Effect of farnesol and biosurfactant, produced by Bacillus subtilis AC7 on different stages of biofilm formation by Candida albicans 40

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SUMMARY

Nosocomial *C. albicans* infections are becoming an increasingly important problem in the clinical practice. Especially people with reduced immune system are more susceptible to the opportunistic pathogen *C. albicans*. Treatment of these types of infections is difficult because of its ability to form a biofilm that has an increased resistance to antifungal therapy. The function of implanted medical devices is also compromised due to biofilm formation (Ramage et al., 2005)

The development of compounds that target biofilm formation could mean a major breakthrough in the treatment of different kind of (life-threatening) infections. Quorum-sensing plays an important role in biofilm formation, because it regulates all phases of biofilm life cycle (Deveau et al., 2011). Farnesol, a quorum-sensing molecule and biosurfactants, which possess antimicrobial and anti-adhesive activities, are getting increased attention as promising compounds which interfere with biofilm formation.

The influence of farnesol alone, of a lipopeptide biosurfactant produced by *Bacillus subtilis* AC7 (BS AC7) alone and farnesol in combination with BS AC7 on different stages of *C. albicans* 40 biofilm formation on silicone disks is evaluated in this thesis. The lipopeptide BS AC7 is produced according to the method described by Rivardo et al. (2009).

Farnesol 100 µM shows the highest percentage of microbial inhibition, when added before 24 hours of biofilm growth. This quorum-sensing molecule also has a less pronounced inhibitory effect on the adhesion of *C. albicans* 40 and on pre-formed biofilm.

Our results show that BS AC7 is able to cause an inhibition of the adhesion of *C. albicans* 40 cells to the silicone disks, while its effect decreases when biofilms are grown for 24 hours.

When farnesol and BS AC7 are combined, an additive inhibitory effect is observed during adhesion of *C. albicans* 40. A low synergistic effect is observed on its pre-formed biofilm. However, farnesol and BS AC7 seem to interfere with each other when they are added before 24 h of *C. albicans* 40 biofilm growth. Because no previous research is done on the effects of farnesol in combination with biosurfactants, it is difficult to find an explanation for the observed effect.

The obtained results form an interesting starting point for further research on the use of farnesol and BS AC7 in the treatment of nosocomial *C. albicans* infections. Future research could focus on the molecular basis of the synergistic/additive effect observed with some conditions and the antagonistic effect with other conditions.
SAMENVATTING

Nosocomiale C. albicans infecties vormen tegenwoordig een zeer belangrijk probleem in de klinische praktijk en hun belang neemt steeds toe. Vooral mensen met een verminderd immuunsysteem vertonen een hoger gevoeligheid voor dit opportunistische pathogeen. De behandeling van deze infecties is moeilijk gezien het vermogen van C. albicans om een biofilm te vormen met een verhoogde resistentie tegen antifungale therapie. Biofilm vorming tast ook de normale werking van inwendige medische apparaten aan. (Ramage et al., 2005)

De ontdekking en ontwikkeling van moleculen die biofilm vorming als target hebben, zou een belangrijke doorbraak kunnen betekenen in de behandeling van verschillende, al dan niet levensbedreigende infecties. Quorum-sensing oefent een belangrijke functie uit in die biofilm vorming, gezien zijn regulerend effect op alle fases van de biofilm levenscyclus (Deveau et al., 2011). De quorum-sensing moleculen farnesol, alsook biosurfactanten, die een antimicrobiële en anti-adhesie werking uitoefenen, krijgen veel aandacht als mogelijke componenten die biofilm vorming verhinderen of verminderen.

Deze thesis gaat de invloed van farnesol alleen, van een lipopeptide biosurfactant geproduceerd door Bacillus subtilis AC7 (BS AC7) alleen, en van farnesol gecombineerd met BS AC7 op de verschillende fasen in C. albicans biofilm vorming op silicone disks na.

Het hoogste percentage inhibitie wordt gevonden wanneer farnesol 100 µM wordt toegevoegd voor 24 uur biofilm groei. Deze quorum-sensing molecule heeft ook een, zij het minder uitgesproken, inhibitoir effect op de adhesie fase van C. albicans 40 en op reeds gevormde biofilm.

Onze resultaten tonen aan dat BS AC7 een inhibitie veroorzaakt van de adhesie van C. albicans 40 cellen op silicone disks. Dit effect neemt af wanneer 24u biofilms worden gevormd.

Een additief inhibitoir effect op C. albicans 40 adhesie en een klein synergistic effect op zijn pre-formed biofilm werd gevonden wanneer farnesol en BS AC7 gecombineerd werden. Farnesol en BS AC7 blijken echter te interferen met elkaar wanneer biofilms voor 24u werden gegroeid.

De gevonden resultaten vormen een interessant beginpunt voor verder onderzoek naar het gebruik van farnesol en BS AC7 in de behandeling van nosocomiale Candida infecties. De moleculaire basis voor het gevonden antagonist effect onder bepaalde condities en het additief/synergistisch effect in andere condities moet verder onderzocht worden.
THANKS TO

I would like to express a word of gratitude to some people that made it possible to complete this thesis. I want to thank Prof. Dr. Apr. H. Nelis for giving me the opportunity to realize the thesis in the laboratorium in Novara and for correcting my thesis. Special thanks I want to express to Prof. Dr. L. Fracchia for the scientific guidance in the microbiological lab and for giving feedback on the thesis. Dr. M. Cavallo and phd student C. Ceresa have helped me every day with fulfilling the experiments successfully and were always ready to answer every question I had. So because of this I want to say thanks to them. My family, boyfriend and friends also deserve a lot of gratitude for their phonecalls, messages, visits and support which made that I didn’t have to miss home. At last I want to say thanks to my new friends from the residence and to my roommate Elke because they made this an unforgettable adventure with a lot of new life experience.
# LIST OF ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>ALS</td>
<td>Agglutinin-like sequence</td>
</tr>
<tr>
<td>C. albicans</td>
<td>Candida albicans</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CSLM</td>
<td>Confocal scanning laser microscopy</td>
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<tr>
<td>CV</td>
<td>Crystal violet</td>
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<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
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<tr>
<td>FPP</td>
<td>Farnesyl pyrophosphate</td>
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<tr>
<td>HIV</td>
<td>Human immunodeficiency virus</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
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<tr>
<td>MDR</td>
<td>Multidrug resistant</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>OSD</td>
<td>Oxidative stress defense</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered saline</td>
</tr>
<tr>
<td>QSM</td>
<td>Quorum-sensing molecule</td>
</tr>
<tr>
<td>rpm</td>
<td>Rotations per minute</td>
</tr>
<tr>
<td>SAC</td>
<td>Surface-active compounds</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>SDA</td>
<td>Sabouraud Dextrose Agar</td>
</tr>
<tr>
<td>YNBD</td>
<td>Yeast Nitrogen Base Dextrose</td>
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1. INTRODUCTION

1.1. BIOFILMS

1.1.1. Definition of biofilm

“Biofilms are defined as structured microbial communities that are attached to a surface and encased in a matrix of exopolymeric material.” (Ramage et al., 2005).

Fungal biofilms form a protected niche, which has important clinical repercussions because they are resistant to antifungal therapy and the cells within biofilms are protected against host immune defenses. The function of indwelling medical devices can be compromised because of the biofilm formation, and the biofilm can act as a reservoir for persistent infections (Ramage et al., 2005). There is a direct correlation between a microorganism’s pathogenicity and its biofilm forming capacity (Thein et al., 2007). It is estimated that biofilms are related to about 65% of human microbial infections (Ramage et al., 2006).

Strict hygienic measures such as hand washing and regular disinfection of equipment and environment take a crucial place in preventive strategies. But disinfection becomes less effective when frequently applied (Dettenkofer et al., 2004, 2007; Kramer et al., 2006, as cited in Falagas & Makris, 2009).

1.1.2. Biofilm formation on medical devices

Almost all kind of surfaces can be subjected to colonization by biofilms (Donlan & Costerton, 2002). Host inflammatory response molecules can facilitate adhesion of cells to the device surface. This promotes biofilm formation (Hall-Stoodley et al., 2004). Urinary catheters (Stickler, 2008), central venous catheters (Petrelli et al., 2006), heart valves (Litzler et al., 2007), voice prostheses (Buijsen et al., 2007), contact lenses (Imamura et al., 2008), hip prostheses (Dempsey et al., 2007) and intrauterine devices (Chassot et al., 2008) all act as a possible target for biofilm formation. Colonization of biomaterials prohibits its normal function. Once a biofilm is formed on these devices, it generally has to be removed to get rid of the infection. The problem is that the patient’s condition, the anatomic location, or underlying disease make this removal often impossible. This has severe consequences for the human health. Contamination can occur before or during implantation of the device by medical personnel (Ramage et al., 2006).
Chemical properties, hydrophobicity and surface roughness of implanted biomaterials have an influence on initial adhesion to the surface. Biomedical devices are normally surrounded by body fluids such as urine, blood, saliva and synovial fluid. Because of this a glycoproteinaceous conditioning film is formed after their implantation, which could lead to completely changed chemical characteristics (Ramage et al., 2006).

1.1.3. Candida albicans and biofilms

1.1.3.1. Candida albicans

*Candida albicans* is a dimorphic fungus. Fungal dimorphism is defined as “an environmentally controlled reversible interconversion of morphology, particularly yeast and mycelial morphologies” (Nickerson et al., 2006).

The dimorphic fungus *Candida albicans* can cause several infections. It is responsible for superficial mucosal conditions such as thrush and vaginitis, but causes also life threatening systemic infections (Berman, 2006). Candidaemia is a bloodstream infection that can develop into disseminated candidiasis, when the internal organs are affected. Both have a very high mortality rate (Sudbery, 2011). This fungus is an opportunistic pathogen, but can also occur as commensal, as it naturally occurs in the gastrointestinal tract. Immunosuppressive therapy, antibiotics, indwelling devices, HIV-infection, diabetes and old age are all predisposing factors for *C. albicans* infections. *C. albicans* has become one of the most important causes of nosocomial infections, because of its ability to invade almost every site on the body. Not only superficial sites, but also deep tissues and organs can be infected by *C. albicans* (Ramage et al., 2001). In infected tissue *Candida* can be found as oval budding yeasts, as continuous septate hyphae or as pseudohyphae as shown in Figure 1.1. (Douglas, 2003).

![Fig. 1.1. Growth forms of Candida albicans with yeast cells (a), pseudohyphae (b) and true hyphae (c) (Berman, 2006)](image_url)
The pseudohyphae have constrictions at the location of septation that are not present in hyphal structures. Hyphae form tube-like filaments and the sides are parallel (Sudbery, 2011). When grown at low temperature and pH, ellipsoid yeast cells occur. Yeast cells grown at 37°C and neutral pH form long, narrow hyphae. External stimuli such as serum lead to the same result. At intermediate temperature and pH, elongated pseudohyphae are seen (Berman, 2006).

Except for the pH and the temperature, the yeast-mycelium dimorphism can be shifted by various chemical and environmental influences, such as glucose levels, nitrogen source, carbon dioxide levels, transition metals, chelating agents, and inoculum size or initial cell density (Nickerson et al., 2006).

*C. albicans* is a diploid, showing no sexual cycle. An important virulence factor of *C. albicans* is its cell wall. It is necessary for protection and growth and is the contact place with the environment. The main components are the polysaccharides: mannan, glucan, and chitin. Chitin and glucan are mainly concentrated on the inner side of the cell wall, while mannan is found throughout the whole cell wall. The outer layer is called the capsule and consists of mannan or mannoprotein. The cell wall is composed of different layers and the thickness can vary. Another predominant virulence factor is adhesion to mucosal membranes which is necessary to infect host tissues. Mannans and mannoproteins play an important role in this adhesion phase. The ability to adhere and the virulence of *C. albicans* are obviously correlated with each other. The secretion of proteinases and phospholipases is a third virulence factor. Mucous membranes all contain secretory Ig A, which is degraded by the carboxyl proteinases produced by *C. albicans*. Proteinases digest proteins such as hemoglobin, keratin, collagen. Phospholipases lead to degradation of cell membranes. These enzymes promote colonization and invasion of the host cells (McCullough et al., 1996; Gokce et al., 2007). Their fast response to external environment changes also contributes to the virulence of *Candida* species (Gokce et al., 2007).

1.1.3.2. *Candida albicans* biofilm formation

Biomaterials normally used in the clinical practice, such as intravascular catheters, heart valves, implanted devices, contact lenses, dentures, and so on, are targets for *Candida* biofilm formation (Seneviratne, 2008). Contact between *Candida* organisms and implanted devices is possible because it is a human commensal (Ramage et al., 2006).

In vivo biofilm formation is influenced by a lot of different factors, such as *Candida* species and strain type, physical traits of the implanted device and the presence of a
conditioning film. Immediately after implantation of the device, host proteins and cells from the surrounding body fluids (tears, saliva, urine or blood) attach to the surface of a device. This layer is called a conditioning film. Planktonic Candida cells can find receptor binding sites provided by conditioning films, in particular serum. Early adhesion in biofilm formation is promoted because of this. Biofilm production and maturation are facilitated by interactions between Candida cells, mucins and fibrinogen, components of saliva and serum. C. albicans can also form a biofilm on biological surfaces (Frade et al., 2009).

C. albicans biofilm typically consists of a thin, basal layer of cells in the yeast form, attached to the surface and an overlying more open layer of filamentous cells in the hyphal form enclosed by an extensive exopolymeric matrix (Kumamoto, 2002, Douglas, 2003). The dimorphic character of C. albicans plays a crucial role in biofilm development. One of the most important virulence factors of C. albicans is this ability to switch from a yeast form to a hyphal form. This transition is induced by several different environmental factors, for example mammalian serum, a temperature of 37°C and neutral pH (Ramage et al., 2002).

The crucial developmental role of this dimorphism appears when wild-type biofilms are compared with biofilms produced by mutant strains incapable of yeast or hyphal growth. The hypha-negative mutant is only able to produce a basal yeast layer, while the yeast-negative mutant forms a thicker, hyphal biofilm, without basal yeast layer. The observation that both mutants are able to form biofilm, suggests that dimorphism is not an absolute obligation for biofilm production. But to obtain the typical architecture of biofilm, it might be necessary (Douglas, 2003).

The immune system does not tolerate invasive hyphal forms and macrophages start a specific immune response against them (Sudbery, 2011).

The distribution of fungal cells and extracellular material gives the mature C. albicans biofilm a highly heterogeneous architecture (Chandra et al. 2001). It is demonstrated with the non-destructive CSLM technique that mature biofilms display a microcolony/water channel structure. This kind of organization provides the possibility of influx of nutrients and efflux of waste products and the formation of micro-niches through the biofilm (Ramage et al. 2001).

The composition of extracellular material, which contains carbohydrate, protein and other unknown components, produced by cells in biofilm, differs from this produced by planktonic cells (Kumamoto. 2002). There is a higher amount of matrix when the biofilm is produced under gentle shaking conditions, producing a flow of liquid over the cell surfaces in comparison to static conditions (Douglas. 2003).
As shown in Fig. 1.2 three developmental phases can be distinguished in *C. albicans* biofilm formation, the early (0-11 h), intermediate (12-30 h) and maturation (38-72) phases. In the early phase, yeast cells attach to the surface and defined microcolonies are formed. During the intermediate phase, a haze-like film of exopolymeric substances is formed over the yeasts, germ-tubes and young hyphae. Finally, fully mature biofilms are formed after up to 48 hours of incubation, consisting of a dense network of yeast cells, hyphae and pseudohyphae encased within the extracellular matrix (Chandra et al., 2001; Douglas, 2003; Seneviratne et al., 2008). Yeast cells are able to detach again and form biofilm in another place (Deveau et al., 2011).

![Fig 1.2. Different steps of biofilm formation with a) attachment of the planktonic cells, b) irreversible adhesion and production of noncellular material, c) maturation and d) detachment and dispersal of biofilm cells.](http://www.intechopen.com/books/scanning-electron-microscopy/scanning-electron-microscopy-sem-and-environmental-sem-suitable-tools-for-study-of-adhesion-stage-a)

Nonspecific factors such as cell surface hydrophobicity and electrostatic forces play a role in the initial attachment, but also specific adhesins that recognize ligands in the conditioning films, such as serum proteins and salivary factors have an influence on the adhesion. Even cell surface proteins encoded by members of the ALS family of adhesin-producing genes and EAP1 may mediate specific adherence (Ramage et al., 2005). Bacteria already growing on the surface can facilitate coaggregation and/or binding of *Candida* cells (Ramage et al., 2006).

The phenotypic properties of the attached cells in biofilms are very different from their planktonic forms. Hereby, cells are able to evade host defences and their susceptibility to antimicrobials and biocides is decreased (Ramage et al. 2001).
β 1,3-glucan is an essential structural component of *Candida* cell walls. This component is also present in the biofilm matrix and might be a partially effective target for antifungal agents. Caspofungin is an inhibitor of the β 1,3-glucan synthesis and its in vitro effect against *C. albicans* biofilms is confirmed (Douglas. 2003).

The overall morphology and architecture of *Candida* biofilms are examined with fluorescence microscopy, scanning electron microscopy (SEM) or confocal scanning laser microscopy (CLSM) techniques. Where fluorescence microscopy analysis is used to give an image of overall biofilm morphology and the emergence of extracellular matrix during biofilm formation, SEM is able to visualize detailed surface topography and morphology. However, sample preparation for this last technique leads to destruction of the native hydrated structural properties because of fixation and dehydration. CSLM does not show this problem and biofilm thickness can be measured. At different depths of biofilm, the three-dimensional structure can be seen by CSLM (Chandra et al. 2008).

Quantification of formed biofilms is done by determination of their metabolic activity or the dry weight of the biofilm (Douglas. 2003). The metabolic activity is measured using a colorimetric assay, based on the reduction of tetrazolium salts by mitochondrial dehydrogenases or ferric reductase. XTT in which cells are incubated, is converted to a water-soluble, colored formazan product by viable cells. The color intensity of the formazan product is measured as optical density directly in the extracellular medium and is proportional to the metabolic activity of cells. Another assay uses MTT for the incubation and a water-insoluble formazan product is then formed. For the measurement, dimethylsulfoxide must be added to lyse the treated cells, before optical density can be evaluated. The dry weight determination is done by scraping the biofilm off the substrate surface and vacuum filtering through a pre-weighed filter. The filter is weighed after drying and the difference in weight in comparison to the pre-weighed filter, shows the dry biomass of the biofilm (Chandra et al. 2008).

1.1.3.3. *Candida* biofilm formation on a central venous catheter

Use of central venous catheters forms the most common cause of nosocomial bloodstream infections. This leads to an important increase in hospital costs, duration of hospitalization and patient morbidity. Biofilms can be found on the outside or the inner lumen of virtually all central venous catheters. This was demonstrated with SEM and transmission electron microscopy (Ramage et al., 2006).

This type of catheter is used to deliver fluids and nutrients, but also cytotoxic drugs. This implanted device can become infected at any time during its use. Contamination of the
infusion fluid or the catheter hub is possible, but mostly organisms are transferred from the patient’s skin or from the hands of the nursing staff. Sometimes organisms get inside through the catheter wound, or the distal tip is contaminated at the time of insertion. If the Candida species are able to penetrate the intestinal mucosa, as they occur in the gastrointestinal tract as commensals, they can reach the catheter tip endogenously. For cancer patients with damaged intestinal mucosa, because of the chemotherapy, this could be a general entry mechanism (Douglas, 2003)

In case of Candida catheter-related infections the catheter should be removed and the patient should take antifungal medicines for at least 14 days after the last positive blood culture and when infection symptoms have disappeared. Prognosis is not good and mortality is high when the central venous catheter cannot be removed (Ramage et al., 2006).

1.1.4. Antifungal resistance of biofilm

The antifungal resistance of C. albicans biofilms is almost 2000-fold higher in comparison to their planktonic cells (Seneviratne et al., 2008). This is becoming an important problem in the treatment of nosocomial infections. Resistance to antifungal drugs can occur before exposure to the drug and is then called primary resistance, but a microorganism can also become resistant in response to the exposure. This type of resistance is called secondary resistance (Jabra-Rizk et al, 2004).

Some mechanisms are considered responsible for the resistance of biofilms against antifungal agents, but the molecular basis for this resistance is not fully understood. Resuspended organisms from disrupted biofilms preserved their antibiotic resistance. This points out that the cells themselves carry the resistance phenotype. The phenotype is reversible because, when grown on solid media, the resuspended organisms lost their resistance (Kumamoto, 2011).

In general, four mechanisms are considered responsible for antifungal resistance of Candida biofilms, including: (a) interference of biofilm matrix on drug penetration; (b) declined growth rate and restricted nutrient availability; (c) expression of resistance genes, especially genes encoding efflux pumps; and (d) presence of ‘persister’ cells (Ramage et al., 2006).

For a long time it has been supposed that the matrix of extracellular polymeric material forms a barrier that prevents drug diffusion. However, susceptibility studies with biofilm produced under statical conditions, containing minimal matrix, and biofilm production in shaking condition, leading to large amounts of matrix, did not show significant differences for
fluconazole, amphotericin B or flucytosine. (Al-Fattani et al. 2004; Douglas, 2001) These results indicate that the matrix and the extent of matrix formation do not play a role in the resistance against antifungal agents (Kumamoto, 2011). But other separate studies suggest that the matrix might play a minor role in drug resistance (Douglas, 2001).

The availability of nutrients is limited, especially at the base of the biofilm, because of which biofilm cells grow slowly. This could cause changes in the composition of the cell surface, which perhaps affects the antifungal susceptibility of the cells. When tested, biofilms were resistant to amphotericin B at all tested growth rates, while planktonic cells showed resistance only at low growth rates. So biofilm resistance seems to be the result of some other characteristic of the biofilm mode of growth than low growth rate alone. (Douglas, 2001).

The phenotype of microorganisms is changed when they attach to a surface and produce a biofilm. ATB-binding cassette (ABC) transporters and major facilitators, encoded by CDR and MDR genes, respectively, are responsible for the efflux of antifungal agents. The expression of these genes is upregulated during formation and development of biofilms (Douglas, 2001). However, high antifungal resistance is still found in mutants missing one or two of these efflux pumps. It is also possible that other hypothetical transporters contribute to antifungal resistance (Kumamoto, 2011).

_**Candida**_ biofilms show anti-oxidative characteristics because they express a higher amount of protein biomarkers associated with oxidative stress defenses (OSD’s) compared with planktonic cells. This increased expression leads to antifungal resistance of _Candida_ biofilms. _C. albicans_ possess the ability to gain resistance against a normally lethal dose of _H_2O_2_ or _O_2^−_ generating agents when exposed before to a nonlethal dose of the same oxidants. OSD’s, which contain both enzymatic and nonenzymatic compounds, are able to restore the oxygen balance by destroying those reactive oxygen species (ROS). Most of the upregulated biomarkers are correlated with yeast-hyphal transition. Pil1p is an interesting biomarker which has an upregulated expression in a mature biofilm. It is also present on the hyphal cell surface, but not on yeast or blastospore forms. Pil1p is a promising target against biofilms because it carries an echinocandin binding domain and it is fungus specific (Seneviratne et al., 2008).

The high resistance of biofilms can also be caused by a small group of persister cells. Antibiotics normally kill planktonic cells and the majority of biofilm cells, but a small fraction of persister cells survives. The immune system is capable of killing planktonic persister cells, but the biofilm persister cells are protected by the matrix and cannot be killed.
When antibiotic concentration is lowered after the symptoms disappear, the persister cells can reform the biofilm and cause a relapse of the infection (Lewis, 2009).

Different molecular mechanisms are responsible for the complex process of drug resistance in *C. albicans* biofilms (Douglas, 2001). So biofilm resistance is multifactorial and cannot be understood by one single mechanism. Echinocandins and liposomal formulations of amphotericin B are recent antifungal agents with a higher activity against *Candida* biofilms (Ramage et al., 2006).

1.2. **FARNESOL**

1.2.1. **Quorum-sensing**

Quorum-sensing plays an important role in *C. albicans* biofilm development (Ramage et al., 2002).

“*Quorum-sensing is a kind of communication between cells of biofilm which release chemical substances that signal the initiation of coordinated cellular differentiation events when they are with enough cells* (Ramage et al., 2005). Unnecessary overpopulation and competition for nutrients can be avoided by this type of cell-cell signaling. The infection process is influenced by this strategy, in particular dissemination and formation of distal sites of infection (Ramage et al., 2005). Quorum-sensing molecules (QSM) cause a complex cell response, depending upon the environmental conditions (Uppuluri et al., 2007).

In vitro, *C. albicans* yeast cells secrete metabolites, including compounds with a possible quorum-sensing function in the medium till stationary phase is reached. Some yeast cells at the bottom of a mature in vitro biofilm arrive in stationary phase. But also mature biofilms are able to synthesize quorum-sensing molecules (Uppuluri et al., 2007).

1.2.2. **Farnesol production**

*C. albicans* secretes an autoregulatory sesquiterpene, named farnesol, which acts as a quorum-sensing molecule and the structure is shown in Fig. 1.3. (Deveau et al., 2011).

Farnesol (1-hydroxy-3,7,11-trimethyl-2,6,10-dodecatriene) shows four isomers and only E,E-Farnesol can act as a quorum-sensing molecule (QSM) (Nickerson et al., 2006).
Farnesol is secreted by *Candida albicans* species in a concentration and temperature independent way. Its secretion is promoted by the presence of serum. This QSM is secreted continuously and levels up to 1 mM farnesol can be reached because of the capsule-like extracellular matrix and farnesol’s lipophilic character (Weber et al., 2010). Diffusion and use of a very common transport system are proposed as possible excretion mechanisms. An alternative pathway from the sterol biosynthetic intermediate farnesyl pyrophosphate (FPP) is followed to produce this QSM (Nickerson et al., 2006). This pathway can be found in the peroxisome and for further metabolism, farnesol has to be transferred out of the peroxisome (Uppuluri et al., 2007). *C. albicans* cell extracts possess enzymes with the ability to defosforylate $[^3]$H-FPP to $[^3]$H-Farnesol (Nickerson et al., 2006). FPP is known as an important precursor in protein prenylation, which is a post-translation modification of proteins (Saidi et al., 2006). When the cells are grown in anaerobic conditions, they do not produce or respond to farnesol (Nickerson et al., 2006). There is no correlation between the place of infection and the farnesol secretion (Weber et al., 2010).

### 1.2.3. Effects and characteristics of farnesol

Farnesol is considered as a virulence factor for the invasion process. It may help in the destruction of the epithelial cell layer of host cells. Farnesol alters the metabolic activity and can also inhibit in vitro *Candida* growth. (Weber et al., 2010).

Strong biofilm formation is prevented by this molecule because it inhibits hyphal growth and the expression of necessary morphology-specific genes. A lot of biofilm development stages are influenced by this QSM, such as the attachment of cells to the substratum, the mature biofilm architecture and the dispersal of cells from biofilm (Deveau et al., 2011).

Farnesol inhibits the yeast-to-mycelium conversion of *C. albicans* and leads to a decrease of biofilm size. It does not matter if farnesol is added before surface-attachment or to preformed mature biofilms (Deveau et al., 2011). Nevertheless some steps of biofilm development may be insensitive to farnesol. The time during which cells can be affected by
farnesol is confined because it does not prevent the elongation of preexisting hyphae. When added after initial attachment during the first stages of biofilm growth, the biofilm size is not impacted by farnesol (Nickerson et al., 2006; Deveau et al., 2011). Cell morphology is not modified in response to farnesol during early stages of hyphal growth. The percentage of cells that are in a hyphal growth stage increase with time. This means that when farnesol is added at time zero, all cells respond to farnesol with inhibition of hyphae formation as consequence. At 90 minutes, all cells are insensitive to farnesol because they are all in the hyphal growth phase (Nickerson et al., 2006). This suggests that farnesol has more potential in preventive strategies than as a therapeutical agent, because of its time dependent effect (Ramage et al 2002). Interestingly, mature biofilm regains its sensitivity to farnesol (Nickerson et al., 2006). Farnesol insensitivity and resistance of young biofilms to farnesol is not fully understood yet. Although it does not show effect on morphology when added to developing biofilms, this QSM does have an impact on other physiological properties. Resistance to oxidative stress and apoptosis in planktonic conditions are influenced by this quorum sensing molecule (Deveau et al., 2011). Temperature and nutrient conditions can change the effect of farnesol (Weber et al., 2010).

For biofilm formation, morphology is very important and because farnesol has an impact on morphology, it is supposed that exogenous farnesol impairs biofilm development. But for endogeneous farnesol, the knowledge about its effects on different phases of biofilm growth and phenotypes as drug resistance is limited (Deveau et al., 2011). Farnesol inhibits the Ras1-adenylate cyclase-protein kinase A signaling pathway, which leads to repression of the hyphal growth. This signaling pathway also impacts other cellular processes, such as stress responses, metabolism and drug resistance (Davis-Hanna et al. 2008). Farnesol causes a concentration-dependent decrease in metabolism of Candida species. (Weber et al., 2010).

1.3. BIOSURFACTANT

Amphipathic compounds, which bring both hydrophilic and hydrophobic characteristics together in one molecule, can be produced by various microorganisms. These amphipathic molecules present surface activities and are called biosurfactants or bioemulsifiers. They reduce surface and interfacial tension at gas-liquid-solid interfaces (biosurfactants) or at solid-liquid interfaces and between immiscible liquids (bioemulsifier). The hydrophobic part of the surface active compounds (SAC) orientates towards the hydrophobic side of the interface, while the hydrophilic parts tends towards the hydrophilic phase or solution. A molecular interfacial layer is formed which lowers surface tension in liquids and also lowers
the interfacial tension between different immiscible liquid phases (Smyth et al., 2010a, 2010b). Biosurfactants have a good biodegradability and a lower toxicity in comparison to synthetic surfactants. This allows their use in food, cosmetic and pharmaceutical industries and the possible replacement of synthetic surfactants by biosurfactants (Muthumasy et al. 2008).

1.3.1. Classification

Classification is based on structural features, the producing organism and molecular mass of SAC. The hydrophilic part mainly consist of an acid, peptide cations, or anions, mono-, di- or polysaccharides. In the hydrophobic part mostly unsaturated or saturated hydrocarbon chains or fatty acids are found (Chen et al., 2010a, 2010b).

1.3.1.1. Low molecular weight compounds

Low molecular weight compounds reduce surface and interfacial tension and lipopeptides and glycolipids are the best studied compounds of this group. Members of the Bacillus species are the main producers of lipopeptides, which can differ in their fatty acid chain and their peptide part (Dastgheib et al., 2008; Jacques, 2010; Thavasi et al., 2011, 2011). *Bacillus subtilis* produces a cyclic lipopeptide, named surfactin because it is the most powerful biosurfactant known so far (Ron & Rosenberg, 2001; Peypoux et al., 1999). It is composed by a seven amino-acid ring structure attached to a fatty-acid chain through a lactone binding (See Fig. 1.4) (Jacques, 2010).

Four isoforms of surfactin can occur, based on the differences in their amino acid sequences and various physiological properties (Shaligram & Singhal, 2010). Surfactin affects the integrity and permeability of membranes and destabilizes them (Bernheimer et al., 1970). Because of this, important membrane functions such as transport and energy generation are compromised. The incorporation of surfactin in the membrane causes dehydratation of the phospholipid polar head groups and the lipid packing becomes looser, which leads to bilayer instability and disruption of the membrane barrier properties. These actions are responsible for the antibiotic action and the other important biological effects of this lipopeptide (Carillo et al., 2003).
Fig. 1.4 Structure of the low molecular weight compound surfactin (Fracchia et al., 2011)

Another lipopeptide compound is lichenysin, with surfactin-like chemical structure and physio-chemical traits (Mcinerney et al., 1990). It is produced by Bacillus licheniformis (Horowitz et al., 1990). Pumilacidin, isolated from Bacillus pumilis is also a surfactin-like compound with interesting antiviral properties (Morikawa et al., 1992; Naruse et al., 1990). Other biosurfactant lipopeptides belong to the iturin, the fengycin or the serrawettin family (Jacques, 2010; Matsuyama et al., 2010). They all demonstrate interesting activities.

Glycolipids are normally mono- or disaccharide compounds which are acylated with long chain fatty acids or hydroxyl fatty acids. The best investigated subclasses are rhamnolipids, mannosylerythritol lipids, sophorolipids and trehalolipids (Fracchia et al., 2011).

1.3.1.2. High molecular weight compounds

High molecular weight biosurfactants do not reduce surface tension so much, but they are able to stabilize emulsions (Neu, 1996; Rosenberg, 2006; Rosenberg & Ron, 1997; Smyth et al., 2010a, 2010b). These biosurfactants are generally polymeric biosurfactants and they are formed by several different bacteria. They are comprised of lipoproteins, proteins, polysaccharides, lipopolysaccharides or complexes consisting of some of these structural types (Ron & Rosenberg, 2001; Rosenberg & Ron, 1997, 1999). The best known high molecular weight compound is emulsan. This bioemulsifier is a lipopolysaccharide produced by Acinetobacter calcoacetiws RAG-1 ATCC 31012 (Rosenberg et al., 1979). RAG-1 emulsan consists of a complex of an anionic heteropolysaccharide and protein (Rosenberg & Kaplan, 1987, as cited in Rosenberg & Ron, 1999). Fatty acids linked through O-ester and N-acyl linkages to the polysaccharide backbone are responsible for the surface activity of RAG-1 emulsan (Belsky et al., 1979, as cited in Rosenberg & Ron, 1999).

Alasan, which consists of a complex of an anionic polysaccharide and a protein, is another high molecular weight compound and is isolated from Acinetobacter radioresistens. It has a
molecular weight of around 1.000 kDa (Navon-Venezia et al., 1995, as cited in Smyth et al., 2010b). These high molecular weight biosurfactants are called bioemulsifiers because of their emulsifying activity. A high number of other polymeric components are still partially or completely uncharacterized (Smyth et al., 2010b). Except for the producing organism and the overall chemical constitution, not much is known about these bioemulsifiers. A number of bioemulsifiers is produced by yeasts and they are used in the food, cosmetic, and petroleum industries (Rosenberg & Ron, 1999). Liposan, containing 83% carbohydrate and 17% protein is one of them. It is produced by Candida lipolytica (Cirigliano & Carman, 1985, as cited in Rosenberg & Ron, 1999). Saccharomyces cerevisiae is the producer of mannanprotein emulsifiers (Cameron et al., 1988, as cited in Rosenberg & Ron, 1999).

1.3.2. Characteristics of microbial surface-active compounds

Biosurfactants reduce the water surface tension. To be efficient, a biosurfactant has to lower the surface tension between pure water and air from 72 mN/m to less than 30 mN/m. Surfactin has the capacity to reduce the surface tension of water from 72 mN/m to 27 mN/m and acts as one of the strongest biosurfactants (Seydlová & Svobodová, 2008). When the compound reaches its critical micelle concentration (CMC), no further decrease in surface or interfacial tension is seen. It is the concentration where the biosurfactant has the highest activity (Desai & Banat, 1997). The CMC is defined as the minimum concentration needed to start micelle formation (Becher, 1965). At this concentration, monomers start to form weak chemical interactions, e.g. hydrophobic, van der Waals and hydrogen bonds. Structured aggregates, such as micelles, vesicles or continuous bilayers are spontaneously formed (Maier, 2003; Raza et al., 2010). The aggregates are fluid-like because no chemical bonds are formed. When electrolyte concentration or temperature is changed, they can easily switch from one state to another (Lin, 1996). The polarity of the solvent is determinative for the aggregate structure of the dissolved surfactant. The polar head groups will point out towards the polar solvent e.g. water, while the hydrophobic tails come together in the middle of the micelle, to protect themselves from the polar solvent. An oil-in-water micelle is formed in this way. In an apolar solvent e.g. oil, the situation is reversed, leading to a water-in-oil micelle (Sobéron-Chávez & Maier, 2010). Environmental factors such as temperature and pH do not influence the biosurfactant surface activities, which is important for industrial and biotechnological applications (Muthusamy et al., 2008).

Another interesting effect for biotechnological use is the formation of stable emulsions. Especially high molecular weight bioemulsifiers show this characteristic. They are able to
stabilize oil-in-water and water-in-oil emulsions. This does not imply that they lower surface tension strongly and act as a good detergent (Dastgheib et al., 2008).

Biosurfactants, produced by a large number of microorganisms are important in many aspects of growth. Their role depends on the chemical structure and surface properties. Some biosurfactants allow the motility of micro-organisms. Bioemulsifiers have a regulating role in the attachment and detachment of microorganisms to and from surfaces (Van Hamme et al., 2006). Additionally they play a role in cell-to-cell interactions such as bacterial pathogenesis, quorum-sensing and biofilm formation, maintenance and maturation. The most widespread function of microbial surface-active compounds is the interaction between microorganisms and insoluble substrates, such as hydrocarbons. The growth of bacteria on hydrophobic water-insoluble substrates is facilitated by some of these components (Neu, 1996; Ron & Rosenberg, 2001; Van Hamme et al., 2006).

1.3.3. Biomedical use of biosurfactants

Because of their safety and their antibacterial, antifungal and antiviral activities, biosurfactants have potential to be used as therapeutic agents. They disrupt membranes which leads to an increase of the membrane permeability, followed by cell lysis and loss of metabolites. These compounds can also influence adhesion properties of microorganisms, by partition at the interfaces (Banat et al., 2010; Cameotra & Makkar, 2004; Rodrigues et al., 2006a; Rodrigues & Teixeira, 2010; Seydlová & Svobodová, 2008; Singh & Cameotra, 2004).

1.3.3.1. Antibacterial activity

The most promising antimicrobial products are lipopeptides. By self-associating and forming a pore-bearing channel or micellar aggregate inside a lipid membrane, they damage microorganisms (Carrillo et al., 2003; Deleu et al., 2008). Surfactin has a nonspecific way of action on both Gram-positive and Gram-negative bacteria. It forms hydrophobic interactions and penetrates into the membrane, causing its disruption (Lu et al., 2007). Fengycin (Vanittanakom et al., 1986) and the iturin compounds (Peypoux et al., 1978), produced by Bacillus subtilis strains, are other amphiphilic surface- and membrane-active compounds with great potential for antimicrobial applications. Daptomycin is another promising antimicrobial lipopeptide that is approved for the treatment of skin infections. It presents a high activity against multiresistant bacteria such as MRSA (Giuliani et al., 2007, as cited in Seydlová &
Interesting antimicrobial effects are also found with glycolipids (reviewed by Fracchia et al., 2011).

1.3.3.2. Antifungal activity

Pathogenic yeasts, e.g. *Candida albicans*, responsible for human mycoses, are attacked by the cellobiose lipid flocculosin, produced by *Pseudozyma flocculosa*. This glycolipid shows no cytotoxic effects against human cell lines. But the antimicrobial activity can be lost under alkaline conditions because of fast deacylation, which explains dubious results concerning their antifungal activity (Mimee et al., 2009). Other glycolipids, such as cellobiose lipids (Kulakovskaya et al., 2009, 2010), rhamnolipids (Debode et al., 2007, Banat et al, 2010) and the cyclic lipopeptides surfactin, iturin and fengycin show different kinds of antifungal activity (reviewed by Fracchia et al., 2011).

1.3.3.3. Anti-adhesion activity

Biosurfactants have the potential to interfere with biofilm formation because they modify the microbial interaction with interfaces (Federle & Bassler, 2003; Merk et al., 2005; Neu, 1996; Rasmussen & Givskov, 2006; Rodrigues et al., 2006b, 2006c; Rodrigues et al., 2007). Biosurfactants show an anti-adhesive activity which correlates with their concentration. At high concentrations they also provide a direct action against microorganisms (Luna et al., 2011). Especially surfactin shows important biofilm controlling effects, when added to the inoculum or pre-coating the surface with surfactin (Pecci et al., 2010). Its activity increases with decrease in temperature (Zeraik & Nitschke, 2010). Because of their anti-adhesive activity they have potential as coating agents for indwelling medical devices. This could lead to a decrease in nosocomial infections by specifically targeting biofilm growth, without needing to use synthetic drugs and chemicals (Fracchia et al., 2011).
2. OBJECTIVES

Many microorganisms show an increased resistance against existing antibiotics, especially when they form biofilms. The search for novel compounds which can inhibit or prevent biofilm formation or even destroy already formed biofilms has become very important nowadays.

This thesis aims to determine the activity of the quorum-sensing molecule farnesol and of the lipopeptide biosurfactant produced by Bacillus subtilis AC7 (BS AC7), and to evaluate the possible synergistic effect of the biosurfactant, and farnesol against C. albicans biofilms. In particular, their influence on Candida adhesion, biofilm formation, and on pre-formed biofilms will be studied on medical-grade silicone disks, using the viable cell counting method.

The following conditions are evaluated:

- Effect of farnesol 100 µM on C. albicans 40 adhesion (addition at time zero)
- Effect of farnesol 100 µM on C. albicans 40 biofilm formation (two conditions: addition at time zero and after 90 minutes of adhesion + addition after 90 minutes of adhesion)
- Effect of farnesol 100 µM on C. albicans 40 pre-formed biofilm (addition after 24 hours biofilm growth)

- Effect of BS AC7 2000 µg/mL on C. albicans 40 adhesion (pre-coating)
- Effect of BS AC7 2000 µg/mL on C. albicans 40 biofilm formation (pre-coating)

- Effect of farnesol 100 µM and 2000 µg/mL BS AC7 on C. albicans 40 adhesion (addition at time zero)
- Effect of farnesol 100 µM and 2000 µg/mL BS AC7 on C. albicans 40 biofilm formation (two conditions: addition at time zero and after 90 minutes of adhesion + addition after 90 minutes of adhesion)
- Effect of farnesol 100 µM and 2000 µg/mL BS AC7 on C. albicans 40 pre-formed biofilm (addition after 24 hours biofilm growth)
3. MATERIALS AND METHODS

3.1 MATERIALS

3.1.1. Instruments

Autoclave: pbi international FEDEGARI AUTOCLAVI SPA
Balance 1: KERN 440-35N (sensitivity: 0.01g)
Balance 2: Sartonius CP324S-OCE (sensitivity: 0.0001g)
Camera: Nikon Coolpix 995
Centrifuge 1: RC5B plus centrifuge, Sorvall, Haverhill, USA
Centrifuge 2: Thermo scientific CL10
LAF unit: Kendro Laboratory products, type HS15 and type HS18, Heraeus, Germany
Magnetic stirrer: ARED heating magnetic stirrer high power VELP scientific
Microscope: Nikon SMZ200 Type 104
PH-meter: HANNA Instruments pH 211 microprocessor pH-meter
Rotary evaporator: IKA RV10 basic
Sonicator: Elma S30H Elmasonic
Tensiometer: KSV Sigma 703D
Vortex: Fisher Scientific TOPMIX FB 15024

3.1.2. Media

**LB broth**: Luria Bertani broth, high salt is a nutritionally rich medium, used for the general cultivation of microorganisms. Twenty-five grams of LB powder (Fluka) are weighed and dissolved in 1 L distilled water using the magnetic stirrer to dissolve all the powder. The pH is adjusted at 7.5 ± 0.2 with 1 M HCl. The broth is sterilized with the autoclave at 121°C for 15 minutes.

**LB agar**: Luria Bertani agar is prepared by adding 25 g of LB powder (Fluka) and 15 g of agar (Fluka) in 1 L distilled water. This medium is sterilized by autoclaving at 121°C for 15 minutes.
**SDA:** Sabouraud Dextrose Agar is a complex medium which promotes fungal growth. Mycological peptone is the nitrogen source, while dextrose acts as an energy source. SDA contains 30 g SD broth (Fluka), 15 g agar (Fluka). These components are dissolved in 1L deionized water. This solution is brought to pH 5.6, which favors fungal growth and prevents contamination by bacteria.

**YNBD:** Yeast Nitrogen Base Dextrose is prepared by weighing 0.67 g of YNB powder (Fluka) and 0.9 g of dextrose (Biolife) and dissolving in 100 mL milli-Q water by using a rotating magnet. The pH is brought to 6.8 with 0.1 M NaOH. This pH is necessary to prevent precipitation and to dissolve the biosurfactant. The solution is then sterilized through a membrane filter (Minisart, Goettingen, Germany) with 0.22 µm pores. Autoclavation is not possible because of degradation of the components. This type of broth is used for the classification of yeasts, based on carbon assimilation and for susceptibility testing of fungi.

### 3.1.3. Solutions

**PBS:** 4 g NaCl (Sigma-Aldrich), 0.1g KCl (Sigma), 0.72 g NaH₂PO₄ (Sigma) and 0.12 g KH₂PO₄ (Sigma) are added to 500 mL distilled water. The magnetic stirrer is used to dissolve this mixture. The pH is adjusted to 7.4 with 1M HCl. The broth is sterilized with the autoclave at 121°C for 15 minutes.

**FBS:** fetal bovine serum, Biochrom AG, stored at -20°C, lot. 0202A, cat. S0115, ISO 900 certified

**Farnesol:** a 50 mM solution of trans, trans farnesol is prepared in a screw thread glass vial by diluting 6.2 µL of stock farnesol (Sigma-Aldrich) in 492 µL methanol under nitrogen flow, because farnesol is oxygen sensitive.

### 3.1.4. Strains

#### 3.1.4.1. *Bacillus subtilis AC7*

Biosurfactant is produced by *Bacillus subtilis AC7*. This strain was isolated from a *Robinia pseudoacacia* stem, collected in Novara. These types of trees naturally occur in the southeastern of the United States, but they are also cultivated in Europe. The biosurfactant produced by this strain is a lipopeptide composed of surfactin and fengicin. Storage is at -80°C in LB broth, supplemented with glycerol (25% V/V). For the production of biosurfactant, the strain is grown on LB agar at 28°C.
3.1.4.2. Candida albicans 40

C. albicans 40, isolated from a central venous catheter, is provided by the hospital “Maggiore della Carità” in Novara. The C. albicans 40 strain is stored at -80°C in Sabouraud dextrose broth with 25% V/V glycerol added. When needed, the strain is thawed and transplanted onto SDA and incubated at 37°C.

3.2 METHODS

3.2.1 Biosurfactant AC7

3.2.1.1 Production

For the biosurfactant production, a loopful of Bacillus subtilis AC7 (see 3.1.4.1) is transferred into 20 mL of LB broth. The Erlenmeyer flask is incubated for 4 hours at 28°C at 120 rpm. Two milliliters of this pre-inoculum are transferred into 500 mL fresh LB broth. The Erlenmeyer flask is then incubated for 24 hours at 28°C at 120 rpm. After transferring the solution into sterile centrifuge tubes, they are centrifuged at 6000 rpm for 20 minutes (Centrifuge 1). The supernatant is acidified to pH 2.2 with 6 M HCl to precipitate the biosurfactant. The supernatant is stored overnight at 4°C for biosurfactant precipitation.

3.2.1.2 Surface tension of the Bacillus subtilis AC7 supernatant

The surface tension is measured with a tensiometer (KSV Sigma 703D, Finland). It is the force that is needed to move a platinum-iridium ring with constant velocity out of a solution into the air. The ring is put in acetone for cleaning and flame sterilized before using it. The tensiometer is first calibrated by measuring the surface tension of 20 mL Milli-Q water (Millipore, Italy) at pH 8. Then, the surface tension of 20 mL LB broth (pH 8) and finally the surface tension of 20 mL non-acidified AC7 supernatant (pH 8) is determined. Each measurement is performed in quadruplicate. When a reduction in surface tension is observed, this indicates the presence of surface active compounds (SAC) in the supernatant.

3.2.1.3 Extraction with organic solvents

Based on the method described by Rivardo et al. (2009) the biosurfactant is extracted three times using an ethylacetate/methanol (4:1) (Sigma-Aldrich) solution. The organic phase is transferred to an Erlenmeyer flask and the remaining water is removed with sodium sulfate (Sigma-Aldrich). Using a rotary evaporator (RV10 IKA basic), the organic fraction is evaporated to dryness under vacuum conditions. Then, a few drops of acetone (Sigma-
Aldrich) are added to recover the raw biosurfactant. Acetone is evaporated at room temperature and the dry biosurfactant is weighed.

### 3.2.1.4 Preparation of biosurfactant AC7 2000 µg/mL solution

A 2000 µg/mL solution of biosurfactant is prepared when needed. One hundred milligrams of biosurfactant AC7 (BS AC7) are dissolved in 30 mL PBS under alkaline conditions. To obtain this condition, a few drops of 1M NaOH are added till pH 9 is reached. The pH is controlled with the pH meter (Hanna-Instruments pH 211 Microprocessor pH meter), which is calibrated with solutions of pH 7.04 and pH 4.01. Finally, the pH is brought to 7 with 1M HCl. PBS is added till the final volume of 50 mL. Membrane filtration (0.22 µm pores) is used to sterilize the solution in a sterile flask. This preparation is stored at 4°C.

### 3.2.1.5 Critical micelle concentration (CMC)

To evaluate the critical micelle concentration, serial dilutions (1:2) of a 2000 µg/mL solution of BS AC7 are prepared with alkaline demineralized water (pH 8). The tensiometer (KSV Sigma 703D, Finland) is first calibrated with the alkaline water (pH 8). The surface tension of 20 mL of each dilution is measured in quadruplicate in the same manner as described in 3.2.1.2. The surface tension is plotted as a function of the BS AC7 concentration. The CMC is obtained from the intercept of the tangents on the concentration-dependent and concentration-independent sections of this curve. The corresponding concentration is the CMC of BS AC7.

### 3.2.2 Washing of the silicone elastomeric disks

This method is based on the method described by Busscher et al (1997). After wearing latex gloves to prevent contamination with the hands, the disks (Tecnextr.) of 15 mm diameter and 1.5 mm thickness are immersed in a 200 mL solution of milli-Q water containing 1.4% RBS solution as detergent. The solution is then sonicated for 5 minutes to remove fatty acids from the surface of the disks. After sonating, the disks are washed in 1 L of milli-Q water by rubbing with the hands over the surface. This step is repeated once. In the next step, the disks are submerged in 50 mL methanol (Sigma-Aldrich) and sonicated for another 5 minutes, again followed by two washing steps in 1 L of milli-Q water. The disks are then placed on a water absorbing paper in a glass Petri dish (diameter of 12 cm) to prevent
that they stick on the surface. Finally, the Petri dish is sterilized by autoclaving at 121°C for 15 minutes.

### 3.2.3 C. albicans 40 biofilm formation

#### 3.2.3.1 Adhesion

According to the method described by Chandra et al. (2008), a pre-inoculum is prepared by dissolving 2-3 colonies of *C. albicans* 40 (see 3.1.4.2) into 25 mL YNBD broth in a 100 mL sterile Erlenmeyer flask. The flask is incubated overnight at 37°C at 140 rpm. After 18-20 hours of incubation, the pre-inoculum is centrifuged in sterile centrifuge tubes (Sterilin LTD) for 10 minutes at 4000 rpm (centrifuge 2). The supernatant is removed and the pellet is resuspended in 25 mL of PBS, using the vortex mixer. This step is followed by centrifuging for 10 minutes under the same conditions. The washing procedure is repeated another time. After this, the pellet is resuspended in 25 mL PBS containing 10% FBS and mixed with the vortex. The suspension is diluted with PBS containing 10% FBS to obtain an OD$_{600}$ 1.0, measured with the spectrophotometer (Biophotometer Eppendorf, Hamburg, Germany). Two milliliters of the *Candida albicans* suspension are then added to each well of a sterile 12-well plate (Costar, NY, USA) and the 12-well plate is incubated for 90 minutes at 37°C in static conditions. Each well is then gently washed three times with PBS to remove non-attached *C. albicans* cells.

#### 3.2.3.2 Biofilm growth

The method as described in 3.2.3.1 is repeated. After 90 minutes incubation, the disks are transferred with a sterile tweezer in a new 12-well plate. In each well, 2 mL of YNBD are added and the plate is incubated for 24 hours at 37°C in static conditions, followed by washing each well three times with PBS.

### 3.2.4 Pre-coating of the disks

The silicone disks are pre-coated with fetal bovine serum (FBS), which forms a conditioning film that promotes early adhesion (Frade et al., 2010).

Cleaned sterile silicone disks are transferred in a 12-well plate using a sterile tweezer. Two milliliters of FBS are added and the disks are submerged in this solution. The 12-well plate is incubated for 24 hours at 37°C at 140 rpm. FBS is removed after 24 hours of
incubation and the disks are washed one time with 2 mL PBS. This pre-coating step is done for all the following experiments.

3.2.5 Influence of farnesol

In these experiments, based on the method described by Deveau et al. (2011), 6 wells of a 12-well plate are used to investigate the influence of farnesol and the other 6 wells are used as controls.

3.2.5.1 Adhesion

A pre-inoculum and a C. albicans OD<sub>600</sub> 1.0 suspension are prepared as described in 3.2.3.1. The suspension is then diluted again to obtain a solution OD<sub>600</sub> 0.2, corresponding to ~10<sup>6</sup> CFU/mL. To confirm this concentration, a serial dilution is prepared from 10<sup>-1</sup> till 10<sup>-5</sup> and 25 µL of the 10<sup>-3</sup>, 10<sup>-4</sup>, 10<sup>-5</sup> dilutions are spread in triplicate onto SDA plates. The plates are incubated overnight at 37°C and the colonies are counted using the stereomicroscope (Nikon SMZ200).

A solution with 12 mL of the C. albicans OD<sub>600</sub> 0.2 suspension and 24 µL farnesol, to obtain a concentration of 100 µM is prepared. Control consists of another solution containing 12 mL of the C. albicans OD<sub>600</sub> 0.2 suspension contacted with 24 µL methanol. Two milliliters of the control solution are added to 6 wells and 2 mL of the farnesol solution are transferred to the other 6 wells. The 12-well plate is then incubated for 90 minutes in static conditions at 37°C. After 90 minutes, each well is gently washed three times with PBS to remove non-attached C. albicans cells.

3.2.5.2 Biofilm formation (addition after 90 minutes of adhesion)

In the same manner as described in 3.2.5.1, a C. albicans OD<sub>600</sub> 0.2 suspension is prepared. Two milliliters of this suspension are added to each well of a 12-well plate with a sterile tweezer. The plate is then incubated for 90 min at 37°C in static conditions. After 90 minutes of incubation, the disks are transferred in a new 12-well plate. Three milliliters of YNBD, containing 100 µM farnesol are then added to 6 wells and 3 ml of YNBD, containing methanol are added to the 6 control wells. The plate is incubated for 24 hours at 37°C at 80 rpm and each well is washed three times with PBS.
3.2.5.3 Biofilm formation (addition at time zero and after 90 minutes of adhesion)

The procedure described in 3.2.5.1 is repeated, adding 24 µL farnesol (final concentration 100 µM) or methanol to 12 mL of the C. albicans OD<sub>600</sub> 0.2 suspension. After 90 minutes of incubation in static conditions, the disks are transferred with a sterile tweezer in a new 12-well plate. They are then submerged in 3 mL of YNBD, containing methanol for the 6 control wells, and 100 µM farnesol for the 6 treated wells. The plate is incubated for 24 hours at 37°C at 80 rpm, followed by washing each well three times with PBS.

3.2.5.4 Pre-formed biofilm

The same method as mentioned in 3.2.5.2 is repeated, but now there is no farnesol or methanol added to the YNBD solution. After 24 hours of incubation at 80 rpm at 37°C, the silicone disks are transferred with a sterile tweezer to a new 12-well plate. They are submerged in 3 mL of YNBD, containing methanol for the 6 control wells, and 100 µM farnesol for the 6 treated wells. The plate is again incubated for 24 hours at 37°C at 80 rpm. Each well is then gently washed three times with PBS.

3.2.6 Influence of biosurfactant AC7 2000 µg/mL

In these experiments, 6 wells of a 12-well plate are used to investigate the influence of BS AC7 and the other 6 wells are used as controls.

3.2.6.1 Adhesion

After pre-coating sterile, cleaned disks with FBS, 2 mL BS AC7 are added in 6 wells and 2 mL PBS in 6 control wells. The 12-well plate is then incubated for 24 hours at 37°C at 140 rpm. Subsequently, BS AC7 and PBS are removed. A C. albicans OD<sub>600</sub> 0.2 suspension is prepared using the method described in 3.2.5.1. Two milliliters of this Candida suspension is added in each well. The plate is incubated for 90 minutes at 37°C in static conditions, followed by washing each well three times with PBS.

3.2.6.2 Biofilm formation

The same procedure as followed in 3.2.6.1. is repeated but after 90 minutes of incubation, the disks are transferred in a new 12-well plate. Three milliliters YNBD is added in each well.
and the plate is incubated for 24 hours at 37°C at 80 rpm. After 24 hours, each well is washed three times with PBS.

3.2.7 Synergistic effect of farnesol and biosurfactant AC7

To know the influence of BS AC7 and its possible synergistic effect with farnesol on biofilm formation, the disks are coated with 2 mL of 2000 µg/mL BS AC7 and added in 6 wells. Two milliliters of PBS are added in each of the other 6 wells. The 12-well plate is then incubated for 24 hours at 37°C at 140 rpm. After 24 hours, BS AC7 is removed. The distribution of the 12-well plate for the following experiments is shown in Fig. 3.1. The experiments are performed in triplicate for each condition.

![Fig 3.1 Distribution of the 12-well plate: control wells (grey), disks treated with farnesol (red), disks pre-coated with BS (white), disks pre-coated with BS and treated with farnesol (yellow)](image)

3.2.7.1 Adhesion

After coating the disks with BS AC7 or PBS, a C. albicans suspension at OD₆₀₀ 0.2 is prepared (see 3.2.5.1). Two milliliters of this suspension, containing methanol are added in the control wells (grey in Fig. 3.1) and to the BS wells (white in Fig. 3.1) and 2 mL of this suspension, containing 100 µM farnesol are added to the farnesol wells (red in Fig. 3.1) and to the farnesol + BS wells (yellow in Fig. 3.1). The 12-well plate is then incubated for 90 minutes in static conditions at 37°C. Subsequently, each well is washed three times with PBS.

3.2.7.2 Biofilm formation (addition after 90 minutes of adhesion)

The procedure described in 3.2.7.1 is repeated, but 2 mL of the C. albicans suspension with OD₆₀₀ 0.2 (see 3.2.5.1) is added to each well, without farnesol or methanol. After 90 minutes of adhesion, the disks are transferred in a new 12-well plate. They are submerged in 3 mL of YNBD containing methanol for the control and BS wells and 3 mL YNBD with 100 µM farnesol for the farnesol and farnesol + BS wells (see Fig. 3.1). The plate is then
incubated for 24 hours at 37°C at 140 rpm, followed by washing each well three times with PBS.

3.2.7.3 Biofilm formation (addition at time zero and after 90 minutes of adhesion)

The method described in 3.2.7.1 is repeated and after 90 minutes of incubation, the disks are transferred in a new 12-well plate. Three milliliters of YNBD, containing methanol are added in the control and BS wells and 3 mL of YNBD, containing 100 µM farnesol are added in the farnesol and farnesol + BS wells (see Fig. 3.1). The plate is incubated for 24 hours at 37°C at 80 rpm and each well is then washed three times with PBS.

3.2.7.4 Pre-formed biofilm

The silicone disks, coated with BS or PBS, are submerged in 2 mL of a C. albicans OD_{600} 0.2 suspension (see 3.2.5.1) and incubated for 90 minutes at 37°C in static conditions. After 90 minutes of adhesion, the disks are transferred to a new 12-well plate. After this, 3 mL of YNBD are added in each well and the plate is incubated for 24 hours at 37°C at 80 rpm. In a next step, the disks are transferred to another 12-well plate and submerged in 2 ml of YNBD containing methanol for the control and BS wells, and containing 100 µM farnesol for the farnesol and farnesol + BS wells. Subsequently, the plate is incubated for 24 hours at 37°C in static conditions. Each well is then subjected to three washing steps with PBS.

3.2.8 Quantification by plate counting

After the necessary incubation time and three times washing with PBS, the disks are transferred with a sterile tweezer in sterile tubes containing 10 mL PBS. Each tube is subjected for four minutes to a specific treatment consisting of alternation of sonicating for 30 seconds and mixing for 30 seconds with vortex. Then, a serial dilution is prepared from 10^{-1} till 10^{-3} and 25 µL of these solutions are spread in triplicate onto SDA plates. The plates are incubated for 18-20 hours at 37°C and the colonies are counted using the stereomicroscope (Nikon SMZ200).

The following formula is used to calculate the number of cells, which is expressed as mean CFU/mL.

\[
N= \text{colony forming units/mL in primary dilution} = \frac{\sum C}{(V(n_1 + 0.1\times n_2) + d)}
\]  
(1)
N = colony forming units/mL in primary dilution.
Σ C = Sum of colonies on the considered plates.
V = Volume of inoculum plated, expressed in mL.
n₁ = number of plates considered for the first dilution.
n₂ = number of plates considered for the second dilution.
d = factor of dilution corresponding with the first dilution.

\[ CFU/disk = N \times V_2 \] (2)

N = colony forming units/mL in the first dilution (1)
V₂ = Volume of the first dilution.

The results are expressed as logarithmic values of CFU/disk.

**3.2.9 Percentage inhibition on different stages of biofilm formation**

The percentage of inhibition on the different stages of biofilm formation is calculated according to following formula:

\[ \% \text{Microbial inhibition} = \left[ 1 - \left( \frac{x_1}{x_2} \right) \right] \times 100 \] (3)

x₁ = CFU/disk for farnesol, BS AC7, or both together (depending on the performed experiment)

x₂ = CFU/disk for control

**3.2.10 Statistical analysis**

The standard deviation is calculated and visualized by error bars in the graphs. Data are analysed with the Wilcoxon test for the experiments with farnesol alone. The ANOVA test is used to analyse data from the experiments with farnesol and biosurfactant together. The Wilcoxon test is the equivalent of the Mann-Whitney U test and is a nonparametric test that is used to determine if the difference between the means of 2 groups of experimental values, obtained under different conditions, is statistically significant.
The ANOVA test is a statistical test to determine if the means of several groups are all equal or not.

The statistical program R (R Development Core Team, http://www.R-project.org) is used to carry out the statistical analysis.

The level of statistical significance is indicated by the sign *

* = $p < 0.05$ ; ** = $p < 0.01$ ; *** = $p < 0.001$
4. RESULTS

4.1. SURFACE TENSION OF *BACILLUS SUBTILIS AC7* SUPERNATANT

The tensiometer is calibrated with milli-Q water (pH 8) and a mean surface tension of 74.96 mN/m is measured. A mean surface tension of 63.61 mN/m is observed for the LB broth (pH 8), while for the supernatant of the *Bacillus subtilis AC7* inoculum (pH 8), a mean surface tension of 31.73 mN/m is measured, thus indicating the production of surface-active compounds.

4.2. CRITICAL MICELLE CONCENTRATION

The surface tension is visualised in the graph (Fig. 4.1) as a function of the biosurfactant AC7 concentration. A more or less constant surface tension value of 35 mN/m is observed with concentrations of BS AC7 from 2000 µg/mL up to 300 µg/mL, visualised by the concentration independent blue line. This stable surface tension, even with higher concentration of BS AC7 can be explained by the formation of structural aggregates, e.g. micelles. For concentrations lower than 300 µg/mL, a faster increase in surface tension is observed and this is visualized by the concentration dependent red line. The CMC is obtained from the intercept of the tangents on the concentration-dependent and concentration-independent sections of this curve. The CMC value for BS AC7 is calculated as 208.5 µg/mL.

![CMC graph](image-url)

**Fig. 4.1.** Plot of the surface tension (mN/m) as a function of the BS AC7 concentration (µg/mL).
4.3. **C. ALBICANS 40 BIOFILM FORMATION**

4.3.1. **Adhesion and biofilm growth**

In this experiment, the silicone disks are submerged in a *C. albicans* OD$_{600}$ 1.0 suspension, containing 10% FBS and the plate is incubated for 90 minutes for the adhesion. The mean log$_{10}$ CFU/disk for adhesion is 6.47. Subsequently the silicone disks are transferred in a new 12-well plate and incubated for 24 hours for biofilm growth. The mean log$_{10}$ CFU/disk is 7.37 (Fig 4.2).

![Fig. 4.2. Log$_{10}$ mean CFU/disk for *C. albicans* 40 after 90 minutes adhesion and 24 hours biofilm formation.](image)

4.4. **FARNESOL**

The distribution of the control wells and farnesol wells in the 12-well plate for the following experiments is shown in Fig. 4.3.

![Fig. 4.3. Distribution of the 12-well plate: control wells (grey) and farnesol treated wells (white).](image)

4.4.1. **Effect of farnesol on *C. albicans* 40 adhesion**

In this experiment, silicone disks, pre-coated with FBS, are treated with farnesol 100 µM added to the *C. albicans* OD$_{600}$ 0.2 suspension. The mean log$_{10}$ CFU/disk for control disks is
5.45, whereas the value for farnesol treated disks is 5.26. Farnesol 100 µM shows an inhibition of *C. albicans* adhesion to silicone disks of 36% in comparison to the control wells containing methanol, as shown in Fig. 4.4. The results are statistically significant as shown in Table 4.1.

Fig. 4.4. Effect of farnesol 100 µM on *C. albicans* 40 adhesion in comparison to control disks (0 µM). The percentage of inhibition is shown above the graph in red. The symbol ** indicates the degree of statistical significance (p < 0.01) (see 3.2.9).

Table 4.1. Statistical analysis. The experimental results are analysed using the Wilcoxon test with significance level of 5%

<table>
<thead>
<tr>
<th>Farnesol concentration (µM)</th>
<th>Wilcoxon test</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.002165</td>
</tr>
</tbody>
</table>

4.4.2. Effect of farnesol on *C. albicans* 40 biofilm formation (addition after 90 minutes of adhesion)

Silicone disks, pre-coated with FBS are contacted with a *C. albicans* OD<sub>600</sub> 0.2 suspension and incubated for 90 minutes. Then, farnesol 100 µM in YNBD is added before the biofilm growth, and compared with control wells containing methanol in YNBD. The mean log<sub>10</sub> CFU/disk for control disks is 7.12, while it is 6.23 for farnesol treated disks. An inhibition of 86% on the *C. albicans* 40 biofilm formation is found with farnesol 100 µM in comparison to the controls containing methanol (Fig. 4.5) and this result is statistically significant (Table 4.2).
Fig. 4.5. Effect of farnesol 100 µM in comparison to control disks (0 µM) on *C. albicans* 40 biofilm formation. The percentage of inhibition is shown above the graph in red. The symbol ** indicates the degree of statistical significance (p < 0.01) (see 3.2.9).

Table 4.2. Statistical analysis. The experimental results are analysed using the Wilcoxon test with significance level of 5%.

<table>
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<th>Farnesol concentration (µM)</th>
<th>Wilcoxon test</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0.007937</td>
</tr>
</tbody>
</table>

4.4.3. Effect of farnesol on *C. albicans* 40 biofilm formation (addition at time zero and after 90 minutes of adhesion)

After pre-coating with FBS, the silicone disks are treated with farnesol 100 µM added in a *C. albicans* OD$_{600}$ 0.2 suspension and incubated for 90 minutes. Then, disks are again treated with farnesol 100 µM in YNBD and incubated for 24 hours. Control disks are treated with methanol in a *C. albicans* OD$_{600}$ 0.2 suspension (90 minutes) and with methanol in YNBD (24 hours).

Fig. 4.6. Effect of farnesol 100 µM in comparison to control disks (0 µM) on *C. albicans* 40 biofilm formation. The percentage of inhibition is shown above the graph in red. The symbol ** indicates the degree of statistical significance (p < 0.01) (see 3.2.9).
A value of 7.47 log\textsubscript{10} CFU/disk is observed for control disks and a value of 6.11 log\textsubscript{10} CFU/disk is observed for farnesol treated disks. Farnesol shows a 95\% inhibition of biofilm formation by \textit{C. albicans} 40 when compared to the control disks (Fig. 4.6). This result is statistically significant (Table 4.3).

<table>
<thead>
<tr>
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<th>Wilcoxon test</th>
</tr>
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<tbody>
<tr>
<td>100</td>
<td>0.002165</td>
</tr>
</tbody>
</table>

4.4.4. Effect of farnesol on \textit{C. albicans} 40 pre-formed biofilm

Silicone disks pre-coated with FBS are contacted with a \textit{C. albicans} OD\textsubscript{600} 0.2 suspension and incubated for 90 minutes. After adding fresh YNBD, the disks are incubated for further 24 hours. Farnesol 100 µM in YNBD is then added and the plate is incubated for another 24 hours. The mean log\textsubscript{10} CFU/disk is 8.11 for control disks and 7.99 for farnesol treated disks. Compared to controls, incubated with methanol in YNBD, the treated disks show an inhibition of 24\% on pre-formed biofilm as shown in Fig. 4.7. Statistical significance is proven (Table 4.4).

![Graph showing effect of farnesol on pre-formed biofilm](image)

**Fig. 4.7.** Effect of farnesol 100 µM on \textit{C. albicans} 40 pre-formed biofilm compared to control disks (0 µM farnesol). The percentage of inhibition is shown above the graph in red. The symbol ** indicates the degree of statistical significance (p < 0.01) (see 3.2.9).

<table>
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<th>Farnesol concentration (µM)</th>
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<tbody>
<tr>
<td>100</td>
<td>0.004922</td>
</tr>
</tbody>
</table>
4.5. BIOSURFACTANT

4.5.1. Effect of biosurfactant AC7 (pre-coated disks) on *C. albicans* 40 adhesion

Silicone disks pre-coated with FBS and coated with BS AC7 for 24 hours, are contacted with a *C. albicans* OD<sub>600</sub> 0.2 suspension and incubated for 90 minutes. For controls, a value of 5.53 log<sub>10</sub> CFU/disk is observed, whereas this value is 5.24 log<sub>10</sub> CFU/disk for disks pre-coated with BS AC7. An inhibition of 48% of *C. albicans* 40 adhesion is found for disks pre-coated with BS AC7 in comparison to control disks that are coated with PBS (Fig. 4.8). This result is statistically significant (Table 4.5).

![Graph showing inhibition of C. albicans adhesion](image)

Fig. 4.8. Effect of 2000 µg/mL BS AC7 (pre-coated disks) on *C. albicans* 40 adhesion in comparison to control disks, pre-coated with PBS (0 µg/mL BS AC7). The percentage of inhibition is shown above the graph in red. The symbol * indicates the degree of statistical significance (p < 0.05) (see 3.2.9).

Table 4.5. Statistical analysis. The experimental results are analysed using the Wilcoxon test with significance level of 5%.

<table>
<thead>
<tr>
<th>BS AC7 concentration (µg/mL)</th>
<th>Wilcoxon test</th>
</tr>
</thead>
<tbody>
<tr>
<td>2000</td>
<td>0.02857</td>
</tr>
</tbody>
</table>

4.5.2. Effect of biosurfactant AC7 (pre-coated disks) on *C. albicans* 40 biofilm formation

In this experiment, the silicone disks, pre-coated with FBS and coated with BS AC7 2000 µg/mL for 24 hours, are contacted with a *C. albicans* OD<sub>600</sub> 0.2 suspension and incubated for 90 minutes. Subsequently, the disks are submerged in YNBD and the plate is incubated for 24 hours. The mean log<sub>10</sub> CFU/disk is 7.79 for control disks and 7.67 for disks pre-coated with BS AC7. An inhibition of 25% on *C. albicans* 40 biofilm formation is observed for disks pre-
coated with BS AC7 in comparison to control disks coated with PBS (Fig. 4.9). This result is statistically significant (Table 4.6).

![Bar chart showing inhibition of C. albicans biofilm formation](image)

**Fig. 4.9.** Effect of 2000 µg/mL BS AC7 (pre-coated disks) on *C. albicans* 40 biofilm formation in comparison to control disks, pre-coated with PBS (0 µg/mL BS AC7). The percentage of inhibition is shown above the graph in red. The symbol * indicates the degree of statistical significance (p < 0.05) (see 3.2.9).

**Table 4.6.** Statistical analysis. The experimental results are analysed using the Wilcoxon test with significance level of 5%.

<table>
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<tbody>
<tr>
<td>2000</td>
<td>0.02857</td>
</tr>
</tbody>
</table>

### 4.6. EFFECT OF FARNESOL AND BIOSURFACTANT COMBINED

The distribution of the 12-well plate for the following experiments is shown in Fig. 4.10.

![Distribution of 12-well plate](image)

**Fig 4.10.** Distribution of the 12-well plate: control wells (grey), disks treated with farnesol (red), disks pre-coated with BS (white), disks pre-coated with BS AC7 and treated with farnesol (yellow).
4.6.1. Effect of farnesol and biosurfactant AC7 (pre-coated disks) on C. albicans 40 adhesion

All silicone disks are pre-coated with FBS. Control disks, coated with PBS (grey in Fig. 4.10) and BS disks, coated with BS AC7 2000 µg/mL (red in Fig. 4.10), are contacted with a C. albicans OD_{600} 0.2 suspension containing methanol. Farnesol disks, coated with PBS (white in Fig. 4.10) and farnesol + BS disks, coated with BS AC7 2000 µg/mL (yellow in Fig. 4.10), are contacted with a C. albicans OD_{600} 0.2 suspension containing farnesol 100 µM. The plate is incubated for 90 minutes. The mean log_{10} CFU/disk is 5.23 for control disks, 5.08 for farnesol disks, 5.03 for BS AC7 disks and 4.84 for farnesol + BS AC7 disks. The results show an inhibition of adhesion of 29% with farnesol alone, of 37% with BS alone, and of 59% with farnesol and BS AC7 together, in comparison to the controls (Fig 4.11). All results are statistically significant with a p-value of 0.0002314.

One-way analysis of variance (ANOVA):  
p-value = 0.0002314

Fig. 4.11. Effect of farnesol 100 µM, biosurfactant AC7 2000 µg/mL (pre-coated disks) and farnesol 100 µM + AC 7 2000 µg/mL on C. albicans 40 adhesion compared to control disks. The percentage of inhibition is shown above the graph in red. The p-value amounts 0.0002314 (ANOVA). The symbol *** indicates the degree of statistical significance (p < 0.001) (see 3.2.9).

4.6.2. Effect of farnesol and biosurfactant AC7 (pre-coated disks) on C. albicans 40 biofilm formation (addition after 90 minutes of adhesion)

After pre-coating silicone disks with FBS, control disks (grey in Fig. 4.10) and farnesol disks (white in Fig. 4.10) are coated with PBS, while BS AC7 disks (red in Fig. 4.10) and farnesol + BS AC7 disks (yellow in Fig. 4.10) are coated with BS AC7 2000 µg/mL for 24 hours. Subsequently, all disks are treated with a C. albicans OD_{600} 0.2 suspension. The plate
is incubated for 90 minutes. Thereafter, farnesol disks and farnesol + BS AC7 disks are submerged in YNBD containing 100 µM farnesol, while control disks and BS AC7 disks are submerged in YNBD with methanol added. The plate is then incubated for 24 hours. Controls show a mean log_{10} CFU/disk of 7.75, farnesol disks show a value of 6.73, while for BS AC7 disks and farnesol + BS AC7 disks a value of respectively 7.67 and 7.54 log_{10} CFU/disk is observed. The results show an inhibition of biofilm formation of 91% with farnesol alone in comparison to the controls, of 17% with BS AC7 alone, and of 38% with farnesol and BS AC7 together, in comparison to the controls (Fig 4.12). Statistical significance of the results is proven with a p-value of 1.309 * 10^{-10}.

One-way analysis of variance (ANOVA):
p-value = 1.309e-10

Fig. 4.12. Effect of farnesol 100 µM, BS AC7 2000 µg/mL (pre-coated disks) and farnesol 100 µM + BS AC7 2000 µg/mL on C. albicans 40 biofilm formation compared to control disks. The percentage of inhibition is shown above the graph in red. The p-value amounts 1.309 * 10^{-10} (ANOVA). The symbol *** indicates the degree of statistical significance (p < 0.001) (see 3.2.9).

4.6.3. Effect of farnesol and biosurfactant AC7 (pre-coated disks) on C. albicans 40 biofilm formation (addition at time zero and after 90 minutes of adhesion)

All silicone disks are pre-coated with FBS. A C. albicans OD_{600} 0.2 suspension containing methanol is added to control disks, coated with PBS (grey in Fig. 4.10) and BS disks, coated with BS AC7 2000 µg/mL (red in Fig. 4.10). Farnesol disks, coated with PBS (white in Fig. 4.10) and farnesol + BS AC7 disks, coated with BS AC7 2000 µg/mL (yellow in Fig. 4.10), are contacted with a C. albicans OD_{600} 0.2 suspension containing 100 µM farnesol. The plate is incubated for 90 minutes. Subsequently, control disks and BS AC7 disks are submerged in
YNBD containing 100 µM farnesol, while farnesol and farnesol + BS AC7 disks are submerged in YNBD containing methanol. The plate is then incubated for 24 hours. The mean log\textsubscript{10} CFU/disk is 7.73 for control disks, 6.64 for farnesol disks, 7.71 for BS AC7 disks and 7.42 for farnesol + BS AC7 disks. The results show an inhibition of biofilm formation of 92% with farnesol alone, of 5% with BS AC7 alone, and of 51% with farnesol and BS AC7 together, in comparison to the controls (Fig 4.13). All results are statistically significant with a p-value of 1.204 * 10\textsuperscript{-7}.

One-way analysis of variance (ANOVA):
p-value = 1.204e-07

Fig. 4.13. Effect of farnesol 100 µM, BS AC7 2000 µg/mL (pre-coated disks) and farnesol 100 µM + BS AC7 2000 µg/mL on C. albicans 40 biofilm formation compared to control disks. The percentage of inhibition is shown above the graph in red. The p-value amounts 1.204 * 10\textsuperscript{-7} (ANOVA). The symbol *** indicates the degree of statistical significance (p < 0.001) (see 3.2.9).

4.6.4. Effect of farnesol & biosurfactant AC7 (pre-coated disks) on C. albicans 40 pre-formed biofilm

After pre-coating silicone disks with FBS, control disks (grey in Fig. 4.10) and farnesol disks (white in Fig. 4.10) are coated with PBS, while BS AC7 disks (red in Fig. 4.10) and farnesol + BS AC7 disks (yellow in Fig. 4.10) are coated with BS AC7 2000 µg/mL for 24 hours. Subsequently, all disks are treated with a C. albicans OD\textsubscript{600} 0.2 suspension. The plate is incubated for 90 minutes, thereafter, all disks are submerged in YNBD and incubated for 24 hours. Then, control disks and BS AC7 disks are submerged in YNBD containing methanol while farnesol and farnesol + BS AC7 disks are submerged in YNBD containing 100 µM farnesol. The plate is incubated another 24 hours. Controls show a mean log\textsubscript{10} CFU/disk of 8.16, farnesol disks show a value of 8.08, while for BS AC7 disks and farnesol + BS AC7 disks a value of respectively 8.05 and 7.91 log\textsubscript{10} CFU/disk is observed. The results show an
inhibition on pre-formed biofilm of 17% with farnesol alone, of 23% with BS AC7 alone, and of 44% with farnesol and BS AC7 together, in comparison to the controls (Fig 4.14). All results are statistically significant with a p-value of 0.008298.

Fig. 4.14. Effect of farnesol 100 µM, BS AC7 2000 µg/mL (pre-coated disks) and farnesol 100 µM + BS AC7 2000 µg/mL on C. albicans 40 pre-formed biofilm compared to control disks. The percentage of inhibition is shown above the graph in red. The p-value amounts 0.008298 (ANOVA). The symbol *** indicates the degree of statistical significance (p < 0.001) (see 3.2.9).
5. DISCUSSION

This thesis aimed to determine the activity of the quorum-sensing molecule farnesol and of the lipopeptide biosurfactant, produced by *Bacillus subtilis* AC7 (BS AC7) on *C. albicans* 40 biofilms. Another aspect focused on the evaluation of the possible synergistic effect of the biosurfactant AC7 and farnesol against *C. albicans* 40 biofilm. In particular, their influence on *Candida* adhesion, biofilm formation (farnesol added at different time points) and on pre-formed biofilm was studied on medical-grade silicone disks. The *Candida albicans* 40 strain had previously been isolated from a central venous catheter.

The susceptibility of *Candida* biofilms to antifungal agents can be investigated by determining the decrease in metabolic activity, by determining the cell viability through measuring the colony forming units or by the crystal violet assays (CV) (Chandra et al., 2008). In this thesis, the viable cell counting method is used to determine the percentage of inhibition on different stages of biofilm formation because of its high precision. Medical-grade silicone disks are used to mimic catheter material. The disks are coated with FBS to promote adhesion and to imitate *in vivo* conditions of biofilm formation (Deveau et al., 2011).

According to manufacturer’s instructions, YNBD broth should be prepared with pH 5.4, but Krom *et al.* (2007) showed that biofilms grown at pH 7 were the most dense ones and the biomass lowered with decreasing pH.

5.1. SURFACE TENSION OF *BACILLUS SUBTILIS* AC7 SUPERNATANT AND CMC OF BS AC7

Since *Bacillus subtilis* AC7 is grown in LB broth, this medium is used as reference for the determination of the surface tension reduction. A reduction of surface tension from 63.61 mN/m for the LB broth (pH 8) to 31.73 mN/m for AC7 supernatant is observed. This indicates the presence of surface-active compounds in the supernatant, which lower surface tension. Desai & Banat (1997) described that an efficient biosurfactant is able to lower the surface tension between pure water and air from 72 mM/m to less than 30 mN/m.

The CMC of BS AC7 is 208.5 µg/mL, while the CMC of lipopeptides, such as surfactin, is situated between 20.00 and 160.0 µg/mL (Desai & Banat, 1997). The higher value that is observed, can be explained by the fact that the biosurfactant produced in this thesis is not as pure as that obtained in previous extractions carried out in the lab, where it showed a CMC value of 43.6 µg/ml.
5.2. FARNESOL

The method, used to determine the effect of farnesol on different stages of biofilm formation is based on the protocol described by Deveau et al. (2011).

Uppuluri et al. (2007) found that a concentration of 100 µM farnesol caused a 82% loss of cell viability for exponential phase cells. But when farnesol was added to 3 days old cells, only little loss of cell viability was seen. Possible explanations for this farnesol resistance can be that older cells have a thicker, more non-porous cell wall or that actively growing cells are more sensible for any disturbance (Uppuluri et al. 2007).

Jabra-Rizk et al. (2006) showed that farnesol (300 µM) had the highest inhibitory effect when it was incubated with biofilm at time zero. With the XTT method, a reduction in absorbance value of 60-80% was seen for C. albicans. The colorometric assay did not show a reduction in absorbance values when farnesol was added after 2-4 h of adhesion, while a decrease in absorbance values was found when it was added to 24 h pre-formed biofilms (Jabra-Rizk et al., 2006). Ramage et al. (2002) had similar results, with the highest effect of farnesol at time zero and on pre-formed biofilms, while he showed a decreased effect when he added farnesol after an initial attachment of 1 h. Both authors used XTT for biofilm quantification, while we used the viable cell counting method and they did not grow biofilms on silicone disks. In our experiments, farnesol 100 µM showed the highest inhibitory effect when added after 90 minutes of adhesion (86%) or when added at time zero and then added again after 90 minutes of adhesion (95%). Both experiments were evaluated after 24 h. A lower inhibitory effect is found when farnesol was added at time zero (36%) and was evaluated after 90 minutes (Candida adhesion) or when it was added to pre-formed biofilm (24%). These results are similar to the results of Ramage et al. (2002). The only difference occurred when farnesol is added after initial attachment of 90 minutes, which suggests that farnesol can have an effect even when hyphal formation is already initiated. Jabra-Rizk et al. (2006) showed that when the percentage killing of C. albicans is investigated with colony counts, the highest percentage (around 92%) is found when cells are exposed to farnesol 100 µM for 24 hours in comparison to exposure for 2 hours (around 40%). These results are similar to the results found in our experiments.

It can be concluded that farnesol succesfully lowers the potential of C. albicans 40 cells to adhere to silicone disks and to form biofilms on it.
5.3. BIOSURFACTANT AC7

The silicone elastomeric disks are coated with BS AC7 according to the method described by Rivardo et al. (2009).

The chemical characterization of BS AC7, previously performed by LC-MS/MS by the Laboratory of Analytical Chemistry (Prof. Gianna Allegrone) of the Department of Pharmaceutical Sciences in Novara, indicated that it is composed of 94% surfactin and 6% fengycin.

Biosurfactant AC7 shows no antimicrobial activity. This was proven by previous work done in the Laboratory of Microbiology in Novara. The MIC test showed that different concentrations of BS AC7 did not kill planktonic Candida cells. On the contrary BS AC7 is able to lower adhesion of C. albicans 40 cells to silicone disks of 48%, suggesting that when a decrease of C. albicans cells is observed on the silicone elastomeric disks, this is not caused by killing of planktonic cells. When the silicone disks, coated with BS AC7, were incubated for 24 hours, a lower percentage of inhibition (25%) is observed. BS AC7 is attached to the silicone disks in a non-covalent manner. We hypothesize that this causes a removal of some BS AC7 during the transfer from one 12-well plate to another, while non-attached cells are not removed by this handling. A biofilm is formed on the BS AC7 coated disks and because of the weight of growing biofilm, the BS AC7 coating can be damaged. This could explain the lower percentage of inhibition during 24 hours of biofilm growth with BS AC7 pre-coating.

Previous experiments, done in the Laboratory of Microbiology in Novara already showed that BS AC7 does not have a high influence on C. albicans biofilm formation, unlike on adhesion.

A lot of research has already been done to investigate the anti-adhesive effects of different biosurfactants against a variety of micro-organisms. Luna et al. (2011) described similar anti-adhesive activity (57%) on C. albicans cells with Lunasan, a biosurfactant produced by Candida sphaerica UCP0995.

Fracchia et al. (2010) observed an inhibition of adhesion of two C. albicans strains (CA-2894 and DSMZ 11225) by another biosurfactant, BS CV8LAC, produced by Lactobacillus CV8LAC. An inhibition of 82% of C. albicans CA-2894 cell adhesion was observed by pre-coating with 312.5 µg/mL biosurfactant CV8LAC. Pre-coating with a concentration of 625 µg/mL BS CV8LAC caused an 86% decrease in adhesion of C. albicans DSMZ 11225 cells. It was also shown that the CV8LAC biosurfactant did not show anti-microbial activity, but only had anti-adhesive effects. Flat-bottomed polystyrene 96-well microtiter plates were used for this experiment and quantification was done by the crystal violet method. The anti-
adhesive effect of different biosurfactants depends on their concentration, on the tested microorganism (Rufino et al., 2011) and on the characteristics of the surface on which the cells attach (Frade et al., 2009).

All these results suggest the potential of biosurfactants as effective agents to combat colonization of medical devices by pathogenic microorganisms.

5.4. FARNESOL AND BIOSURFACTANT

To our knowledge, no previous work has been performed on the possible synergistic effect of farnesol and biosurfactants. These results are preliminary and form an interesting starting point for further experiments. Generally, an additive effect is concluded on when the activity of a combination of two compounds is equal to the sum of the effects of each compound tested separately at the same concentration as in the combination, while a synergistic effect occurs when the activity of the combination is higher than the sum of the effects. Antagonism is observed when the combination decreases the activity of one of the compounds.

The results show an inhibition of adhesion of 29% with farnesol alone, of 37% with BS AC7 alone, and of 59% with farnesol and BS AC7 together. This suggests an additive effect of farnesol (100 µM) and BS AC7 (2000 µg/mL) on Candida albicans adhesion. We consider the effect as additive even though the strict criterion for additive effect is not completely fulfilled. A low synergistic effect was observed on pre-formed biofilm with an inhibition of 17% with farnesol alone, of 23% with BS AC7 alone, and of 44% with farnesol and BS AC7 together. However, an antagonistic effect was observed on biofilm formation with addition of farnesol after 90 minutes of adhesion (inhibition of 91% with farnesol alone, of 17% with BS AC7 alone, and of 38% with farnesol and BS AC7 together) and with addition of farnesol both at time zero and after 90 minutes of adhesion (inhibition of 92% with farnesol alone, of 5% with BS AC7 alone, and of 51% with farnesol and BS AC7 together). It is difficult to find an explanation for this antagonistic effect, because no previous work has been done on this subject and because we find a low synergistic and additive effect during other stages of biofilm formation. Apparently, farnesol 100 µM and BS AC7 interfere with each other during different stages of biofilm formation. These are the stages were farnesol 100 µM normally has the highest inhibitory effect. Further research has to be done to understand and explain the mechanisms for this observed interference.
5.5. FUTURE PERSPECTIVES

In the future, further research has to be done to find the molecular reason for the antagonistic effect of farnesol and BS AC7 during some stages of biofilm formation and the synergistic/additive effect during other stages.

It can be investigated if farnesol in a lower concentration than 100 µM also shows an antagonistic effect when combined with the same concentration of BS AC7 during different stages of biofilm formation, or if a synergistic/additive effect is observed during all stages of biofilm formation.

In our experiments, BS AC7 was attached to the silicone elastomeric disks in a non-covalent manner, which may explain its lower inhibitory effect when biofilm is grown for 24 hours in comparison to 90 minutes. In the future, other disks can be used to which BS AC7 is attached in a covalent way. This could prevent the removal of BS AC7 when transferred from one plate to another.

Being BS AC7 composed of surfactin and fengycin, another perspective will be to coat silicone elastomeric disks with surfactin only or fengycin only and to study their possible synergistic effect with farnesol.
6. CONCLUSIONS

The influence of farnesol, a quorum-sensing molecule, of the lipopeptide BS AC7, produced by *Bacillus subtilis* AC7, and of the combination of farnesol and BS AC7 on different stages of *C. albicans* 40 biofilm formation was investigated in this thesis.

Farnesol, at a concentration of 100 µM, shows an inhibitory effect on different stages of biofilm formation by *C. albicans* 40 on silicone elastomeric disks. The highest percentage of microbial inhibition by farnesol 100 µM is observed when it is added for 24 hours during biofilm growth. Lower percentages of inhibition are observed on *C. albicans* 40 adhesion and on pre-formed biofilm.

*Bacillus subtilis* AC7 is able to produce a potent biosurfactant which reduces surface tension from 63.61 mN/m for LB broth to 35 mN/m. The biosurfactant, produced by *Bacillus subtilis* AC7 consists of 96% surfactin and 4% fengycin. The lipopeptide BS AC7 (2000 µg/mL) causes a significant reduction adhesion of *C. albicans* 40 cells to the silicone disks at 90 minutes, indicating its anti-adhesive activity. A lower percentage of inhibition is observed with an incubation time of 24 hours.

When 100 µM farnesol and 2000 µg/mL BS AC7 are combined, an additive inhibitory effect on *C. albicans* 40 adhesion and a low synergistic effect on pre-formed biofilm are observed. However, these two compounds seem to interfere with each other during 24 hours *C. albicans* 40 biofilm growth.

These results suggest the potential use for these two compounds as preventive or therapeutical agents for *C. albicans* biofilms and infections. Further experiments should investigate the molecular reason for the synergistic/additive effect and for the antagonistic effect under different conditions. Other experiments should also be done with lower concentrations of farnesol, with covalent binding of BS AC7 to silicone disks, or with surfactin or fengycin only.
7. BIBLIOGRAPHY


http://www.faidherbe.org/site/cours/dupuis/canthar4.htm (16/04/2013)


