the inhibitory activity is significantly enhanced compared to the two compounds tested alone, suggesting a synergistic effect. These findings are supported also by the comparative ultra-structural analysis performed by SEM and CLSM on microorganisms grown on test and control samples. The analysis of quantitative SEM data on the percentage of polymer surface covered by biofilm shows lower values in treated samples in respect to controls. No phenotypical differences are indeed found in blastoconidia, hyphal morphology and budding locations.
These results suggest that CV8LAC and AC7 biosurfactants, thanks to their ability to change the surface characteristics, alone or in combination with quorum-sensing molecules or antifungal compounds, can be considered as potential inhibitors of biofilm growth on medical insertional materials.

Inhibition of *Candida albicans* adhesion on medical-grade silicone by a *Lactobacillus*-derived biosurfactant

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Summary

The study aimed at investigating the ability of biosurfactant produced by a *Lactobacillus brevis* isolate (CV8LAC) to inhibit adhesion and biofilm formation of *Candida albicans* on medical-grade silicone elastomeric disks (SEDs). Biosurfactant activity was evaluated at physiological conditions, by means of co-incubation and pre-coating assays. Additionally, biosurfactant extract was tested for antifungal susceptibility against *C. albicans* in both planktonic and sessile form. Biofilm covered surface and hyphae and blastospores occurrence were quantified by scanning electron microscopy (SEM) and image analysis. Biosurfactant did not inhibit growth of *C. albicans* in both planktonic and sessile form. Nevertheless, co-incubation with 2000 μg ml^{-1} biosurfactant significantly reduced biofilm formation on SEDs surface by 89%, 90% and 90% after 24, 48 and 72 h of incubation. Fungal

adhesion and biofilm formation to pre-coated SEDs was reduced by 62%, 53%, 50% and 44% after 1.5, 24, 48 and 72 h. SEM showed a significant reduction of biofilm covered surface in pre-coated disks but no differences in the production of hyphae or blastospores, except at 1.5 h of incubation. This study demonstrated that CV8LAC biosurfactant has the ability to counteract significantly the initial deposition of *C. albicans* to silicone surfaces and to effectively slow biofilm growth. The anti-adhesive properties of the CV8LAC biosurfactant suggest a potential role of the coating for preventing fungal infection associated to silicone medical devices.

Introduction

Transplantation procedures, immunosuppression, the use of chronic indwelling devices, and prolonged intensive care unit stays have increased the prevalence of fungal implant-related infections. Even with current antifungal therapy, *Candida* species causes 10% of the overall infections of intravenous catheters and cardiac devices prosthetic valves as well as 21% of the total cases of urinary catheters infection with a mortality rate of 20-40% and highly expensive costs [1]. Nowadays, *Candida albicans* is the fungus most frequently associated to medical devices-related infections [2-5] and its ability to form biofilms has a profound impact on the capacity to cause human disease. The presence of biofilm, in fact, protects the microorganism from host defences and reduces significantly its susceptibility to antifungal agents [6].

The early phase of *Candida* biofilm formation, consisting in the adherence of yeast cells to biomaterials [7], is determined by a complex interplay of hydrophobicity, electrostatic interactions, and presence of specific receptor sites on the cell surface [8]. Macromolecular components (such as saliva, urine, or blood) adsorbed onto biomaterial surfaces may change the electrical properties and the hydrophobicity of the surface, favoring microbial colonization. Altering the chemical-physical

properties of this conditioning film could represent a preventive strategy to limit pathogens adhesion and biofilm formation on medical implantable materials [9].

Biosurfactants are amphipathic metabolites produced by a wide variety of microorganisms, with both hydrophilic and hydrophobic moieties within the same molecule [10]. It has been hypothesized that the adsorption of biosurfactants to a substratum surface may reduce its hydrophobicity, thus interfering with microbial adhesion and desorption processes [11].

Lactobacilli, which are part of the human and animal commensal intestinal flora, have recently caught the attention of medical and scientific researchers due to the extraordinary health enhancing benefits they exhibit [12-14]. These bacteria produce a wide variety of secondary metabolites with anti-microbial activity, such as hydrogen peroxide, lactic acid, and bacteriocins. In addition, lactobacilli interfere with the adhesion of different pathogens on epithelial cells of urogenital and intestinal tracts [15] and on a variety of medical devices producing adhesion inhibitors, such as biosurfactants [14].

Biosurfactants produced by lactic acid bacteria have been shown to reduce the initial adhesion of different bacterial pathogens and, to a lesser extent, of yeasts to polystyrene [16,17], silicone rubber [18, 19], surgical implants [20] and voice prostheses [21,22].

The crude biosurfactant produced by *Lactobacillus brevis* CV8LAC was previously found to be an excellent anti-adhesive agent against culture collection strains of *C. albicans* on polystyrene plates [23].

In this study, the ability of CV8LAC biosurfactant to inhibit adhesion and biofilm formation of a *C. albicans* strain, isolated from explanted central venous catheter, was evaluated on silicone elastomer at different times of biofilm growth, in physiological conditions, by means of the viable cell counting method and the biofilm was visualized and quantified by scanning electron microscopy (SEM).

Materials and Methods

Strains

Lactobacillus brevis CV8LAC strain was isolated from a fresh cabbage obtained from a producer of biological fruit and vegetables in a rural area of Piedmont, Italy [23]. *Candida albicans* 40 (DSM 29204), used for biofilm assays, is a wild strain, clinically isolated from a central venous catheter. *L. brevis* CV8LAC strain was cultivated in MRS broth without Tween 80® (Sigma-Aldrich) and stored at −80°C in the same medium supplemented with 25% glycerol. *C. albicans* 40 strain was cultivated in Yeast Nitrogen Base broth (Sigma-Aldrich) with 50 mmol l^{-1} dextrose (Biolife) (YNBD) and stored at −80°C in Sabouraud dextrose broth (Sigma-Aldrich) supplemented with 25% glycerol.

Biosurfactant production and extraction

For biosurfactant (BS) production, a seed culture was prepared by transferring a loop of *L. brevis* CV8LAC strain from a MRS agar (supplemented with 0.1% Tween 80®) overnight culture into 20 ml of MRS broth without Tween 80® and incubated at 28°C for 4 h at 140 rpm. Thereafter, 2 ml were inoculated in 500 ml of the same broth in a 2 l flask and incubated again at 28°C for 24 h at 140 rpm. The culture broth was then centrifuged at 6000×g for 20 min and the supernatant collected. For BS extraction, the supernatant was acidified to pH 2 with 6 mol l^{-1} HCl, stored overnight at 4°C and extracted three times with ethyl acetate: methanol (4:1) (Sigma-Aldrich) according to the method described by Rivardo *et al.* [24]. The remaining water in the organic phase was removed by anhydrous sodium sulfate. The organic phase was evaporated to dryness under vacuum condition and acetone was added to recover raw CV8LAC BS. Acetone was, then, evaporated and CV8LAC BS collected and weighted.

Antifungal susceptibility testing against Candida albicans planktonic cells

The effect of CV8LAC BS on planktonic cells of *C. albicans* 40 was evaluated according to EUCAST guidelines [25]. Briefly, CV8LAC BS solution (4000 µg ml^{-1}) was diluted in a 96-well microtiter plate (Bioster) with PBS to obtain final concentrations of CV8LAC BS ranging from 3.9 µg ml^{-1} to 2000 µg ml^{-1} in a final volume of 100 µl per well. *C. albicans* 40 suspension at the concentration of 1-5×10^5 Colony Forming Unit per ml (CFU ml^{-1}) was prepared in sterile double-strength RPMI 1640 medium (Sigma-Aldrich) buffered with 3-(N-morpholino) propanesulfonic acid buffer (MOPS) (Sigma-Aldrich) and supplemented with D-glucose (2% final) pH 7.0. One-hundred microliters of inoculum were then added to each well and the microtiter plate was incubated without agitation at 37°C for 24 h. Quadruplicate assays were performed for all CV8LAC BS concentrations used for test. After the incubation, the absorbance at 450 nm was determined for each well.

Medical-grade silicone elastomeric disks preparation

Two different sizes of medical-grade silicone elastomeric disks (SEDs) (TECNOEXTR S.r.l., Italy) were used in the study: 15 mm in diameter, 1.5 mm in thickness for experiments in 12-well culture tissue plates and 10 mm in diameter, 1.5 mm in thickness for experiments in 24-well culture tissue plates. Each silicone disk was cleaned, sterilized and conditioned before use according to the method described by Busscher *et al.* [18]. Briefly, disks were submerged in 200 ml of distilled water supplemented with 1.4% (v/v) of RBS 50, sonicated for 5 min at 60 kHz using Elma S30H (Elmasonic, VWR International) and rinsed in 1 l of MilliQ water for two times. Then, disks were submerged in 20 ml of MeOH (99%) (Sigma-Aldrich), rinsed twice, dried under a sterile hood and steam sterilized for 15 min at 121°C. Before use, each silicone disk was aseptically transferred into 2 ml of Fetal Bovine Serum (FBS) (Sigma-Aldrich), incubated at 37°C for 24 h at 140

rpm and rinsed with PBS to provide for a serum protein coating simulating blood contact during clinical use.

Antifungal susceptibility testing of Candida albicans biofilm

The antifungal effect of CV8LAC BS on *C. albicans* 40 preformed biofilm was carried out according to Chandra *et al.* [26], with minor changes. Briefly, *C. albicans* 40 cells were grown for 24 h at 37°C in 25 ml of YNBD at 140 rpm. After centrifugation and two washings with PBS, the pellet was resuspended in the same buffer and standardized to 1×10^7 CFU ml^{-1}. One milliliter of this fungal suspension was added to each well of 24-well plates (Greiner bio-one) containing a silicone disk. After 1.5 h (adhesion phase) in static conditions at 37°C, the disks were placed into new plates containing, in each well, 1 ml of YNBD and incubated for 24 h at 37°C at 90 rpm (biofilm growth phase). Mature biofilms were transferred into new plates with different concentrations of CV8LAC BS ranging from 3.9 µg ml^{-1} to 2000 µg ml^{-1} and incubated for other 24 h at 37°C in static conditions. The antifungal activity of CV8LAC BS was evaluated by the XTT (Sigma-Aldrich) colorimetric assay. Disks covered with 48 h old *C. albicans* 40 biofilm were transferred into new plates containing 1 ml of PBS, 12.5 µl of XTT solution (1 mg ml^{-1}) and 1 µl of menadione solution (1 mmol-Sigma-Aldrich). Plates were covered with aluminum foil and incubated at 37°C for 5 h at 90 rpm. Triplicates assays were performed for all CV8LAC BS concentrations used for the test. From each well, 150 µl were transferred to a 96-well plate and the absorbance was measured at 490 nm using a Ultramark microplate imaging system (Bio-Rad).

Anti-adhesion and anti-biofilm assays

Co-incubation assays

C. albicans 40 cells were grown for 24 h at 37°C in 25 ml YNBD at 140 rpm. After centrifugation and two washings with PBS, the pellet was resuspended in the same

buffer and standardized to 2×10^7 CFU ml^{-1}. One milliliter of the fungal suspension was added to each well of a 12-well plates (Greiner bio-one) containing a silicone disk together with either 1 ml of a 2× CV8LAC BS solution (4000 µg ml^{-1}) (test samples) or PBS (control). After 1.5 h, the disks were placed into new plates containing in each well either 2 ml of YNBD with 0 µg ml^{-1} (control group) or 2000 µg ml^{-1} CV8LAC BS (test group). After incubating the samples for 24, 48 and 72 h at 37°C with gentle shaking, the supernatants were discarded and the disks were washed three times with PBS to remove non-adherent cells. The biofilm inhibition activity of CV8LAC BS was evaluated by means of the viable cell counting method reported below, and expressed as Colony Forming Unit per disk (log_{10} CFU/disk). Assays were carried out in triplicate. Results were expressed as mean values and standard deviations.

Pre-coating assays

Silicone disks were submerged in 2 ml of CV8LAC BS solution at the concentration of 2000 µg ml^{-1} (test group) or in PBS only (control group). All samples were incubated at 37°C for 24 h at 140 rpm, and then gently rinsed with PBS to remove non-adherent CV8LAC BS.

C. albicans 40 cells were grown for 24 h at 37°C in 25 ml of YNBD at 140 rpm. After centrifugation and two washings with PBS, the pellet was resuspended in the same buffer and standardized to 1×10^7 CFU ml^{-1}. Two milliliters of fungal suspension were added to each well of a 12-well plate containing a disk pretreated with 2000 µg $ml^{-1}$of CV8LAC BS (test group) or PBS (control group). After 1.5 h of adhesion phase, six disks for each group were washed three times with PBS to remove non-adherent cells. The other samples were transferred in new plates containing 2 ml of YNBD and incubated at 37°C with gentle shaking for testing longer incubation times at 24, 48 and 72 h. After the incubation period, disks were washed three times with PBS to remove non-adherent cells. The biofilm inhibition

activity of CV8LAC BS after 1.5 h and 24, 48, 72 h incubation was evaluated with the viable cell counting method described below and expressed as $\log_{10}$ CFU/disk. Assays were carried out in triplicate and experiments were repeated three times. Results were expressed as mean values and standard deviations.

Viable cell counting

The effect of CV8LAC BS on *C. albicans* 40 adhesion and biofilm formation was assessed by determining the viable cell counts after biofilms had been rinsed for three times with PBS to remove non adherent cells. Briefly, disks were inserted into 50 ml tubes containing 10 ml PBS and subjected to four cycles of sonication (30 s) and stirring (30 s). The sonication fluid with detached cells was serially diluted in PBS and each dilution was plated onto Sabouraud Dextrose Agar (Sigma-Aldrich), in triplicate. Agar plates were incubated for 24 h at 37°C and colonies were then enumerated. Results were expressed as $\log_{10}$ CFU/disk.

SEM analyses

A specific set of samples was realized according to the method applied in the pre-coating assay for a qualitative and quantitative analysis of *C. albicans* 40 biofilm formation by scanning electron microscopy (SEM). After the specific incubation time, each disk was washed three times in PBS to remove the culture medium and non-adherent cells from the silicone substrate, fixed by immersion in aldehydic solution (2.5% glutaraldehyde in 0.1mol l^{-1} phosphate buffer) for 24 h at 4°C, washed twice in distilled water, dehydrated by immersion in ascending alcohol solutions (70%, 90% and 100% ethanol, 10 min each) and dried overnight under a laminar flow cabinet. Finally, dried samples were glued to SEM sample holder by double bonding carbon tape and gold sputtered.

SEM analyses were conducted in a XL30 ESEM FEG (Fei-Eindhoven, NL) scanning electron microscope at a 10 KV beam voltage. A preliminary qualitative

investigation of the *Candida* biofilm was performed at a magnification ranging from 100× to 10000×. A representative selection of images at 1000× was stored by collecting the secondary electrons signal revealing fine morphological details of cells and extracellular matrix.

For quantitative purposes, two sets of images were also acquired per each sample. A first set of nine different fields of view was obtained by collecting the backscattered electrons signal at a magnification of 40×, thus guaranteeing the observation of a total area of 64 mm^2 at the silicone disk centre. To distinguish between biofilm covered surface and exposed silicone, compositional contrast was stressed and images were processed according to a protocol adapted from Bressan *et al.* [27]. Briefly, high resolution digital images (1936×1452 pixels) were acquired and then threshold by an automated routine implemented in ImageJ (NIH, US). The number of dark pixels associated to *Candida* biofilm and bright pixels representing the silicone surface was obtained. Percent area of the silicone disk covered by C*andida* biofilm (BA%) was computed calculating the percent ratio of dark pixels over the whole pixels number of the image.

To obtain quantitative data on hyphae and blastospores occurrence on *Candida* biofilm, a second set of five SEM images per sample was acquired by collecting the signal from secondary electrons at a magnification of 1000×. The enumeration of the two features of interest was performed according to a protocol adapted from Lucas *et al.* [28]. A squared grid of 15 µm element size was superimposed to each acquired imaged and the observation of each single cross point allowed to quantify the occurrence of hyphae or blastospores. Percentage of hyphae and blastospores was computed over the total number of evaluated cross points.

Statistical analysis of data

Statistical analysis and graphs were elaborated by means of the statistical program R (R Development Core Team, http//www.R-project.org).

ANOVA was used to compare optical densities of *C. albicans* 40 planktonic cells and pre-formed biofilm at different CV8LAC BS concentrations.
Welch Two Sample t-test was performed to investigate the effect of CV8LAC BS in co-incubation and pre-coating assays on biofilm formation at different incubation times. Results were considered to be statistically significant when $P<0.05$. In this case, the R package dupi R was used to estimate $\log_{10}$ CFU/disk from colony counts [29].
Chi-square test with Yates' continuity correction was used to compare proportions of hyphae and blastospores at the different time points.

Results

Antifungal susceptibility testing on Candida albicans planktonic cells and biofilm

CV8LAC BS susceptibility testing was carried out on *C. albicans* 40 planktonic cells and pre-formed biofilm by means of EUCAST and XTT methods, respectively. Optical densities at 450 nm and 490 nm versus the biosurfactant concentration were plotted in Fig. 1.
One-way ANOVA was used to evaluate the role of CV8LAC BS concentration. While on planktonic cells a significant stimulating effect could be detected ($P=6.5\times10^{-4}$), on preformed biofilm no significant activity (either inhibitory or stimulating) could be established ($P=0.89$).
By further inspection of the role of CV8LAC BS concentration on planktonic cells, we tried to locate the concentration cutoff that minimizes the P-value and best discriminates (by separating concentrations in 2 groups) the effect. We were able to isolate a first concentration group (ranging from 0 µg ml^{-1} to 62.5 µg ml^{-1} CV8LAC BS) where optical densities of planktonic cells co-incubated with biosurfactant were comparable to that of control (no biosurfactant added) and a second group (from 125 µg ml^{-1} to 2000 µg ml^{-1} CV8LAC BS), with higher optical densities,

where we observed a stimulating activity of CV8LAC BS starting from the concentration of 125 μg ml^{-1} ($P=3.8\times10^{-8}$).

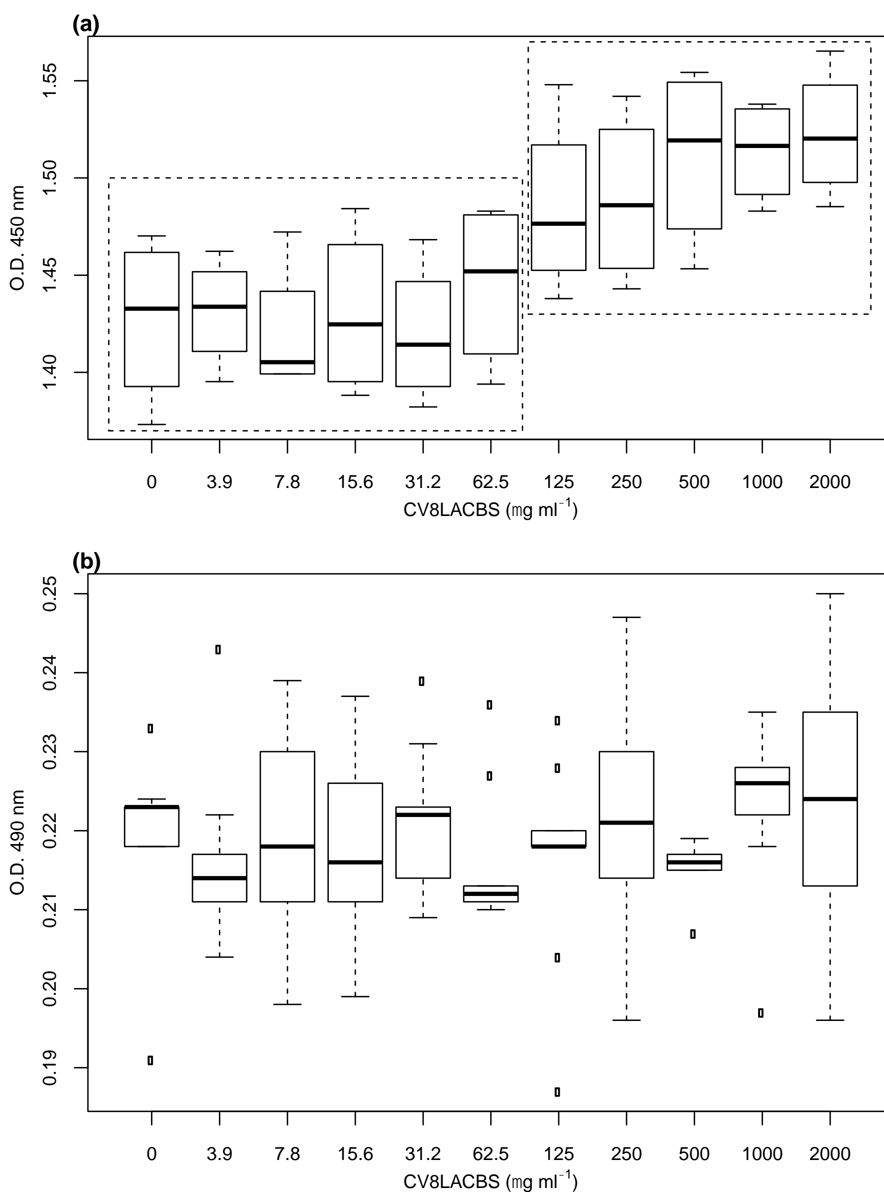

Figure 1. CV8LAC BS activity at 24 h on (a) *C. albicans* 40 planktonic cells and (b) pre-formed biofilm, measured by optical density. In (a) the dashed boxes separate two data groups significantly different from each other (One-way ANOVA, $P< 0.05$).

Anti-biofilm activity of CV8LAC BS

The anti-biofilm assays were carried out using a CV8LAC BS concentration of 2000 µg ml^{-1}. Previous studies suggested that this was the lowest concentration (data not shown).

Table 1 summarizes the results of the pre-coating and co-incubation assays, carried out at different times (1.5 h, 24 h, 48 h and 72 h), expressed as means and standard deviations for log_{10} CFU/disk. In addition, for each time and assay, the results of the Welch Two Sample t-test comparing CV8LAC BS treated and control samples are shown. The results are reported as P-values and 95% confidence intervals for the differences. The final column of Table 1 indicates the percentages of inhibition calculated as $(1-10^{\mu})*100$, where µ is the difference in log_{10} CFU/disk of BS treated and control samples.

Experimental setting	Time (h)	Control		CV8LACBS		95% confidence interval	P	Inhibition measures	
		Mean	SD*	Mean	SD			µ†	Percentage of inhibition (%)‡
Co-incubation									
	24	7.50	0.09	6.52	0.29	[-1.16, -0.78]	5.4×10^{-8}	-0.97	89
	48	7.67	0.08	6.67	0.08	[-1.07, -0.93]	2.6×10^{-19}	-1.00	90
	72	7.71	0.06	6.72	0.09	[-1.05, -0.93]	2.2×10^{-18}	-0.99	90
Pre-coating									
	1.5	6.10	0.21	5.68	0.24	[-0.61, -0.23]	1.4×10^{-4}	-0.42	62
	24	7.57	0.24	7.24	0.28	[-0.56, -0.10]	7.0×10^{-3}	-0.33	53
	48	7.56	0.09	7.26	0.23	[-0.46, -0.14]	1.2×10^{-3}	-0.30	50
	72	7.60	0.13	7.35	0.22	[-0.41, -0.09]	4.0×10^{-3}	-0.25	44

*SD: standard deviation

†$\mu = (log_{10}\, CFU/disk_{CV8LACBS} - log_{10}\, CFU/disk_{Control})$

‡Percentage of inhibition = $(1-10^{\mu})*100$

Table 1. Mean *C. albicans* 40 concentrations, expressed as log_{10} CFU/disk, recovered on silicone disks, 95% confidence interval, P values and inhibition measures, in co-incubation and pre-coating assays with CV8LAC BS.

In co-incubation experiments, the number of adherent cells on control disks was 7.50±0.09 log_{10} CFU/disk after 24h, and increased to 7.67±0.08 and 7.71±0.06 log_{10} CFU/disk after 48 and 72 h of incubation, respectively. The number of adherent cells on BS treated disks was 6.52±0.29 log_{10} CFU/disk after 24 h, and

increased to 6.67±0.08 and 6.72±0.09 log_{10} CFU/disk after 48 and 72 h of incubation, respectively.

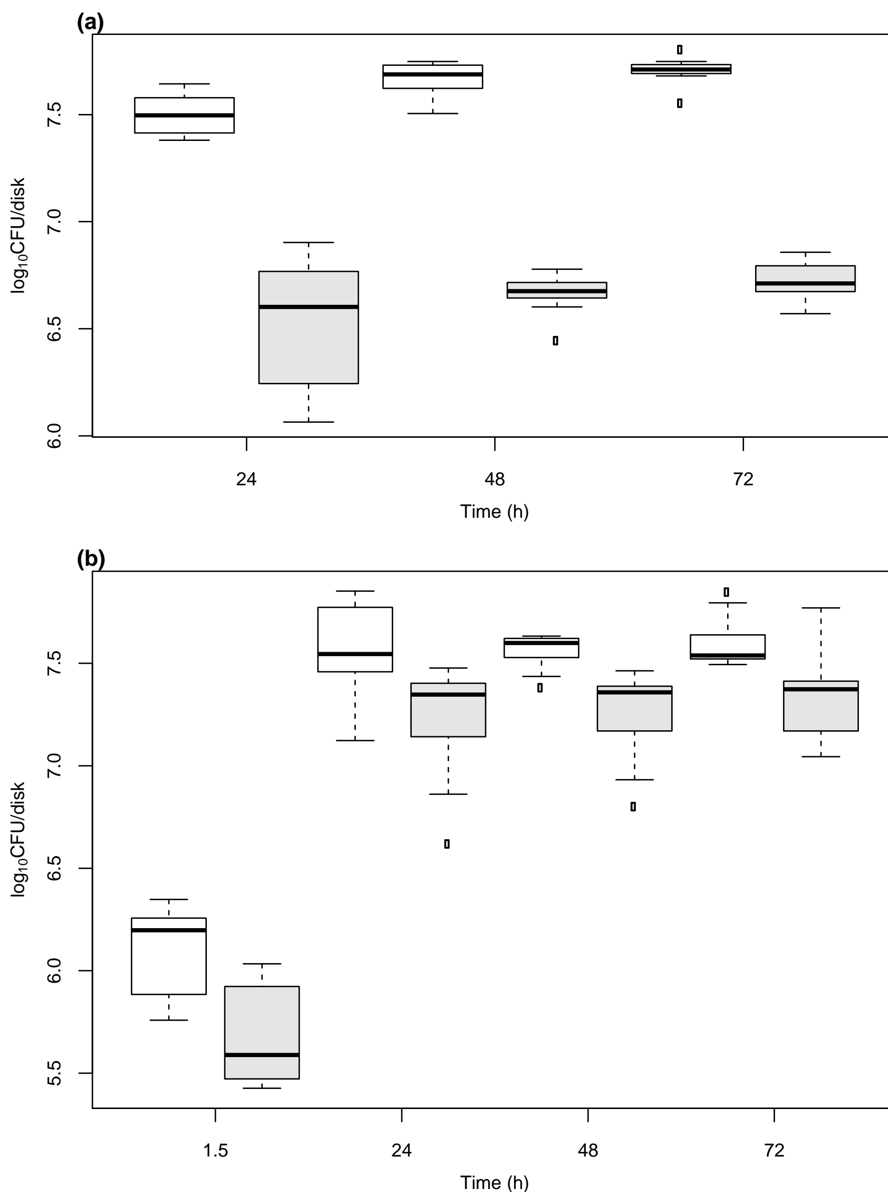

Figure 2. (a) Inhibition of *C. albicans* 40 biofilm formation on silicone disks by CV8LAC BS in co-incubation assays and (b) inhibition of *C. albicans* 40 adhesion and biofilm formation on silicone disks pre-coated with CV8LAC BS, evaluated at 1.5, 24, 48 and 72 h. White boxes: control disks; grey boxes: CV8LAC BS treated or co-incubated disks.

Co-incubation with CV8LAC BS at the concentration of 2000 µg ml^{-1} induced a significant reduction ($P<5.4\times10^{-8}$ at least) of biofilm formation in respect to controls by a $\log_{10}$ factor ~1 (i.e. about 90%) at all times of incubation.

In pre-coating assays, the number of adherent cells on control disks was 6.10±0.21 $\log_{10}$ CFU/disk after 1.5 h and it gradually increased to 7.57±0.24, 7.56±0.09 and 7.60±0.13 $\log_{10}$ CFU/disk after 24, 48 and 72 h, respectively. On BS pre-coated disks the number of adherent cells was 5.68±0.24 $\log_{10}$ CFU/disk at 1.5 h and then it gradually increased to 7.24±0.28, 7.26±0.23 and 7.35±0.22 $\log_{10}$ CFU/disk after 24, 48 and 72 h, respectively. With respect to controls, fungal adhesion to SEDs treated with 2000 μgml^{-1} BS was significantly reduced by 62% at 1.5 h ($P<1.4\times10^{-4}$) and biofilm formation after 24, 48 and 72 h of incubation was inhibited by an average of 50% ($P<7.0\times10^{-3}$ at least), respectively.

CV8LAC BS activity against *C. albicans* 40 adhesion and biofilm formation on silicone disks in co-incubation and pre-coating assays at different incubation times is displayed in Fig. 2. Both panels show clearly that fungal adhesion and biofilm formation on treated disks is lower (at each incubation time) that on untreated disks. The difference is more evident in co-incubation assays (see again last column in Table 1). To be noted that in the pre-coating assay (Fig 2(b)) at time 1.5 h fungal counts are very low compared to later times as *C. albicans* 40 is in the initial phase of adhesion.

SEM analyses

Qualitative analysis of the biofilm microstructure on high magnification SEM images evidenced a less compact structure of the biofilm in treated samples in respect to controls at 24, 48 and 72 h of incubation (Fig. 3). Moreover, a complex three-dimensional structure with long hyphae arranged in a multilayered network was found in controls at 48 and 72 h of incubation. Differently, treated samples did

not developed a thick hyphal network in the tested conditions. No phenotypical differences were found between microorganisms growth on test or control samples showing in both cases ovoid spherical blastoconidia with polarly or random located budding and true hyphae formation. No difference in the amount of extracellular material was documented.

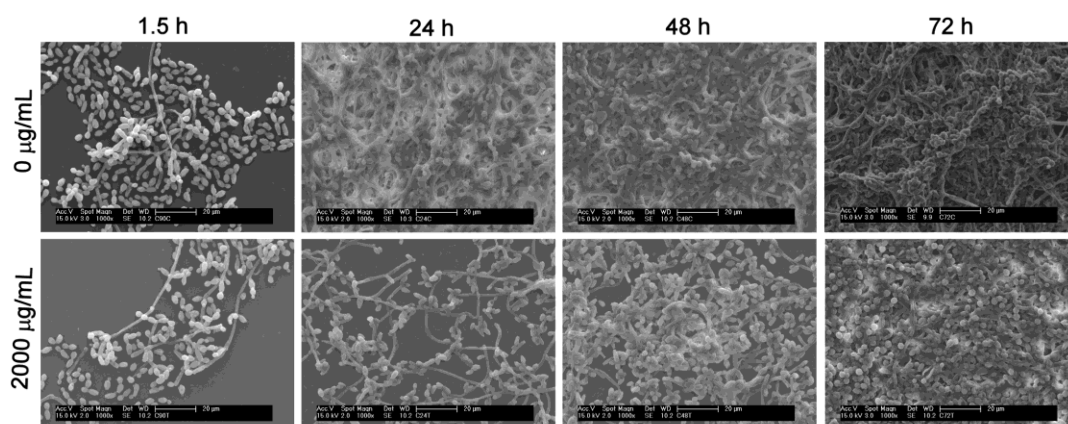

Figure 3. Scanning electron micrographs of CV8LAC BS treated (lower row) and control (upper row) silicone surfaces after *C. albicans* 40 adhesion phase (1.5 h) and at 24, 48 and 72 h of incubation. Original magnification 1000×.

The quantification of BA% on SEDs showed differences between treated samples and controls (Table 2). After 1.5 h of incubation, the 33% of the control disks surface was covered by *C. albicans* 40 cells whereas, in the treated disks, only the 14% of the surface was occupied.

Time (h)	Biofilm covered surface (%)			
	Control		CV8LAC BS	
	Mean	SD*	Mean	SD
1.5	33	15	14	4
24	99	0	73	13
48	99	0	88	11
72	100	0	95	8

Table 2. Mean *C. albicans* 40 biofilm covered surface evaluated by SEM and ImageJ (NIH, U.S.) on CV8LAC BS pre-coated and control disks

During the biofilm formation phase, control disks were totally covered by *C. albicans* biofilm (99% at 24 and 48 h, 100% at 72 h) whereas disks treated with CV8LAC BS showed a BA% of 73%, 88% and 95%, respectively at 24, 48 and 72 h.

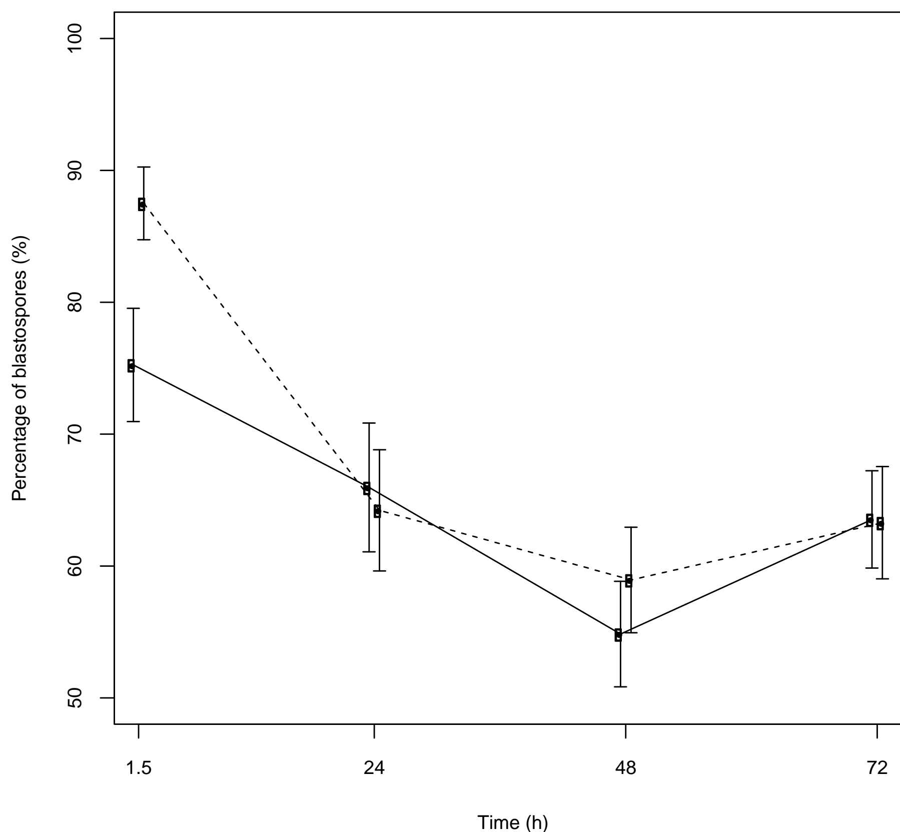

Figure 4. Percentage of blastospores detected on silicone disks pre-coated or not with CV8LAC BS. The dotted line represents control samples while the solid line represents treated samples. The vertical bars indicate the standard deviations of the percentages.

Fig. 4 represents the percentage of blastospores in control and treated samples with respect to total number of hyphae and blastospores, as a function of incubation time. The percentage of hyphae is not reported being the complement to 100%. No statistically significant difference between treated samples and controls was found,

except at time 1.5 h where a lower proportion of blastospores ($P=0.02$) was detected in treated SEDs with respect to untreated ones. No differences in the production of hyphae or blastospores within the biofilm were associated with BS pre-coating in respect to controls at 24, 48 and 72 h.

Discussion

The high demand for new antimicrobial and antifungal agents due to the increased resistance of pathogenic microorganisms has drawn attention to biosurfactants as a new source of antimicrobial and antifungal drugs [24]. Biosurfactants have the ability to disrupt membranes leading to cell lysis and metabolite leakage by inducing changes in physical membrane structure or by disrupting protein conformations altering important membrane functions such as transport and energy generation [30, 31]. In addition, biosurfactants form a film that changes wettability and surface energy of the original surface affecting the adhesion properties of microorganisms [32].

In a previous work, Fracchia *et al*. [23] observed that CV8LAC crude biosurfactant was able to reduce the water surface tension from 70.92 mN m^{-1} to 47.68 mN m^{-1} and its Critical Micellar Concentration (CMC) was 106 μgml^{-1}. Furthermore, CV8LAC BS decreased the adhesion of two biofilm-producer strains of *C. albicans* derived from culture collections (DSMZ 11225, CA-2894) of more than 80% in pre-coated polystyrene microtiter plates.

In this work, the activity of CV8LAC biosurfactant against the adhesion and biofilm formation of a nosocomial *C. albicans* isolate was assessed on medical-grade silicone, at physiological conditions (37°C), in the presence of a proteinaceous solution, i.e. fetal bovine serum, to mimic blood contact during clinical use in internal body conditions. The efficacy of the CV8LAC BS to inhibit *C. albicans* biofilm formation was first evaluated in co-incubation experiments, and subsequently after its absorption on the surface of silicone disks, in order to

imitate a functional coating condition. In co-incubation assays, CV8LAC BS induced a significant reduction of biofilm formation (about 90%) at all incubation times. In pre-coating experiments, the highest performance was observed during *C. albicans* adhesion phase, whereas during the biofilm formation phase, the inhibition was lower (about 50 %) but still significant.

Previous studies assessed the effect of biosurfactants from lactic acid bacteria against the initial deposition and adhesion of *Candida albicans* strains. Busscher *et al.* [18] demonstrated that pre-adsorption of biosurfactants from *Streptococcus thermophilus* to silicone rubber with a salivary conditioning film was effective against *C. albicans* GB 1/2 adhesion. In particular, relative initial cell deposition rate and *C. albicans* GB 1/2 cells adhering at 4 h on silicone rubber were respectively 69% and 77%, compared to controls (100%). In another study, a biosurfactant obtained from *Lactobacillus acidophilus* at a concentration of 1 mg ml^{-1} reduced the initial deposition of *C. albicans* strains of about 50% but it did not show anti-adhesive activity after 4 h in pre-coating experiments in a silicone-rubber bottom plate (Velraeds *et al*. [19]. A study from Rodrigues *et al.* [21] reported that the adsorption of a biosurfactant obtained from the probiotic bacterium *L. lactis* 53 on silicone rubber reduced *C. albicans* deposition rate as well as the number of cells adhering on the surface after 4 h. The Authors also showed that *C. albicans* initial deposition and adhesion to silicone rubber treated with a biosurfactant from *Streptococcus thermophilus* A were reduced of 65% and 67-70%, respectively [22]. Similarly, our results suggest that CV8LAC BS has the ability to counteract successfully the initial adhesion of *C. albicans* on the pre-coated silicone surface. Noteworthy, we found that CV8LAC biosurfactant activity persisted at further incubation times, where a significant reduction of biofilm growth was observed.

It has been supposed that biosurfactants influence bacterial-surface interactions thanks to their ability to change surface tension and bacterial cell-wall charge, affecting both cell-to-cell and cell-to-surface interactions [33]. Our results support the opinion that lactobacilli-derived biosurfactants remarkably affect these

interactions and, as a result, the surface is made less supportive for bacterial adhesion.

Cultural data were also in agreement with SEM microscopic observation of the biofilm on silicone disks. The analysis of quantitative SEM data on the percent of polymer surface covered by biofilm showed lower values in treated samples in respect to controls. Namely, pre-coated silicone disks were characterized by a BA% reduction of 57% after 1.5 h and of 26%, 11% and 4.5% after 24h, 48h and 72h respectively when compared to control disks. Quantitative data were based only on a bi-dimensional evaluation of the biofilm extension. The correct interpretation of these results should consider also the information obtained from the qualitative high magnification analysis, giving information on the three-dimensional development of the biofilm. We noted a complex and well developed three dimensional structure controls at 48 and 72 h of incubation. This suggest that differences in BA% between test samples and control at 48 and 72 were probably underestimated by bi-dimensional image analysis. Other real three-dimensional imaging techniques, such as confocal laser microscopy, should be considered to provide data for a more exhaustive evaluation of the biofilm volume [26].

As many biosurfactants have been reported to show antifungal activity [16, 17], the effect of CV8LAC BS on *C. albicans* 40 planktonic cells and pre-formed biofilm was also evaluated. Interestingly, no antifungal activity was observed in both experiments at concentrations up to 2000 µg ml^{-1}, suggesting that CV8LAC BS inhibited pathogen adhesion without affecting cell viability in planktonic and sessile form. These findings are in agreement with data reported by Velraeds *et al.* [19] and were supported also by the comparative ultra-structural analysis performed by SEM on microorganisms grown on test and control samples. No phenotypical differences were indeed found in blastoconidia, hyphal morphology and budding locations.

Surprisingly, a raising number of *C. albicans* 40 planktonic cells was observed when the concentration of CV8LAC BS increased. Possibly, the non-purified

extract of biosurfactant might contain other compounds used by the microorganism as a source of nutrients.

The decreased activity of CV8LAC BS on biofilm formation in pre-coating assays compared to co-incubation assays may be due to the strategy chosen to coat the silicone surface, as this was simply done by physical absorption. The biosurfactant molecules are attached to the surface by weak bounds (hydrogen bounds, Van der Walls forces) and not by covalent bounds. Hence, during the washing steps involved in the experimental setting, the biosurfactant might have been gradually removed from silicone, leading to activity loss. Physical or chemical surface modification will be tested in the future to bind more effectively the biosurfactant to the silicone and to improve and prolong anti-biofilm properties.

Pinto *et al*. [34] have recently modified a Poly(dimethyl siloxane) surface by argon plasma treatment followed by the coating with different biosurfactants isolated from probiotic strains, to evaluate how these compounds affect the PDMS surface properties. Tested materials had no toxicity and were found to be non-hemolytic, indicating that the proposed approach may be effective and open new possibilities for the application of these surfaces in the biomedical field.

Considering the wide selection of methods nowadays available for bonding biomolecules onto medical grade polymers, the anti-adhesive properties of the CV8LAC BS deserves a promising role as an anti-adhesive product for medical devices (catheters, prosthesis, stents, etc.) to effectively limit colonization and prevent *C. albicans* infections.

Acknowledgments

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Inhibition of *Candida albicans* adhesion on silicone by a lipopeptide biosurfactant from *Bacillus subtilis* AC7

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Summary

This study investigated the properties and the activity of a lipopeptide biosurfactant produced by *Bacillus subtilis* AC7 against adhesion and biofilm formation of *Candida albicans* on medical-grade silicone elastomeric disks (SEDs). Biosurfactant AC7 is composed mainly of surfactin and of a lower percentage of fengycin, it reduces water surface tension to 30.6 nN m^{-1} and its critical micelle concentration is 31.9 μg ml^{-1}. Co-incubation with 2 mg ml^{-1} biosurfactant significantly reduced adhesion and biofilm formation of three *C. albicans* strains on SEDs in a range of 67–69% after 1.5 h and of 56–57% after 24h of incubation. On pre-coated SEDs, fungal adhesion and biofilm formation were reduced by 57–62% and 46–47%, respectively. Additionally, biosurfactant AC7 did not inhibit growth of *C. albicans* strains in both planktonic and sessile form. This study illustrates that biosurfactant AC7 contrasts significantly the initial deposition of *C. albicans* and slows biofilm growth, suggesting a potential role of the coating for preventing fungal adhesion to silicone medical devices.

Introduction

During the last years, a significant increase in the incidence of human fungal infection has been observed. *Candida* species are the major problem, especially in immunocompromised patients [1-3]. Invasive candidiasis presents a high global mortality rate, ranging from 36% to 63% in different patient groups [4,2], and represents a significant problem in terms of patient management and healthcare costs in the public health system [5].

Of the *Candida* species isolated from humans, *Candida albicans* is the most frequently associated with the formation of biofilms on a wide variety of medical devices [6-9]. The ability of *C. albicans* to form biofilms has a profound impact on its capacity to cause human disease [10]. Biofilms, the most prevalent type of microbial growth in nature, consist in a structured surface-associated cell communities embedded in an extracellular matrix that have distinct phenotypes compared to their planktonic cell counterparts [11]. The presence of biofilm protects the microorganism from host defences and reduces significantly its susceptibility to antifungal agents [12]. Furthermore, the tenancy with which *C. albicans* infects indwelling medical devices necessitates, in almost all the cases, their removal. For these reasons the development of new technologies based on the control of the *Candida sp.* biofilm growth, represents the major breakthrough in the clinical practice and preventive medicine. The search has drawn attention on microbial metabolites, recognized as a major source of compounds endowed with potent biological activities [13]. Among these, 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 [14-17].

Biosurfactants are amphipathic metabolites with both hydrophilic and hydrophobic moieties within the same molecule which allow them to exhibit surface activities at interfaces [18]. In particular, lipopeptides, have steadily gained increased

significance for their potential commercial application in pharmaceutical industries thanks to their promising biological characteristics such as antibacterial, antifungal and anti-adhesive activities [19]. Adsorption of biosurfactants to a substratum surface modifies its hydrophobicity, interfering with microbial adhesion and desorption processes [20]; in that sense, pre-coating catheters and other medical implantable materials with biosurfactants could be used as a preventive strategy to inhibit pathogenic biofilm growth, thus reducing the use of pharmaceuticals and antibiotics [21-23].

In this study, lipopeptide biosurfactant AC7 (AC7 BS) was characterized and its ability to inhibit adhesion and biofilm formation of tree different *C. albicans* strains was evaluated on silicone elastomer at different times of biofilm growth, in physiological conditions, by means of the crystal violet staining and the viable cell counting methods.

Materials and Methods

Strains

The endophytic biosurfactant producing strain AC7 was isolated from the inside of stems of *Robinia pseudoacacia* and was genotypically identified as *Bacillus subtilis*. For biofilm assays, the strain *Candida albicans* IHEM 2894, isolated from human tongue, was purchased from The Belgian Co-ordinated Collections of Microorganisms (BCCM), *Candida albicans* 40 (DSM 29204) and 42 (DSM 29205) are two wild strains, clinically isolated from central venous catheter and urinary catheter, respectively. Strain AC7 was stored at − 80°C in a Luria Bertani (LB) broth (Sigma-Aldrich) supplemented with 25% glycerol and grown on LB plate 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.* [24]. Surface tension of a 0.5 mg ml^{-1} AC7 BS solution 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 mg ml^{-1} to 0.5 mg ml^{-1}). Surface tension of each dilution was determined in triplicate. The CMC was assessed from the intercept of two 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 mg ml^{-1} AC7 BS solution was evaluated by measuring E_{24} 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 solution (0.5 mg ml^{-1}) was adjusted to different pH values (3-11) with 1 M NaOH or 1M HCl. After 1 h, the surface tension and the ability to form emulsion were measured. Measurements were carried out in triplicate.

Chemical characterization of AC7 BS

The chemical characterization of the crude extract was developed according to the method described by Pecci *et al*. [25] and slightly modified. Liquid chromatography-mass spectrometric (LC-ESI-MS) analyses were performed with a Surveyor HPLC coupled on line with a LCQ DECA XP Plus (Thermo Finnigan) Ion Trap mass spectrometer equipped with an ESI source. Separations were performed on the analytical Luna 5 µm C18(2), 150 × 4.6 mm (Phenomenex, Torrance, CA) protected with a C18-Security Guard cartridge, 4 × 3.0 mm (Phenomenex). The mobile phase components were: A = water, 1% formic acid; B = acetonitrile; the lipopeptides were eluted according to the following linear

gradient: A: B (70 : 30) for 4 min, then A : B (0 : 100) over 20 min and then 100% B over 6 min at flow rate of 0.8 ml min^{-1}. The mass spectrometer was operated in positive ESI (electrospray ionization) mode with a spray voltage of 5.30 kV. The capillary temperature was maintained at 350°C and nitrogen was used as nebulizing gas at 30 arbitrary units. Alternatively, liquid chromatography-tandem mass spectrometry (LC/ESI-MS/MS) modalities were applied to the selected precursor ions, following the conditions set during the infusion analysis.

Antifungal susceptibility testing against Candida albicans planktonic cells

The effect of AC7 BS on planktonic cells of *C. albicans* strains was evaluated according to EUCAST guidelines [26]. Briefly, 100 µl of AC7 BS solutions (from 0.06 mg ml^{-1} to 6 mg ml^{-1}) were added in a 96-well microtiter plate (Bioster). *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 RPMI 1640 medium (Sigma-Aldrich) buffered with 3-(N-morpholino)propanesulfonic acid buffer (MOPS) (Sigma-Aldrich) and supplemented with D-glucose (2% final concentration), pH 7.0. One-hundred microliters of inoculum were then added to each well to obtain final concentrations of AC7 BS ranging from 0.03 to 3 mg ml^{-1} in a final volume of 200 µl/well. Finally, the microtiter plate was incubated without agitation at 37°C for 24 h. Triplicates assays were performed for all AC7 BS concentrations used for test. After the incubation, OD_{450} was determined for each well using a Ultramark Microplate Imaging System (Bio-Rad).

Medical-grade silicone elastomeric disks preparation

Two different sizes of medical-grade silicone elastomeric disks (SEDs) (TECNOEXTR S.r.l., Italy) were used in the study: 15 mm in diameter, 1.5 mm in thickness for experiments in 12-well culture tissue plates and 10 mm in diameter, 1.5 mm in thickness for experiments in 24-well culture tissue plates. Each silicone

disk was cleaned, sterilized and conditioned before use, according to the method described by Busscher *et al*. [27]. Briefly, disks were submerged in 200 ml of distilled water supplemented with 1.4% (v/v) of RBS 50, sonicated for 5 min at 60 kHz using Elma S30H (Elmasonic, VWR International) and rinsed in 1 l of MilliQ water for two times. Then, disks were submerged in 20 ml of MeOH (99%) (Sigma-Aldrich), rinsed twice, dried under the hood and steam sterilized for 15 min at 121°C.

Antifungal susceptibility testing of Candida albicans biofilm

C. albicans cells were grown for 24 h at 37°C onto SDA plates. Cells were suspended in PBS with 10% Fetal Bovine Serum (FBS) and standardized to 1×10^7 CFU ml^{-1}. One milliliter of this fungal suspension was added to each well of 24-well plates (Greiner bio-one) containing a silicone disk. After 1.5 h (adhesion phase) in static conditions at 37°C, each disk was placed in 1 ml of YNBD 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 other 24 h at 37°C in static conditions. The antifungal activity of BS was evaluated by the XTT (Sigma-Aldrich) colorimetric assay. Disks were submerged in 1 ml of PBS containing 12.5 µl of XTT solution (1 mg ml^{-1}) and 1 µl of 1 mM menadione solution (Sigma-Aldrich). Plates were incubated at 37°C for 5 h at 90 rpm. Triplicates assays were performed for all AC7 BS concentrations used for the test. From each well, 150 µl were transferred to a 96-well plate and the absorbance was measured at 490 nm.

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

Co-incubation assays

Five hundred microliters of a double concentrated *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× AC7BS 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 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 h and 24 h by crystal violet (CV) staining. The supernatants were discarded and the disks were washed three times with PBS to remove non-adherent cells. Afterwards, disks were dried at 37°C for 2 h and submerged into 2 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. Assays were carried out in triplicate and the experiments were repeated two times. Results were expressed as mean values and standard deviations.

Pre-coating assays

Silicone disks were submerged in 2 ml of AC7BS solution at concentrations ranging from 0.5 to 3 mg ml^{-1} (test groups) or in PBS only (control group) at 37°C for 24 h at 140 rpm. Two milliliters of *C. albicans* suspensions, standardized to 1×10^7 CFU ml^{-1}, were added to each well of 12-well plates containing disks pretreated with AC7 BS (test groups) or PBS (control group). After 1.5 h of incubation (adhesion phase), the disks were transferred into 2 ml of 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 h and 24 h with CV staining as indicated previously.

Furthermore, the anti-adhesion and anti-biofilm activity of AC7 BS pre-coating was evaluated by means of the viable-cell counting method at the concentration of 2 mg ml^{-1}. Silicone disks and *C. albicans* suspensions were prepared as described in the previous experiment. After 1.5 h or 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 included into melted SDA (Sigma-Aldrich) 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.

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 followed by Tukey Honest Significant Difference (HSD) was used to compare optical densities of planktonic cells and pre-formed biofilm at different AC7 BS concentrations for the three *C. albicans* strains.

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 $p < 0.05$. In this case, the R package dupi R was used to estimate log_{10} CFU/disk from colony counts [28].

Results

Critical micelle concentration and stability study of AC7 BS

CMC was evaluated on the AC7 BS crude extract. AC7 BS solution at a concentration of 0.5 mg ml^{-1} reduced the surface tension of alkaline distilled water from 72.4 to 30.6 mN m^{-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}.

AC7 BS (0.5 mg ml^{-1})			
pH	Surface tension (mN m^{-1})		E_{24h} (%)
	Mean	SD[a]	
3.0	49.01	0.35	0
4.0	49.40	0.29	0
5.0	37.53	0.39	0
6.0	28.45	0.10	9.3
7.0	30.50	0.17	60.5
8.0	30.95	0.18	60.5
9.0	30.96	0.14	60.5
10.0	31.13	0.22	60.5
11.0	30.76	0.32	60.5

[a]SD: standard deviation

Table 1. Surface tension and E_{24} of AC7 BS as a function of pH. Surface tension of alkaline distilled water was 72.4 mN m^{-1}.

AC7 BS at a concentration of 0.5 mg ml^{-1} showed an E_{24} of 60%. The E_{24} was not altered by heat/cold treatments. In particular, the extract maintained the emulsification property even after treatment at 100°C for 1 h, at 121°C for 15 min and at – 80°C for 24 h, and the emulsion remained indefinitely stable. Furthermore, the head/cold treatments did not alter the ability of AC7 BS solution to decrease

water surface tension. Studies on the pH stability of AC7 BS demonstrated that it was stable over a wide pH range (Table 1).

The highest surface activity was found between pH 6.0 (28 mN m^{-1}) and pH 11.0 (30.76 mN m^{-1}). The E_{24} of AC7 BS was not altered at pH values from 7 to 11, but it was strongly reduced at pH 6 (9.3%) and absent at pH 3-5.

Chemical characterization of AC7 BS

The LC/ESI-MS analysis of the crude extract showed the presence of the homologues of two lipopeptide families, surfactin and fengycin, respectively. Surfactin family resulted 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. Fengycin family resulted 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% of surfactin and 2% of fengycin.

Antifungal susceptibility testing on Candida albicans planktonic cells and biofilm

AC7 BS susceptibility testing was carried out on *C. albicans* 40, 42, IHEM 2894 planktonic cells and pre-formed biofilms by means of EUCAST and XTT methods, respectively. Optical densities at 450 nm and 490 nm versus the biosurfactant concentration were plotted in Figure 1. ANOVA analysis indicated that both OD_{450} and OD_{490} were not significantly associated to biosurfactant concentrations, showing that no antifungal activity against planktonic cells or biofilms was detected for any of the strains. For biofilm, two-way ANOVA showed that OD_{490} was significantly different for the three strain (p = 0.006371) but not for AC7 BS

concentrations. Tukey's HSD method showed that the strain *C. albicans* IHEM 2894 is a stronger biofilm producer.

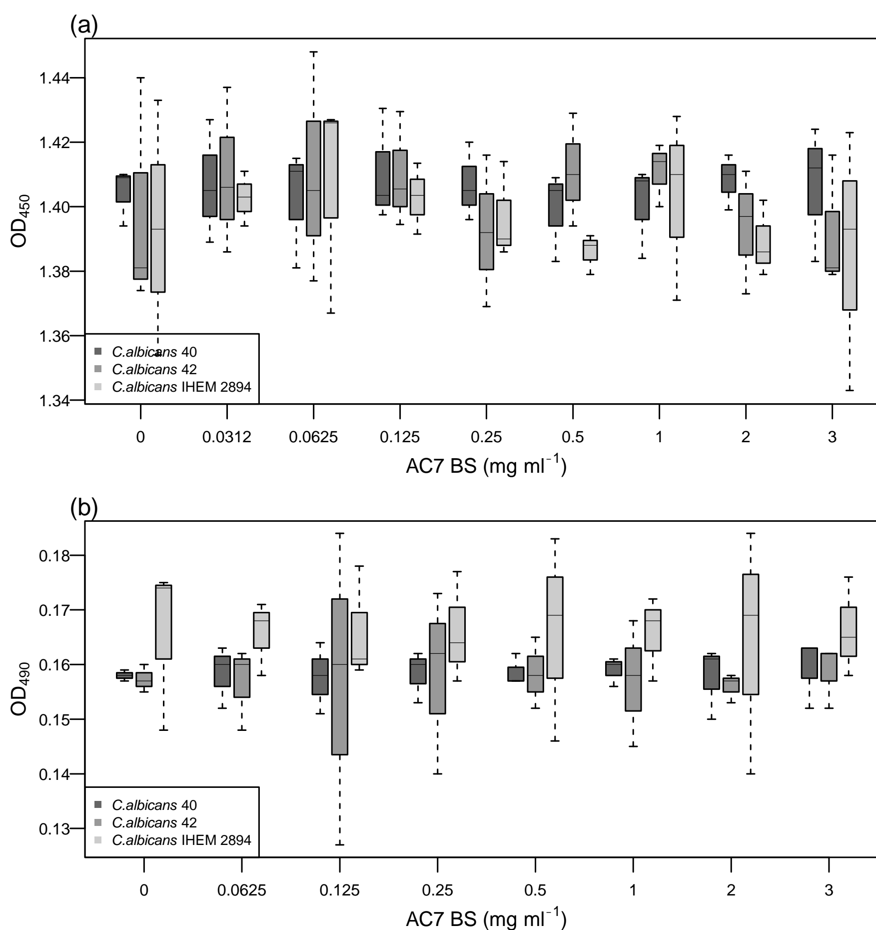

Figure 1. AC7 BS activity at 24 h on (a) *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 planktonic cells and (b) pre-formed biofilm, measured by OD_{450} and OD_{490}, respectively.

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 mg ml^{-1} to 3 mg ml^{-1} in co-incubation and in pre-coating conditions were evaluated by the CV staining method. Figures 2 and 3 show the OD_{570} as a function

of the biosurfactant concentration. The different scales on the y-axes are due to the progressive biofilm formation.

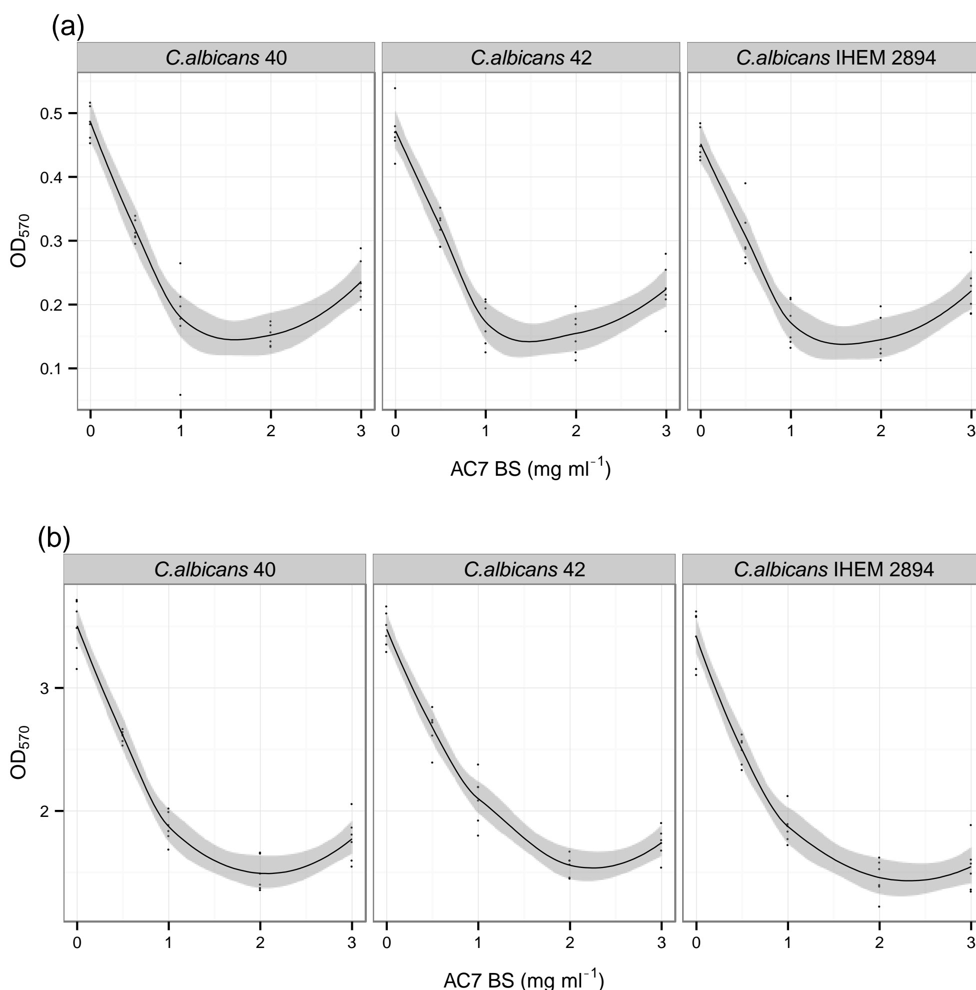

Figure 2. Inhibition of *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 adhesion and biofilm formation on silicone disks by different concentrations of AC7 BS in co-incubation assays, respectively at (a) 1.5 h and (b) 24 h incubation evaluated by means of the CV method. The inhibition of adhesion and biofilm formation is measured by OD_{570}; each scatterplot includes a Loess curve (local regression curve) and a Loess confidence region (95%).

In the co-incubation experiment, the trend is always realized by a U–shaped curve (Figure 2). 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 concentrations, 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. At the highest concentration (3 mg ml^{-1}), *C. albicans* adhesion and biofilm formations 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 1.5h, and between 56% and 57% at 24 h.

Two-way ANOVA shows that at time 1.5 h adhesion is significantly dependent on concentration (but not on the strain) while at time 24 h biofilm formation is significantly dependent on concentration and on the strain. In particular, Tukey's HSD test reveals a significant reduction (both at time 1.5 h and at time 24 h) with a p value adjusted for multiple comparison $< 10^{-9}$.

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 2. Percentages of inhibition of *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 adhesion and biofilm formation in co-incubation and pre-coating experiments at different AC7 BS concentrations.

Pre-coating assays were carried out at first by CV staining using AC7 BS concentrations ranging from 0.5 mg ml^{-1} to 3 mg ml^{-1} (Figure 3). In this case, as well, the trend is always realized by a U–shaped curve. The adhesion and biofilm formation of the three *C. albicans* strains to SEDs were progressively reduced as a function of biosurfactant concentrations, with a minimum reached at the

concentration of 2 mg ml^{-1} at both incubation times. As previously, at the highest concentration (3 mg ml^{-1}), adhesion and biofilm formations 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 1.5h, and between 47% and 50% at 24 h.

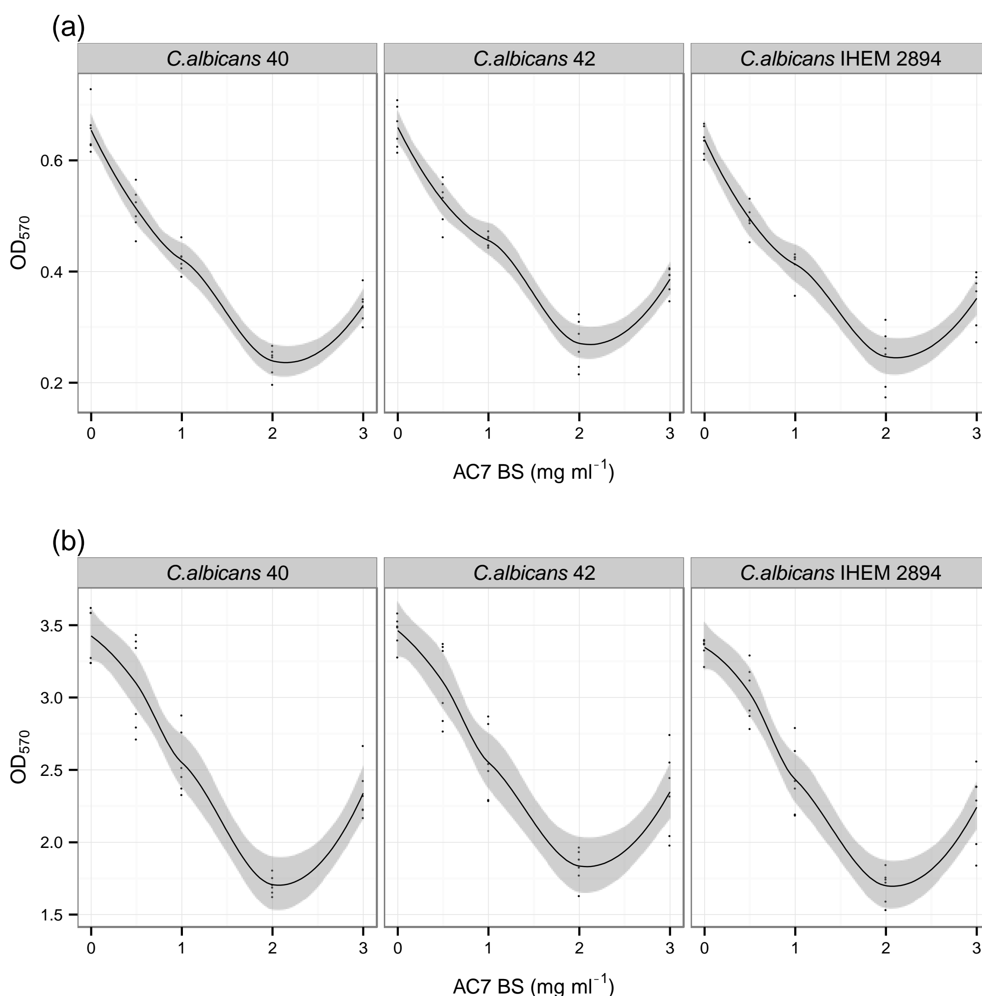

Figure 3. Inhibition of *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 adhesion and biofilm formation on silicone disks by different concentrations of AC7 BS in pre-coating assays, respectively at (a) 1.5 h and (b) 24 h incubation evaluated by means of the CV method. The inhibition of adhesion and biofilm formation is measured by OD_{570}; each scatterplot includes a Loess curve (local regression curve) and a Loess confidence region (95%).

Two-way ANOVA shows that at 1.5 h adhesion is significantly dependent on AC7 BS concentration and also on the strain while at 24 h biofilm formation is significantly dependent on concentration (but not on the strain). In particular, Tukey's HSD test reveals a significant reduction (both at time 1.5 h and at time 24 h.) with a *p* value adjusted for multiple comparison $<10^{-9}$.

Time (h)	Strain	Control (C)		AC7 BS		95% confidence interval	*p*	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

[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 = $(1-10^{\mu})*100$

Table 3. Mean *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 concentrations recovered on silicone disks, 95% confidence interval, *p* values and inhibition measures, in pre-coating assays with AC7 BS (2 mg ml^{-1}).

The effect of SEDs pre-coating with AC7 BS concentration of 2 mg ml^{-1} was further investigated by the viable cell counting method. Table 3 summarizes 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 $(1-10^{\mu})*100$, where μ is the difference in $\log_{10}$ CFU/disk of BS treated and control samples. The number of adherent vital cells on control disks after 1.5 h was 6.66 $\log_{10}$ CFU/disk for *C. albicans* 40, 6.66 $\log_{10}$ CFU/disk for strain 42 and 6.67 $\log_{10}$ CFU/disk for strain IHEM 2894, and increased to 7.63 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.63 $\log_{10}$ CFU/disk for strain 42 and 7.63 $\log_{10}$ CFU/disk for strain IHEM 2894 after 24 h. On AC7 BS pre-

coated disks, the number of adherent cells at 1.5 h was 6.24 $\log_{10}$ CFU/disk for *C. albicans* 40, 6.29 $\log_{10}$ CFU/disk for strain 42 and 6.26 $\log_{10}$ CFU/disk for strain IHEM 2894 and it increased to 7.35 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.36 $\log_{10}$ CFU/disk for strain 42 and 7.35 $\log_{10}$ CFU/disk for strain IHEM 2894 after 24h. 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).

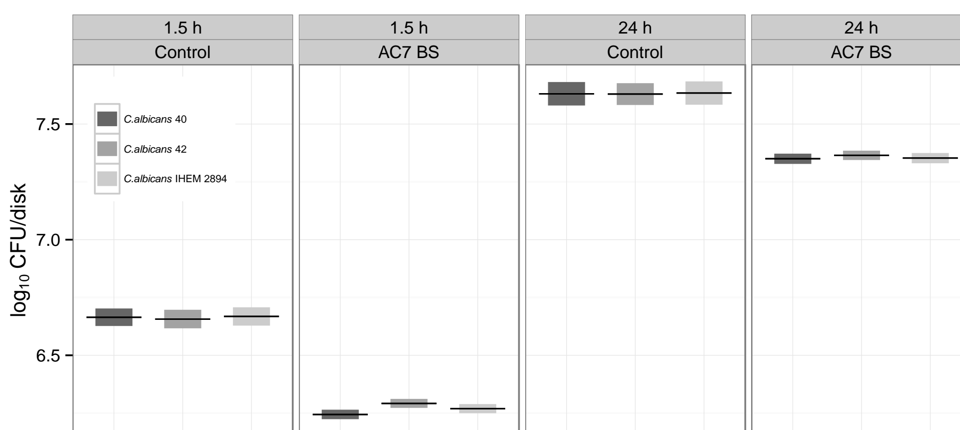

Figure 4. Inhibition of *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 adhesion (1.5 h) and biofilm formation (24 h) on silicone disks by AC7 BS (2 mg ml^{-1}) in pre-coating assays evaluated by means of the viable-cell counting method.

AC7 BS activity against *C. albicans* 40, 42 and IHEM 2894 adhesion and biofilm formation on silicone disks in pre-coating assays is displayed in Figure 4. The comparative boxplots show clearly that fungal adhesion and biofilm formation on treated disks is significantly lower (at both incubation times) than on untreated disks. The difference is more evident at time 1.5 h. To be noted that, at time 1.5 h, fungal counts are very low compared to 24 h as *C. albicans* stains are in the initial phase of adhesion. Two-ways ANOVA confirms that *C. albicans* viable counts are

significantly dependent on the disk treatment (untreated or AC7 BS pre-coated) and on incubation time ($p < 10^{-15}$).

Discussion

Candida albicans is one of the most important nosocomial pathogens frequently involved in implanted device-associated infection [29] and represents a serious public health problem with important medical and economic consequences [30,31]. There is, therefore, the need of biomaterials with antimicrobial-coated surfaces for the inhibition of the microbial adhesion and the eradication of biofilms. To obtain surfaces with permanent antimicrobial properties, polymeric surfaces have been modified with functional coating layers via direct covalent coupling of different type of molecules [32]. Main drawbacks of antimicrobial coatings arise from time limited effectiveness and potential toxicity towards human cells [33,34].

In this context, biosurfactants have recently emerged as a potential new generation of anti-adhesive and antimicrobial agents with enhanced biocompatibility [14]. Among biosurfactants, lipopeptides form the most widely reported class with antimicrobial activity due to their ability to disrupt phospholipidic membranes and to affect cell to surface interactions by decreasing hydrophobicity and, thus, interfering with cell deposition processes and microbial adhesion [21].

In this study, the endophytic strain *Bacillus subtilis* AC7 was found to be a good biosurfactant and bioemulsifier producer. Surface tension, CMC and emulsification capacity of AC7 BS were comparable to those observed for other lipopeptide biosurfactants [35,36]. Moreover, AC7 BS showed a high stability over a wide range of temperatures and pH, similarly to what observed by Ghojavand et al. [37] for the biosurfactant produced by *B. subtilis* PTCC 1696 and by Kim et al. [38] for the lipopeptide biosurfactant from *B. subtilis* C9. Chemical analysis of the crude extract revealed the presence of surfactin and fengycin, as found for other lipopeptide biosurfactants [24,25,39,40].

As lipopeptide biosurfactants have been often reported to show antifungal activity [41-44], the effect of AC7 BS on *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 planktonic cells and pre-formed biofilms was evaluated. Interestingly, no antifungal activity was observed, in both experimental conditions, at concentrations up to 3 mg ml^{-1}, suggesting that AC7 BS inhibited pathogen adhesion without affecting cell growth. Similarly, biosurfactants from *Lactobacillus brevis* CV8LAC [45] and from *Bacillus licheniformis* V9T14 [46] were reported to have anti-biofilm but not antimicrobial activity.

The anti-adhesion and anti-biofilm activity of AC7 BS against the three different *C. albicans* strains was evaluated on medical-grade silicone, at physiological conditions (37 °C), in the presence of a proteinaceous solution, i.e. fetal bovine serum, to mimic blood contact during clinical use in internal body conditions.

The efficacy of AC7 BS, at different concentrations, to inhibit *C. albicans* biofilm formation on SEDs was first evaluated in co-incubation experiments, and subsequently after its absorption on the surface of silicone disks, in order to imitate a functional coating condition. AC7 BS treatment resulted in a significant reduction of the total adherent cells and biofilm biomass compared to controls for all the 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 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 CV and the viable cell counting based 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 *C. albicans* adhesion phase, whereas during the biofilm formation phase, the inhibition was lower but still significant.

Janek *et al.* (2012) demonstrated that a cyclic lipopeptide, named Pseudofactin II, lowered the adhesion of two *C. albicans* strains on different types of surfaces. 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 at a lower extent (29-39%). In addition, both the pretreatment of silicone urethral catheters with pseudofactin II and the inclusion of the biosurfactant in the growth medium caused an efficient reduction of *C. albicans* biofilm growth. Very recently, Rautela et al. [44] evaluated the influence of lipopeptides from *Bacillus amyloliquefaciens* strain AR2 on *Candida 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.

In this study, the highest percentages of inhibition of *C. albicans* adhesion and biofilm formation on silicone were observed in the AC7 BS co-incubation assay. The lower activity of AC7 BS in pre-coating assays may be due to the strategy chosen to coat the silicone surface. By a physical absorption strategy, biosurfactant molecules are not covalently bound to surfaces but simply attached to the surface with hydrogen bounds and Van der Walls forces. Hence, during the transferring steps involved in the experimental setting, the biosurfactant film might have been gradually removed from silicone, leading to activity loss. Physical or chemical surface modification will be tested in the future to bind more effectively the biosurfactant to the silicone and to improve and prolong anti-biofilm properties.

Pinto et al. (2011) have recently modified a Poly(dimethyl siloxane) surface by argon plasma treatment followed by the coating with different biosurfactants

isolated from probiotic strains, to evaluate how these compounds affect the PDMS surface properties. Tested materials had no toxicity and were found to be non-hemolytic, indicating that the proposed approach may open new possibilities for the application of these surfaces in the biomedical field.
In conclusion, the lipopeptide AC7 BS, thanks to its anti-adhesive properties, could represent a potential candidate to effectively limit colonization of medical devices and prevent *C. albicans* infections.

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Inhibition of *Candida albicans* biofilm formation by lipopeptide biosurfactant AC7 and farnesol

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Unpublished results

Summary

The aim of the present study was to investigate the ability of AC7 lipopeptide biosurfactant (AC7 BS), alone or in combination with farnesol, to ihnibit *Candida albicans* biofilm formation on medical-grade silicone elastomeric disks in simulated physiological conditions. AC7BS significantly reduced the adhesion of three *C. albicans* strains up to 42% at 1.5 h and biofilm formation up to 35% and 29% at 24 h and 48 h, respectively. Farnesol significantly reduced adhesion up to 46% at 1.5 h and biofilm formation up to 81% and 28% at 24 h and 48 h, respectively. AC7BS in combination with farnesol significantly reduced adhesion up to 74% at 1.5 h and biofilm formation up to 93% and 60% at 24 h and 48 h, respectively. SEM and CLSM confirmed cultural results. In particular, SEM showed a significant reduction of biofilm covered surface.

Lipopeptide biosurfactant AC7 is able to reduce significantly *C. albicans* cell adhesion and biofilm formation on medical grade silicone disks. Moreover, when AC7 BS is used in combination with farnesol a synergistic effect is obtained. These results suggest that AC7 BS, thanks to its ability to change surface characteristics, alone or in combination with quorum-sensing molecules, can be considered a potential inhibitor of biofilm growth on medical insertional materials.

Introduction

Candida albicans is part of the normal human flora, and it grows on mucosal surfaces in healthy individuals. In susceptible hosts, this organism causes approximately 50% of cases of candidemia and at least 80% of cases of oropharyngeal and vulvovaginal candidiasis [1]. Nowadays *C. albicans* is the fungus most frequently associated with the formation of biofilms on a wide variety of medical devices (such as venous or urinary catheters, endotracheal tubes, dental prostheses, and other indwelling devices) [2]. This ability has a profound impact on *C. albicans* capacity to cause human disease [3]. Development of new technologies based on the control of the *Candida* spp. biofilm growth is foreseen as a major breakthrough in the clinical practice and preventive medicine. *In vitro*, experiments showed that formation of *C. albicans* biofilm is a complex process involving multiple regulatory mechanisms. Initially, yeast cells adhere to a surface and form microcolonies. Afterwards, these cells form filaments and produce an extracellular matrix until a mature biofilm with a real three-dimensional structure is formed [3,4]. The presence of biofilm may protect the microorganism from host defenses, as well as significantly reduce its susceptibility to antifungal agents [5].

It has been demonstrated that quorum-sensing plays an important role in *C. albicans* biofilm development [6]. *C. albicans* planktonic cells secrete an autoregulatory sesquiterpene, named E,E-Farnesol which acts as a quorum-sensing molecule (QSM) [7]. A lot of biofilm development stages are influenced by this

QSM, such as the attachment of cells to the substratum, the biofilm architecture and the dispersal of cells from biofilm. In particular, biofilm formation is limited by farnesol which inhibits hyphal growth and the expression of morphology-specific genes, regulating, yeast-to-mycelium conversion thus leading to a decrease of biofilm size [8]. However, the use of farnesol is not definitive in avoiding fungal adhesion and biofilm development on device surfaces, demanding for new or integrative approaches in prevention and treatment of *Candida* biofilm formation.

Recent studies have drawn attention to bacterial antagonistic products [9]. Among these, biosurfactants have gained importance thanks to their interesting biological properties, such as antibacterial, antifungal and anti-adhesive activities [10-14]. Biosurfactants are amphipathic compounds, with both hydrophilic and hydrophobic moieties present within the same molecule which allow them to exhibit surface activities at interfaces [15]. Numerous investigations have lead to the discovery of many interesting chemical and biological properties of biosurfactants and several pharmaceutical and medical applications have been envisaged [15-17]. In particular, their ability to destabilize membranes by disturbing their integrity and permeability leading to metabolite leakage and cell lysis [18-24], as well as their propensity to partition at the interfaces, modifying surface properties and thus affecting microorganisms adhesion, are important functions for antimicrobial and antibiofilm applications [25].

Aim of the present study was to investigate the ability of a lipopeptide biosurfactant AC7 (AC7 BS) in combination with farnesol to inhibit different stages of *Candida albicans* biofilm development on medical-grade silicone elastomeric disks, at physiological conditions, by means of the viable cell counting method. Futhermore, adherent cells and biofilm were visualized and quantified by scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM).

Materials and Methods

Strains

The endophytic biosurfactant producing strain AC7 was isolated from the inside of stems of *Robinia pseudoacacia* and was genotypically identified as *Bacillus subtilis*. For biofilm assays, the strain *Candida albicans* IHEM 2894, isolated from human tongue, was purchased from The Belgian Co-ordinated Collections of Microorganisms (BCCM), *Candida albicans* 40 (DSM 29204) and 42 (DSM 29205) are two wild strains, clinically isolated from central venous catheter and urinary catheter, respectively. Strain AC7 was stored at -80°C in a Luria Bertani (LB) broth (Sigma-Aldrich) supplemented with 25% glycerol and grown on LB plate 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.

Biosurfactant production and extraction

For biosurfactant (BS) production, a seed culture was prepared by transferring a loop of *B. subtilis* AC7 strain from a LB agar overnight culture into 20 ml of LB broth and incubated at 28°C for 4 h at 140 rpm. Thereafter, 2 ml were inoculated in 500 ml of the same broth in a 2 l flask and incubated again at 28°C for 24 h at 140 rpm. The culture broth was then centrifuged at 6000 × g for 20 min and the supernatant collected. For BS extraction, the supernatant was acidified to pH 2 with 6N HCl, stored overnight at 4 °C and extracted three times with ethyl acetate : methanol (4 : 1) (Sigma-Aldrich) according to the method described by Rivardo et al. [11]. The remaining water in the organic phase was removed by anhydrous sodium sulfate. The organic phase was evaporated to dryness under vacuum condition and acetone was added to recover raw AC7 BS. Acetone was, then, evaporated and AC7 BS collected and weighted.

Medical-grade silicone elastomeric disks preparation

In this study medical-grade silicone elastomeric disks (SEDs) (TECNOEXTR S.r.l., Italy) with 15 mm in diameter, 1.5 mm in thickness were used for experiments in 12-well culture tissue plates. Each silicone disk was cleaned, sterilized and conditioned before use according to the method described by Busscher et al. [26]. Briefly, disks were submerged in 200 ml of distilled water supplemented with 1.4 % (v/v) of RBS 50, sonicated for 5 min at 60 kHz using Elma S30H (Elmasonic, VWR International) and rinsed in 1 l of MilliQ water for two times. Then, disks were submerged in 20 ml of MeOH (99 %) (Sigma-Aldrich), rinsed twice, dried under a sterile hood and steam sterilized for 15 min at 121°C. Before use, each silicone disk was aseptically transferred into 2 ml of Fetal Bovine Serum (FBS) (Sigma-Aldrich), incubated at 37°C for 24 h at 140 rpm and rinsed with PBS to provide for a serum protein coating simulating blood contact during clinical use.

Anti-adhesion and anti-biofilm assays

Silicone disks were submerged in 2 ml of AC7 BS solution at the concentration of 2 mg ml^{-1} (AC7 BS test group) or in PBS only (control group) at 37°C for 24 h at 140 rpm. *C. albicans* cells were grown for 24 h at 37°C in 25 ml of YNBD at 140 rpm. After centrifugation and two washings with PBS, the pellet was resuspended in the same buffer and standardized to 1×10^6 CFU ml^{-1}. Farnesol (Sigma Aldrich) was diluted in pure methanol to obtain a 50 mM working stock solution. Farnesol was added to the standardized suspension of *C. albicans* and to the growth medium to test a final concentration of 100 μM. Two milliliters of fungal suspension were added to each well of a 12-well plate containing silicone disks. After 1.5 h of adhesion phase, the disks were transferred in new plates containing 2 ml of YNBD and incubated at 37°C with gentle shaking for 24 h. Furthermore, the effect of farnesol was also evaluated on preformed biofilms. *C. albicans* biofilms were

formed on AC7 BS test group and control group disks (6 disks per group) for 24 h at 37°C by using the protocol described above. Following biofilm formation, three disks per group were added with farnesol at the final concentration of 100 µM and then incubated for additional 24 h at 37°C.

Assays were carried out in triplicate by means of the viable cell counting method (see following paragraph) and experiments were repeated two times. Results were expressed as mean $\log_{10}$ CFU/disk .

Viable cell counting

The effect of AC7 BS, farnesol and AC7 BS + farnesol on *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 adhesion and biofilm formation was evaluated by the viable cell counting method. After 1.5, 24 and 48 h incubation, disks were washed three times with PBS to remove non-adherent cells, inserted into 50 ml tubes containing 10 ml PBS and subjected to four cycles of sonication (30 s) and stirring (30 s). The disrupted biofilm cells were serially diluted in PBS and 1 ml of each dilution was included into melted SDA (Sigma-Aldrich) using the pour-plate method. Agar plates were incubated for 24 h at 37°C and colonies were then enumerated. Results were expressed as $\log_{10}$ CFU/disk.

Scanning Electron Microscopy analyses

A second set of samples, equivalent to that realized for the viable cell count, was prepared to perform a qualitative and quantitative analysis of *C. albicans* IHEM 2894 biofilm formation by scanning electron microscopy (SEM). At the end of incubation times, each disk was washed three times in PBS to remove the culture medium and the non-adherent cells from the silicone substrate, fixed by immersion in aldehydic solution (2.5% glutaraldehyde in 0.1mM phosphate buffer) for 24 h at 4°C, washed twice in distilled water, dehydrated by immersion in ascending alcohol solutions (70%, 90% and 100% ethanol, 10 min each) and dried overnight

under a laminar flow cabinet. Finally, dried samples were glued to SEM sample holder by double bonding carbon tape and gold sputtered.

SEM analyses were conducted in a XL30 ESEM FEG (Fei-Eindhoven, The Netherland) scanning electron microscope at a 10 KV beam voltage. A preliminary qualitative investigation of the *Candida* biofilm was performed at a magnification ranging from 100× to 10000×. A representative selection of images at 1000× was stored by collecting the secondary electrons signal revealing fine morphological details of cells and extracellular matrix.

For quantitative purposes, two sets of images were also acquired per each sample. A first set of nine different fields of view was obtained by collecting the backscattered electrons signal at a magnification of 40×, thus guaranteeing the observation of a total area of 64 mm^2 at the silicone disk centre. To distinguish between biofilm covered surface and exposed silicone, compositional contrast was stressed and images were processed according to a protocol adapted from Bressan et al. [27]. Briefly, high resolution digital images (1936×1452 pixels) were acquired and then threshold by semi-automated routine implemented in ImageJ (NIH, US). The number of dark pixels associated to Candida biofilm and bright pixels representing the silicone surface was obtained. Percent area of the silicone disk covered by *Candida* biofilm (BA%) was computed calculating the percent ratio of dark pixels over the whole pixels number of the image.

Confocal Laser Scanning Microscopy analyses

A third equivalent set of samples was also realized for the analysis of *C. albicans* IHEM 2894 biofilm by Confocal Laser Scanning Microscopy (CLSM). After incubation, each disk was washed three times in PBS to remove the culture medium and non-adherent cells from the silicone substrate and incubated for 30 min at 37°C in 2 ml of staining solution composed by 2 µl of 10 mM FUN-1 solution (Life Technologies) and 10 µl of Concavalin A (CON-A, Life

Technologies) 5 mg ml^{-1} solution in PBS. Observations were performed with an inverted confocal microscope (Nikon A1, Nikon Corporation, Japan) in wet conditions with the sample positioned upside down, to avoid the opaque silicone disk interference. Samples were scanned using 488 nm and 525 nm excitation wavelengths and collecting emissions at 525/25 nm and 650/100 nm respectively.

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 followed by Tukey's HSD test was performed to investigate the effect of AC7 BS alone, farnesol alone or AC7 BS + farnesol on the three *C. albicans* stains adhesion and biofilm formation in assays carried out by means of the viable cell counting method. The R package dupiR was used to estimate log_{10} CFU/disk from colony counts [28]. Results were considered to be statistically significant when $P<0.05$.

Results and discussion

Anti-biofilm activity of AC7 BS and farnesol

The activity of AC7 BS (2 mg ml^{-1}) and farnesol (100 μM) was evaluated on different stages of *C. albicans* biofilm development on medical-grade silicone, at physiological conditions (37 °C), in the presence of a proteinaceous solution, i.e. fetal bovine serum, to mimic blood contact during clinical use in internal body conditions. Figure 1 show the activity of farnesol alone, AC7 alone and of the combination of AC7 BS with farnesol against *C. albicans* biofilm formation on silicone disks in pre-coating assays at different incubation times. Panels show that fungal adhesion and biofilm formation on AC7 BS, farnesol and AC7 BS +

farnesol treated disks is lower than on untreated disks at all incubation times. According to two-way ANOVA *Candida albicans* biofilm development is significantly dependent on the type of treatment, incubation time and on the type of strain ($P<10^{-5}$).

Table 1 summarizes the results obtained for the three *C. albicans* strains expressed as means $\log_{10}$ CFU/disk and the 95% confidence intervals.

Time (h)	Strain	Control		Farnesol		AC7 BS		AC7 BS + farnesol	
		Mean	95% confidence interval	Mean	95% confidence interval	Mean	95% confidence interval	Mean	95% confidence interval
1.5	*C. albicans* 40	5.63	[5.57, 5.68]	5.40	[5.38, 5.42]	5.41	[5.39, 5.43]	5.04	[5.01, 5.07
	C. albicans 42	5.63	[5.58, 5.68]	5.41	[5.39, 5.44]	5.42	[5.40, 5.44]	5.08	[5.05, 5.11]
	C. albicans IHEM 2894	5.61	[5.56, 5.67]	5.34	[5.32, 5.37]	5.37	[5.35, 5.40]	4.99	[4.96, 5.03]
24	*C. albicans* 40	7.72	[7.67, 7.76]	7.10	[7.07, 7.13]	7.55	[7.49, 7.61]	6.93	[6.89, 6.97]
	C. albicans 42	7.64	[7.58, 7.69]	7.05	[7.02, 7.09]	7.48	[7.42, 7.55]	6.84	[6.80, 6.88]
	C. albicans IHEM 2894	7.77	[7.72, 7.81]	7.06	[7.03, 7.10]	7.59	[7.53, 7.64]	6.63	[6.57, 6.68]
48	*C. albicans 40*	8.13	[8.10, 8.16]	8.03	[8.00, 8.07]	8.01	[7.97, 8.04]	7.83	[7.78, 7.87]
	C. albicans 42	7.98	[7.95, 8.02]	7.89	[7.85, 7.93]	7.87	[7.83, 7.91]	7.71	[7.66, 7.76]
	C. albicans IHEM 2894	8.10	[8.07, 8.13]	7.98	[7.94, 8.02]	7.95	[7.91, 7.99]	7.70	[7.66, 7.75]

Table 1. Mean *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 concentrations, expressed as $\log_{10}$ CFU/disk, recovered on silicone disks and 95% confidence intervals, in anti-adhesion (1.5 h) and anti-biofilm (24 and 48 h) assays.

The number of adherent cells on control disks after 1.5 h was 5.63 $\log_{10}$ CFU/disk for *C. albicans* 40, 5.63 $\log_{10}$ CFU/disk for strain 42 and 5.61 $\log_{10}$ CFU/disk for strain IHEM 2894, it increased to 7.72 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.64 $\log_{10}$ CFU/disk for strain 42 and 7.77 $\log_{10}$ CFU/disk for strain IHEM 2894 after 24 h and to 8.13 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.98 $\log_{10}$ CFU/disk for strain 42 and 8.10 $\log_{10}$ CFU/disk for strain IHEM 2894 after 48 h.

The number of adherent cells on disks treated with farnesol after 1.5 h was 5.40 $\log_{10}$ CFU/disk for *C. albicans* 40, 5.41 $\log_{10}$ CFU/disk for strain 42 and 5.34 $\log_{10}$ CFU/disk for strain IHEM 2894, it increased to 7.10 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.05 $\log_{10}$ CFU/disk for strain 42 and 7.06 $\log_{10}$ CFU/disk for strain IHEM 2894 after 24 h and to 8.03 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.89 $\log_{10}$ CFU/disk for strain 42 and 7.98 $\log_{10}$ CFU/disk for strain IHEM 2894 after 48 h.

The number of adherent cells on AC7BS pre-coated disks after 1.5 h was 5.41 $\log_{10}$ CFU/disk for *C. albicans* 40, 5.42 $\log_{10}$ CFU/disk for strain 42 and 5.37 $\log_{10}$ CFU/disk for strain IHEM 2894, it increased to 7.55 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.48 $\log_{10}$ CFU/disk for strain 42 and 7.59 $\log_{10}$ CFU/disk for strain IHEM 2894 after 24 h and to 8.01 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.87 $\log_{10}$ CFU/disk for strain 42 and 7.95 $\log_{10}$ CFU/disk for strain IHEM 2894 after 48 h.

The number of adherent cells on AC7 BS pre-coated disks treated with farnesol after 1.5 h was 5.04 $\log_{10}$ CFU/disk for *C. albicans* 40, 5.08 $\log_{10}$ CFU/disk for strain 42 and 4.99 $\log_{10}$ CFU/disk for strain IHEM 2894, it increased to 6.93 $\log_{10}$ CFU/disk for *C. albicans* 40, 6.84 $\log_{10}$ CFU/disk for strain 42 and 6.63 $\log_{10}$ CFU/disk for strain IHEM 2894 after 24 h and to 7.83 $\log_{10}$ CFU/disk for *C. albicans* 40, 7.71 $\log_{10}$ CFU/disk for strain 42 and 7.70 $\log_{10}$ CFU/disk for strain IHEM 2894 after 48 h.

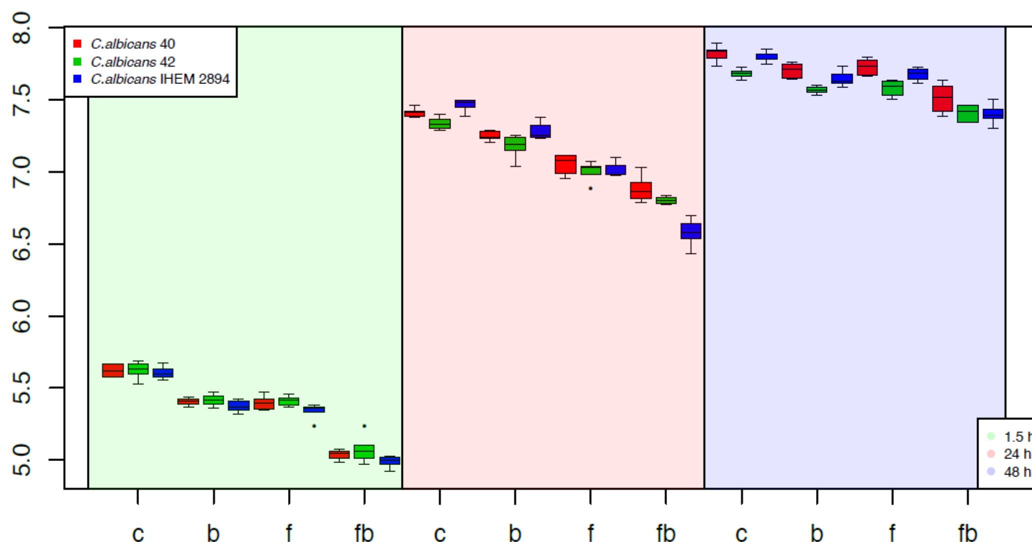

Figure 1. Inhibition of *C. albicans* adhesion and biofilm formation on silicone elastomeric disks, evaluated at 1.5, 24 and 48 h. c: control; b: AC7 BS, f: farnesol; fb: AC7BS + farnesol.

Mean percentages of reduction are reported in Table 2. AC7 BS significantly reduced *C. albicans* strains adhesion in a range between 38.5% and 42% and

biofilm formation in a range between 30.3% and 34.8% respectively. Furthermore, it reduced pre-formed biofilms in a range between 22.9% and 29.1%.

Farnesol significantly reduced *C. albicans* strains adhesion and biofilm formation in respect to control, showing a reduction of viable cell counts in treated samples ranging from 39% to 46.2% after 1.5 h and from 74.1% to 80.4% after 24h, respectively. Furthermore, farnesol reduced pre-formed biofilms in a range between 19.6% and 23.8%.

AC7 BS in combination with farnesol significantly reduced the adhesion of the three *C. albicans* strains in a range between 72.1% and 75.9% and biofilm formation in a range between 83.8% and 92.9%, respectively. Furthermore, the two molecules together reduced pre-formed biofilms in a range between 46.6% and 59.8%.

In general, AC7 BS treatment resulted in a significant reduction of the total adherent cells and biofilm biomass compared to controls for all the three *C. albicans* strains, as evaluated by the viable cell counting method. The highest performance of AC7 BS was observed during *C. albicans* adhesion phase ($P<0.001$), whereas during the biofilm formation phase the inhibition was lower but still significant ($P<0.05$, at least). Previous results obtained in the Laboratory of Microbiology revealed that AC7 BS has no antifungal activity at 2 mg ml^{-1} against planktonic and sessile *C. albicans* cells, suggesting that AC7 BS inhibited pathogen

Strain	1.5 h			24 h			48 h		
	Farnesol	AC7 BS	AC7 BS + farnesol	Farnesol	AC7 BS	AC7 BS + farnesol	Farnesol	AC7 BS	AC7 BS + farnesol
C.albicans 40	40.9	39.3	74.0	75.9	31.7	83.8	19.6	23.9	50.9
C.albicans 42	39.0	38.5	72.1	74.1	30.3	83.9	20.5	22.9	46.6
C.albicans IHEM 2894	46.2	42.0	75.9	80.4	34.8	92.9	23.8	29.1	59.8

Table 2. Percentages of inhibition of *C. albicans* 40, *C. albicans* 42 and *C. albicans* IHEM 2894 adhesion and biofilm formation in respect to untreated controls.

adhesion without affecting cell growth.
BS AC7 was attached to the silicone elastomeric disks by a physical absortion strategy, in a non-covalent manner. It is therefore possible that biosurfactant film might have been gradually removed from the surfaces during the transferring step involved in esperimental setting, leading to a loss of activity.
Recently, Pinto et al. [29] have modified a Poly(dimethyl siloxane) surface by argon plasma treatment followed by the coating with different biosurfactants isolated from probiotic strains, to evaluate how these compounds affect the PDMS surface properties. Tested materials had no toxicity and were found to be non-hemolytic, indicating that the proposed approach may open new possibilities for the application of these surfaces in the biomedical field.
Starting from these encouraging results, AC7 BS will be covalently bound to silicone by physical or chemical surface modification to improve and prolong its anti-adhesive and anti-biofilm properties.
Farnesol showed the highest inhibitory effect at 24 h, during the biofilm formation phase ($P<0.001$). A lower effect was observed when farnesol was evaluated during the adhesion phase ($P<0.001$) or when it was added to pre-formed biofilms ($P<0.01$, at least). Similar results were obtained by Jabra-Rizk et al. and Ramage et al. [6,30]. In both studies, the addition of farnesol at the concentration of 100-300 μM to the initial fungal suspension resulted in a higher reduction of biofilm formation whereas a lower effect, although still significant, was revealed on pre-formed biofilm. The reduced effect of farnesol on pre-formed biofilm could be related to the ability of this molecule to inhibit mycelial development in newly produced cells without affecting the already formed biofilm [30]. Ramage et al. [6] noted that the initial adhesion phase, prior to the addition of farnesol, was important in terms of the ability of farnesol to inhibit biofilm formation. Adherent cell populations that began to germinate naturally were not inhibited by a subsequent addition of farnesol.

The effect of AC7 BS and farnesol was found to be similar during the adhesion phase and on pre-formed biofilm (P>0.05). On the contrary, at 24 h the two compounds were found to perform differently (P<0.001).

To our knowledge, it is the first time that the possible synergistic effect of farnesol and biosurfactant is studied.

AC7 BS in combination with farnesol significantly reduced *C. albicans* adhesion (P<0.001) and biofilm formation (P<0.001). Furthermore, the two compounds together significantly reduced pre-formed biofilm (P<0.001). In addition, the effect obtained by the combination of the two compounds differed significantly from those observed with AC7 BS and farnesol alone.

SEM and CLSM analyses

Qualitative analysis of *C. albicans* IHEM 2894 biofilm microstructure on high magnification SEM images and CLSM images evidenced a less compact structure of the biofilm between samples treated with AC7BS alone, farnesol alone, AC7BS + farnesol and controls. From a comparative evaluation of the fluorescence from FUN-1 (addressing membrane integrity and metabolic capability) and Con-A (addressing cell membrane) it is possible to assess that, for all the samples, the biofilm is characterized by a high vitality

Moreover, a complex three dimensional structure with long hyphae arranged in a multilayered network was found in controls at 24 and 48h of incubation. Differently, treated samples did not developed a thick hyphal network at the tested conditions. No phenotipical differences were found between microorganisms growth on test or control samples showing, in both cases, ovoid spherical blastoconidia with polarly or randomly located budding and true hyphae formation. Difference in the amount of extracellular material was also documented.

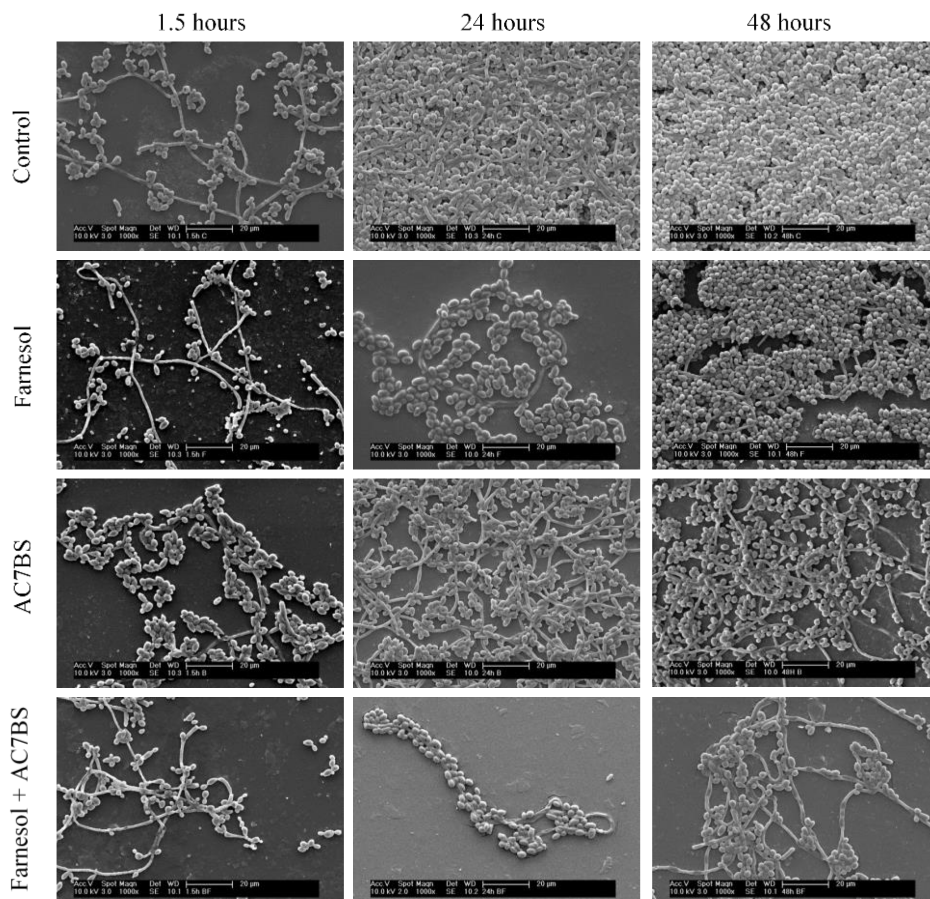

Figure 2. Scanning electron micrographs of silicone elastomeric disks after *C. albicans* IHEM 2894 adhesion phase (1.5 h), biofilm formation (24 h) and pre-formed biofilm (48 h). Original magnification 1000×.

The quantification of the silicone disk area covered by *C. albicans* IHEM 2894 biofilm (BA%) on SEDs showed a similar trend. Differences in BA% between samples treated with AC7BS alone, farnesol alone and AC7BS + farnesol and controls were observed (Table 3). After 1.5h of incubation, 24% of the control disks surface was covered by *C. albicans* cells whereas, only 11.2% of the farnesol treated disks, 11.5% of the BS treated disks, and 8.6%. of AC7 BS + farnesol treated disks. During the biofilm formation phase, control disks were almost totally covered (97%) by *C. albicans* IHEM 2894 biofilm both at 24 and and at 48h,

whereas disks treated with AC7BS showed a BA% of 67% and 39%, disks treated with farnesol of 13% and 68%, disks treated with AC7 BS + farnesol of 7% and 25%, respectively at 24 and 48h.

Time (h)	Biofilm covered surface (%)							
	Control		Farnesol		AC7 BS		AC7 BS + farnesol	
	Mean	SD*	Mean	SD*	Mean	SD*	Mean	SD*
1.5	24	4	11.2	2	11.5	3	8.6	2
24	97	1	13	17	67	10	7	3
48	97	1	68	21	39	36	25	4

Table 3. Percentages of *C. albicans* IHEM 2894 biofilm covered surface evaluated by SEM and ImageJ (NIH, U.S.) on silicone elastomeric disks.

A preliminary evaluation of the film thickness, educible from the multi-stack images acquired from confocal microscope, evidences that after 24 hours the AC7BS + farnesol treated samples are capable of a significant reduction in the biofilm thickness (Table 4). Both farnesol and AC7BS alone have a similar behavior with respect to the control sample, while they seem to express a detectable capacity in controlling the biofilm thickness only after 48 hours.

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

Table 4. Mean *C. albicans* IHEM 2894 biofilm thickness.

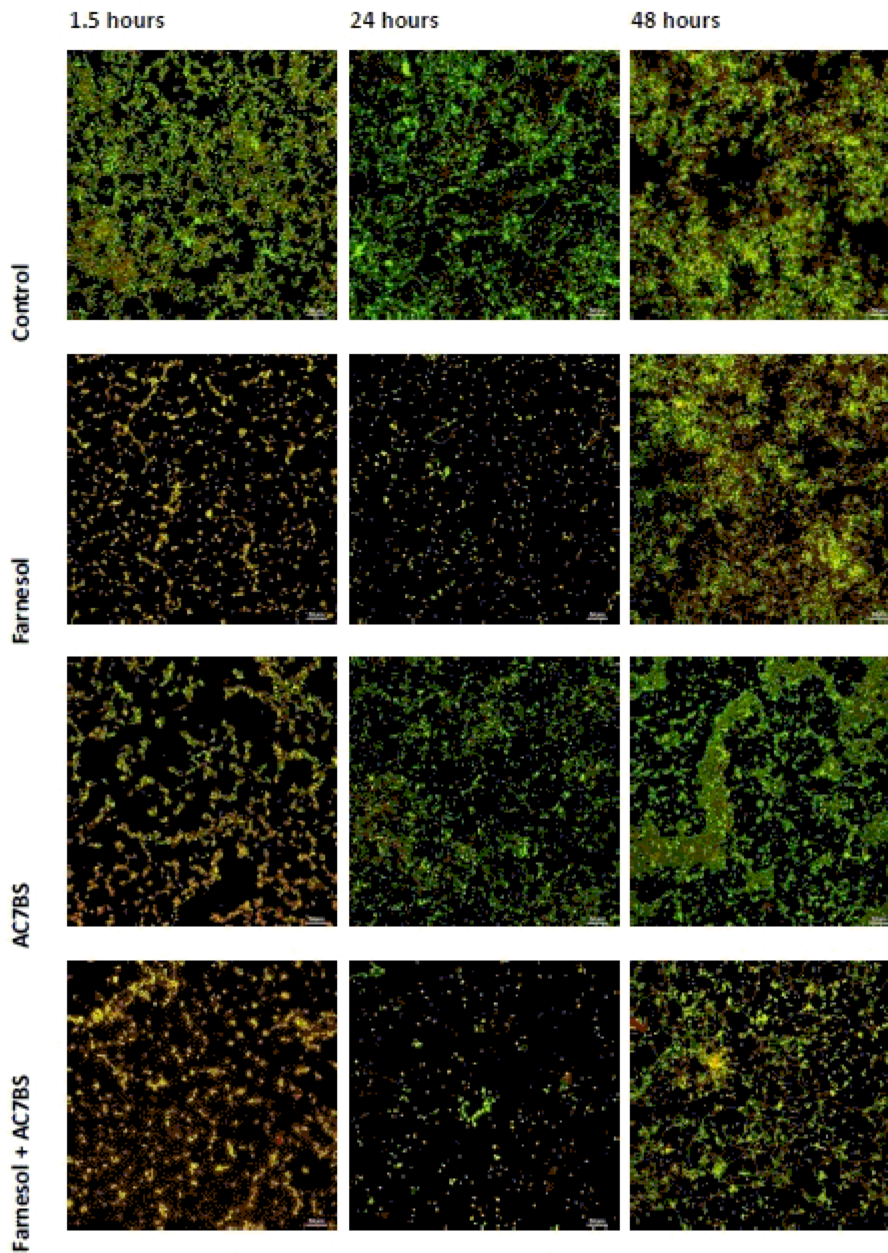

Figure 3. Confocal laser scanning micrographs of silicone elastomeric disks after *C. albicans* IHEM 2894 adhesion phase (1.5 h), biofilm formation (24 h) and pre-formed biofilm (48 h). Scale bar is 100 µm.

Conclusion

Nowadays, the urgent need for new antimicrobial compounds induced by the newly emerged pathogens and increased resistance shown by conventional others remains a major breakthrough in the clinical practice and preventive medicine [9,31]. In this contest, biosurfactants are recognized as compounds endowed with potent biological activities and some of them have been already described as alternatives to synthetic medicines and antimicrobial agents [16,32,33]. Lipopeptide biosurfactant AC7 is able to reduce significantly *C. albicans* cell adhesion and biofilm formation on medical grade silicone disks. Furthermore, it does not inhibit *C. albicans* planktonic cells and biofilm, thus indicating an anti-adhesive but not antifungal activity. In addition, when lipopeptide biosurfactant AC7 is used together with farnesol against C. *albicans* adhesion, growing biofilm or pre-formed biofilm, a synergistic effect is observed. These results suggest that AC7BS, thanks to its ability to change the surface characteristics, alone or in combination with quorum-sensing molecules, can be considered a potential inhibitor of biofilm growth on medical insertional materials.

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Conclusions

Biosurfactants are amphiphilic molecules having hydrophilic and hydrophobic moieties that confer them the ability to exhibit surface activities at interfaces [1]. Particularly, these molecules reduce surface and interfacial tension at gas-liquid-solid interfaces forming a molecular interfacial film that affects the properties (surface energy and wettability) of the original surface [2]. Biosurfactants present numerous and interesting physicochemical and biological features such as antibacterial, antifungal and anti-adhesive activities that make them attractive to a wide range of potential pharmaceutical and biomedical applications [3]. In particular, the ability to destabilize cell membranes by disturbing their integrity and permeability and to affect microorganism adhesion by modifying surface characteristics are valuable properties for their use in the treatment of infection caused by microbial biofilms on medical devices [4,5]. The presence of biofilm protects the microorganism from host defenses and reduces significantly its susceptibility to antimicrobial agents [6]. Furthermore, the tenancy with which microrganisms infect indwelling medical devices necessitates, in almost all the cases, their removal [7].

Candida species are responsible of an increasing number of these infections, especially those involving central and peripheral venous catheters, urinary catheters, tracheal tubes, neurosurgical shunts, voice prostheses, dentures and intrauterine devices [8]. *Candida* spp. cause 10% of the overall infections of intravenous catheters and cardiac devices prosthetic valves as well as 21% of the total cases of urinary catheters infection with a mortality rate of 20-40% [9]. In particular, *C. albicans* represents the fungus most frequently associated with the formation of biofilms on a wide variety of medical devices [10-13]. Quorum-sensing plays an important role in *C. albicans* biofilm development because it regulates all phases of biofilm life cycle. The quorum-sensing molecule farnesol inhibits hyphal growth and the expression of necessary morphology-specific genes

for the yeast-to-mycelium conversion of *C. albicans* leading to a decrease of biofilm size, suggesting a potential use for biofilm restraint [14, 15].

Current biofilm preventive strategies are essentially aimed at increasing material hydrophilicity [16, 17], thus decreasing microbial adhesion and biofilm formation, or at coating medical-device surfaces with antimicrobial agents [18]. Main drawbacks of antimicrobial coatings arise from time limited effectiveness due to oxidative degradation, development of microorganism resistance and potential toxicity towards human cells [19, 20]. From this point of view, it could be useful to increase the efficacy of known antibiotics and biocides with alternative strategies aimed at decreasing bacterial adhesion and reducing the biofilm populations on medical device surfaces.

My PhD research activity has been mainly focused on the study of two bacterial biosurfactants - CV8LAC BS produced by *Lactobacillus brevis* and AC7 BS produced by *Bacillus subtilis* – and their activity against *Candida albicans* biofilm formation.

In a previous work, it has been shown that CV8LAC BS decreased the adhesion of two biofilm-producer strains of *C. albicans* derived from culture collections of more than 80% in pre-coated polystyrene microtiter plates [21]. On the other hand, the use of AC7 BS for the prevention of *Candida albicans* biofilm formation on silicone disks and acrylic resins has been recently reported [22]. Pre-coating with this biosurfactant caused a greater reduction in biofilm cell number and viability than chlorhexidine. In addition, the biosurfactant concentrations used in the anti-adhesive assays were found to be significantly less cytotoxic than chlorhexidine in both fibroblast and keratinocyte in vitro cell models.

In this work, a clinically oriented approach was chosen in order to assess whether the effects previously obtained were still present when applying wild strains on medical grade polymers. To fulfill this aim, we used clinically relevant wild strains (*C. albicans* 40 and 42) isolated respectively from a central venous catheter and a urinary catheter, both made of silicone elastomer that is the very same biomaterial

chosen as substratum for our assays, and a strain isolated from the tongue and obtained from a public collection (*C. albicans* IHEM 2894).

In order to assess the anti-adhesive and anti-biofilm activity of BS on *Candida*, a number of different complementary methods (fungal biomass staining, viable cell counting, microscopic analysis) on multiple strains were used, to deeply address and understand the phenomena we were observing. In particular, first a co-incubation method was used, where biosurfactant solutions were added to microbial inocula or media. Secondly, in order to imitate a functional coating condition, a pre coating method was used, in which biosurfactant was previously adsorbed on the surface of silicone disks.

In collaboration with Prof. Maurizio Rinaldi - Department of Pharmaceutical Sciences, Novara, a deep statistical analysis was carried out with multiple tests allowing to confirm the significance of data. Statistical analysis and graphs were elaborated by means of the statistical program R (R Development Core Team, http//www.R-project.org). One-way or two-way ANOVA were used to compare optical densities of *C. albicans* planktonic cells and pre-formed biofilm at different biosurfactant concentrations. Two-way ANOVA followed by Tukey’s HSD test was used to evaluate the effect of the two biosurfactants on *C. albicans* adhesion and biofilm formation in co-incubation and pre-coating assays carried out by means of CV staining method. Welch Two Sample t-test and two-way ANOVA followed by Tukey’s HSD test were performed to investigate the effect of the two biosurfactants on *C. albicans* adhesion and biofilm formation in co-incubation and pre-coating assays carried out by means of the viable cell counting method. R package Dupi R was used to estimate $\log_{10}$ CFU/disk from colony counts. Chi-square test with Yates’ continuity correction was used to compare proportions of hyphae and blastospores at the different time points.

Results demonstrated that both biosurfactants were able to inhibit significantly the adhesion and, to a lower extent, to slow biofilm formation of different *C. albicans* strains on silicone elastomer, at physiological conditions.

Co-incubation of *C. albicans* 40 with CV8LAC BS induced a significant reduction of biofilm formation in respect to controls by a $\log_{10}$ factor ~1 (i.e. about 90%) at all incubation times (24, 48, 72 h). In addition, in pre-coating assays, fungal adhesion was significantly reduced by 62% at 1.5 h and biofilm formation was inhibited by 53%, 50% and 44% after 24, 48 and 72 h of incubation, respectively.

In co-incubation assays, AC7 BS induced a significant reduction of fungal biomass of the three strains by an average of 68% at 1.5 h, and by an average of 56 % at 24 h in respect to controls. In pre-coating assays, a significant reduction in fungal biomass by an average of 61% at 1.5 h, and of 49% at 24 h was detected. Using the viable cell counting method, the adhesion of the three *C. albicans* stains was significantly reduced by an average of 60% at 1.5 h and biofilm formation was significantly inhibited by an average of 47% after 24 h of incubation.

The anti-adhesion and anti-biofilm activity displayed by CV8LAC and AC7 biosurfactants cannot be attributed to an antifungal action, as the growth of *Candida* planktonic and sessile cells was not inhibited by both biosurfactants. It can be supposed that CV8LAC and AC7 biosurfactants influence bacterial-surface interactions thanks to their ability to change surface tension and bacterial cell-wall charge, affecting both cell-to-cell and cell-to-surface interactions and, as a result, the surface is made less supportive for bacterial adhesion.

The decreased activity of CV8LAC BS and AC7 BS on biofilm formation in pre-coating assays compared to co-incubation assays may be due to the strategy chosen to coat the silicone surface, as this was simply done by physical adsorption. The biosurfactant molecules are attached to the surface by weak bounds (hydrogen bounds, Van der Walls forces) and not by covalent bounds. Hence, during the washing steps involved in the experimental setting, the biosurfactant might have been gradually removed from silicone, leading to activity loss.

In collaboration with the research groups directed by Prof. Giandomenico Nollo at the Department of Industrial Engineering of University of Trento and by Prof. Iole Caola at the Section of Electron Microscopy of Department of Medicine

Laboratory, Azienda Provinciale per i Servizi Sanitari di Trento, SEM and CLSM analyses were conducted on *C. albicans* 40 and *C. albicans* IHEM 2894 adherent cells and biofilms. SEM microscopic and CLSM observation supported cultural data of the biofilm on silicone disks. During the adhesion phase, the number of attached cells on silicone surfaces was significantly reduced on biosurfactant treated disks. A complex three-dimensional structure with long hyphae arranged in a multilayered network was evidenced in controls of mature biofilms. On the contrary, a less compact structure of the biofilm was found in treated samples. No morphological differences were found between microorganisms growth on test or control samples showing, in both cases, ovoid spherical blastoconidia with polarly or randomly located budding and true hyphae formation. Difference in the amount of extracellular material was also documented. Furthermore, the analysis of quantitative SEM data on the percentage of polymer surface covered by biofilm showed lower values in treated samples in respect to controls. CV8LAC BS pre-coated silicone disks were characterized by a percent area of the silicone disk covered by C*andida* biofilm (BA%) reduction of 57% after 1.5 h and of 26%, 11% and 4.5% after 24h, 48h and 72h respectively, when compared to control disks. On the other hand, the pre-treatment of silicone disks with AC7 BS led to a BA% reduction of 52% after 1.5 h and of 31%, and 60% after 24 h and 48 h respectively. Finally, when AC7 BS was used together with farnesol, a synergistic effect was observed. In particular, AC7BS in combination with farnesol 100 μM significantly reduced *C. albicans* adhesion up to 74% at 1.5 h and biofilm formation up to 93% and 60% at 24 h and 48 h, respectively. Concerning BA% reduction, the combined use of AC7 BS and farnesol led to a decrease of 64% after 1.5 h and of 92%, and 74% after 24 h and 48 h respectively, when compared to control disks.

These findings suggest a potential use of biosurfactants in combined therapies (e.g. combination with antifungal drugs). Preliminary results obtained in the Laboratory of Microbiology revealed a synergistic effect by combining the biosurfactant with Amphotericin B.*C. albicans* 40 and 42 represented, in this study, very good models

to evaluate the anti-adhesive and anti-biofilm activity of CV8LAC and AC7 BS on silicone elastomer. This consideration also brought us to deposit the two strains in the DSMZ public collection. Nevertheless, a larger number of *C. albicans* strains is expected to be tested to exhaustively verify anti-adhesion properties of the two biosurfactans in future.

Moreover, the activity of both biosurfactants will be evaluated on clinically relevant biofilm-producer bacterial strains. Recently, preliminary results obtained in the Laboratory of Microbiology have shown that AC7 BS is able to reduce biofilm formation of *P aeruginosa* by 82%, of *S. epidermidis* ATCC35984 by 74% and of *E. coli* CFT073 by 72%, suggesting promising potentialities against bacterial biofilms.

AC7 BS was fully characterized. The LC/ESI-MS analysis, carried out by Prof. Gianna Allegrone - Department of Pharmaceutical Sciences - Novara, revealed the presence of the homologues of two lipopeptide families, principally surfactin family and a lower percentage of fengycin family. Chemical characterization of CV8LAC BS will be carried out in the near future. According to a preliminary TLC analysis, two substances were detected at 254 nm UV light and other two substances, at minor retention factor, were visualized by spraying a specific reagent to detect sugars.

Finally, physical or chemical surface modification will be tested in the future to bind the biosurfactants more efficiently to the silicone surface in order to improve and prolong anti-biofilm properties. Considering the wide selection of methods nowadays available for bonding biomolecules onto medical grade polymers, the anti-adhesive properties of the CV8LAC and AC7 BS deserve a promising role as anti-adhesive products to effectively limit colonization and prevent *C. albicans* infections on medical devices.

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