Universidade do Minho
Escola de Engenharia

Fernanda Isabel Antunes Gomes

New therapeutic strategies against *Staphylococcus epidermidis* biofilms

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Dissertation for PhD degree in Biomedical Engineering

Supervisors:
Professor Rosário Oliveira
Co-Supervisor
Doctor Pilar Teixeira

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“Share your knowledge. It’s a way to achieve immortality.”

Dalai Lama

NEW THERAPEUTIC STRATEGIES AGAINST *Staphylococcus epidermidis* BIOFILMS

| ABSTRACT

S*taphylococcus epidermidis* was previously regarded as an innocuous commensal microorganism on the human skin. However, nowadays it is seen as an important opportunistic pathogen and ranks first among the causative agents of nosocomial infections on indwelling medical devices. Infections with this leading pathogen are characterized by biofilm development on devices (heart valves, catheters, contact lenses, etc.) and this factor is considered the main virulence mechanism of *S. epidermidis*. Biofilm cells are organised into structured communities enclosed within a matrix of extracellular material. These cells are phenotypically different from planktonic or suspended cells; notably, they resist host defences and display a significantly decreased susceptibility to antimicrobial agents.

Since biofilm-associated infections are frequently resistant to conventional antimicrobial therapy, the aims of this doctoral work were to study new therapeutic strategies for treatment of infections caused by *S. epidermidis*. To this end, the susceptibility of planktonic and biofilm cells to farnesol, a possible antimicrobial agent against *S. epidermidis*, as well as the effect of farnesol on structure and composition of biofilm matrix were studied. The comparison of the effect of this compound with antibiotics traditionally used in the treatment of *S. epidermidis* infections such as vancomycin, tetracycline and rifampicin, and the determination of the presence of synergy of farnesol when combined with the antibiotics previously mentioned and with *N*-acetylcysteine (NAC) were other goals of the work. Moreover, the postantimicrobial effect (PAE) of farnesol and of the antibiotics mentioned above were determined and compared. Antibiotics combination represents a therapeutic option in the treatment of *Staphylococcus* infections, as a result of the increasing appearance of multi-resistant microorganisms. Taking this into consideration, this work also aimed at studying the effect of a wide range of antibiotics alone and in combination. Finally, the last purpose of the described work was the study of the genetic expression of some genes responsible for virulence of *S. epidermidis* biofilm cells, *icaA* (virulence gene - PNAG producing) and *rsbU* (stress regulator), after being exposed to various treatment conditions.

The results showed that farnesol caused a significant reduction of cellular viability of planktonic cells and a less pronounced effect was observed on biofilm cells. The quantification of extracellular polymers and the visualization of biofilms treated with farnesol under confocal microscopy, support the hypothesis that farnesol causes disruption of the cytoplasmic membrane and consequently release of cellular content. Additionally to cell death, farnesol seems also to destroy the biofilm structure and the biofilm matrix reducing the amount of poly-N-acetylglucosamine (PNAG) exopolysaccharide in the biofilm matrix. This general impairment of the biofilm caused by farnesol may be a potential help to the human immune system to eradicate focus of *Staphylococcus epidermidis* infections. With few exceptions, none of the antibiotics tested and NAC worked in synergy with farnesol. In some cases, farnesol was as effective as the antibiotics tested, being a possible alternative to antibiotics. Furthermore, farnesol has demonstrated to have a pronounced PAE comparatively to the antibiotics tested. All our results suggest farnesol as a potential antimicrobial therapeutic agent against *S. epidermidis* infections. Another potential alternative to antibiotics may be the use of NAC as a therapeutic agent, since it had a pronounced antimicrobial effect on both planktonic cells and biofilms. To overcome the problem of resistance to antibiotics, we also tested the susceptibility of biofilm cells to double combinations of antibiotics, and some combinations demonstrated to be effective against *S. epidermidis* biofilms, namely those containing rifampicin. Two of these combinations were rifampicin+clindamycin and rifampicin+gentamicin. These two combinations induced a lower genetic expression of *icaA* and *rsbU* genes, responsible for PNAG/PIA production and consequently can reduce biofilm formation recidivism, in comparison with rifampicin alone. This seems to be an additional advantage of the combinatorial therapy over monotherapy.

NOVAS ESTRATÉGIAS TERAPÊUTICAS CONTRA BIOFILMES DE *Staphylococcus epidermidis*

| RESUMO

Staphylococcus epidermidis foi anteriormente considerado um microorganismo comensal inócuo presente na pele humana. Porém, hoje em dia é visto como um importante patogénico oportunista e ocupa o primeiro lugar entre os agentes causadores de infecções hospitalares associadas ao uso de dispositivos médicos. As infecções causadas por este patogénico são caracterizados pelo desenvolvimento de biofilmes na superfície desses dispositivos implantados no doente (válvulas cardíacas, catéteres, lentes de contacto, etc.), sendo este factor considerado o principal mecanismo de virulência desta bactéria. As células em biofilme estão organizadas em comunidades estruturadas, envolvidas por uma matriz constituída por material extracelular. Estas células são fenotipicamente diferentes das células planctónicas ou suspensas; nomeadamente porque têm maior resistência às defesas do hospedeiro e exibem uma susceptibilidade diminuída aos agentes antimicrobianos.

Uma vez que as infecções associadas a biofilmes são frequentemente resistentes à terapia antimicrobiana convencional, os objectivos deste trabalho de doutoramento consistiram no estudo de novas estratégias terapêuticas para o tratamento de infecções provocadas por *S. epidermidis*. Para isso, estudou-se a susceptibilidade de células planctónicas e em biofilme a outros agentes antimicrobianos tais como o farnesol, bem como o efeito desta molécula sobre a estrutura e a composição da matriz do biofilme. A comparação do efeito deste composto com antibióticos tradicionalmente utilizados no tratamento de infecções causadas por *S. epidermidis*, como por exemplo a vancomicina, tetraciclina e rifampicina, e a determinação da presença de sinergia do farnesol quando combinado com os antibióticos previamente citados e com *N*-acetilcisteína (NAC) foi outro dos objectivos do trabalho. Foi também determinado o efeito pós-antimicrobiano (PAE) do farnesol e dos antibióticos anteriormente mencionados. Devido ao crescente aparecimento de microrganismos multi-resistentes tem-se recorrido à combinação de antibióticos como uma opção terapêutica no tratamento de infecções por estafilococos. Assim, este trabalho teve também como objectivo o estudo do efeito de uma ampla gama de antibióticos isolados e em combinação. Finalmente, o último propósito do trabalho consistiu no

estudo da expressão de alguns genes, responsáveis pela virulência de células de S*. epidermidis* em biofilme, *icaA* (gene de virulência – produção de PNAG) e *rsbU* (regulador de stress), após terem sido expostas a diferentes condições de tratamento.

Verificou-se que o farnesol causou uma redução significativa da viabilidade das células planctónicas mas um efeito menos pronunciado em células em biofilme. A quantificação de polímeros extracelulares e a visualização com microscópio confocal dos biofilmes tratados com farnesol apoiam a hipótese de que o farnesol causa o rompimento da membrana citoplasmática das células e, consequentemente, a libertação de conteúdo celular. Para além de provocar morte celular, o farnesol também parece destruir a matriz e a estrutura do biofilme, reduzindo a quantidade do exopolissacarídeo, poli-N-acetilglicosamina (PNAG) na matriz do biofilme. Este enfraquecimento geral do biofilme provocado pelo farnesol pode ser uma potencial ajuda para o sistema imunológico humano na erradicação do foco de infecção por *Staphylococcus epidermidis*. Excepto raras excepções, nenhum dos antibióticos testados e NAC apresentaram sinergia com o farnesol. Em alguns casos, o farnesol teve uma eficácia semelhante à dos antibióticos testados, sendo uma possível alternativa a estes agentes antimicrobianos. Além disso, o farnesol demonstrou ter um PAE elevado relativamente aos antibióticos testados. Todos estes resultados sugerem que o farnesol pode ser utilizado como um potencial agente terapêutico antimicrobiano no combate a infecções provocadas por *S. epidermidis*. Outra potencial alternativa aos antibióticos poderá ser o uso de NAC como agente terapêutico visto este apresentar um pronunciado efeito antimicrobiano quer em células planctónicas quer em biofilmes. Para superar o problema da resistência aos antibióticos, testou-se também a susceptibilidade dos biofilmes a combinações duplas de antibióticos, tendo-se verificado que algumas combinações demonstraram ser muito eficazes contra biofilmes de *S. epidermidis*. Duas destas combinações foram rifampicina+clindamicina e rifampicina+gentamicina. Estas duas combinações induziram uma menor expressão dos genes *icaA* e *rsbU*, responsáveis pela produção de PNAG/PIA e podem consequentemente reduzir a reincidência de formação de biofilme, em comparação com a rifampicina isolada. Esta parece ser uma vantagem adicional da terapia combinatória em relação à monoterapia.

| SCIENTIFIC OUTPUT

| PAPERS IN PEER REVIEWED JOURNAL:

Gomes FI, Teixeira P, Azeredo J and Oliveira R (2009) Effect of farnesol on planktonic *versus* biofilm cells of *Staphylococcus epidermidis*. *Curr Microbiol* **59**(2): 118-22. (Chapter II)

Gomes F, Teixeira P, Cerca N, Azeredo J and Oliveira R. Effect of farnesol on structure and composition of *Staphylococcus epidermidis* biofilm matrix. Submitted for publication. (Chapter III)

Gomes F, Leite B, Teixeira P, Cerca N, Azeredo J and Oliveira R. Farnesol as antibiotics adjuvant in *Staphylococcus epidermidis* control *in vitro*. *Am J Med Sci*. In press. (Chapter IV)

Gomes F, Leite B, Teixeira P, Azeredo J and Oliveira R. Effect of farnesol in combination with *N*-acetylcysteine against *Staphylococcus epidermidis* planktonic and biofilm cells. Submitted for publication. (Chapter V)

Gomes F, Cerca N, Teixeira P, Ceri H and Oliveira R. Combination of antibiotics against *in vitro Staphylococcus epidermidis* biofilms. Submitted for publication. (Chapter VI)

| PROCEEDINGS AND ABSTRACTS:

Gomes F, Cerca N, Teixeira P, Ceri H and Oliveira R (2010) Effect of single *versus* antibiotic combinations on *Staphylococcus epidermidis* biofilm viability and on genetic expression of some virulence genes. *Biofilms 4 International Conference*, Winchester, United Kingdom, 1-3 September.

Gomes F, Cerca N, Teixeira P, Azeredo J and Oliveira R (2009) The comparative effect of farnesol and antibiotics against *Staphylococcus epidermidis*. *5th ASM Conference on Biofilms*, Cancun, Mexico, 15-19 November, p. 52.

Gomes F, Cerca N, Teixeira P and Oliveira R (2009) Antimicrobial activity of farnesol against *Staphylococcus epidermidis*. *Eurobiofilms 2009 - First European Congress on Microbial Biofilms*, Rome, Italy, 2-5 September, p. 83-84.

Gomes F, Cerca N, Teixeira P and Oliveira R (2009) Farnesol as a prospective antimicrobial agent against *Staphylococcus epidermidis. 19th European Congress of Clinical Microbiology and Infectious Diseases*, Helsinki, Finland, 16-19 May, Abstract on CD-ROM- P1086.

Gomes FI, Teixeira P and Oliveira R (2008) Synergy of farnesol and antibiotics against planktonic *versus* biofilm cells of *Staphylococcus epidermidis. Biofilms III*, Munich, Germany, 6-8 October, p. 144.

Gomes FI, Teixeira P and Oliveira R (2008) Effect of farnesol on planktonic versus biofilm cells of *Staphylococcus epidermidis*. *18th European Congress of Clinical Microbiology and Infectious Diseases*, Barcelona, Spain, 19-22 April, abstract on CD-ROM.

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| NOMENCLATURE

| SYMBOLS AND ABBREVIATIONS

AAP	Accumulation associated protein
AB	Alamar Blue
AMPs	Antimicrobial peptides
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
BHI	Brain heart infusion
BSA	Bovine serum albumin
CBD	Calgary biofilm device
cDNA	Complementary desoxyribonucleic acid
CEF	Cefazolin
CEPH	Cephalothin
CFU	Colony forming units
CLIND	Clindamycin
CLSI	Clinical and laboratory standards institute
CoNS	Coagulase-negative staphylococci
CSLM	Confocal scanning laser microscopy
CT	Cycle threshold
CVC	Central venous catheter
CV	Crystal violet
DAPI	4',6-diamidino-2-phenylindole
DNA	Desoxyribonucleic acid
EPS	Extracellular polymeric substances
FAME	Fatty acid modifying enzyme
FCT	Fundação para a Ciência e Tecnologia
GENT	Gentamicin
GlcNac	*N*-acetylglucosamine
IgG	Immunoglobulin G
LD_{50}	Lethal dose 50%

L/D	Live/Dead
LEVO	Levofloxacine
MBC	Minimal bactericidal concentration
MBEC	Minimal biofilm eradication concentration
MIC	Minimum inhibitory concentration
MSCRAMM	Microbial surface components recognizing adhesive matrix molecule
NAC	*N*-acetylcysteine
NCCLS	National committee for clinical laboratory standards
OD	Optical density
P	Significance value
PCR	Polymerase chain reaction
PGA	Poly-gama-glutamic acid
PIA	Polysaccharide intercellular adhesin
PMS	Phenazine methosulphate
PNAG	Poly-N-acetylglucosamine
PS	Peak serum concentration
PSM	Phenol-soluble modulin
QPCR	Quantitative real-time polymerase chain reaction
RIF	Rifampicin
RNA	Ribonucleic acid
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT	Reverse transcriptase
RT-PCR	Real-time polymerase chain reaction
SEM	Scanning Electron Microscopy
SPSS	Statistical Package for the Social Sciences
TET	Tetracycline
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
UTI	Urinary tract infection
VANC	Vancomycin
WGA	Wheat germ agglutinin

XTT	2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide

I. GENERAL INTRODUCTION

| *Staphylococcus* spp - *Staphylococcus epidermidis*

The genus Staphylococcus contains 35 species, all of which are part of normal skin and mucous membrane flora of humans and animals. The coagulase enzyme producing *Staphylococcus aureus* is the most important pathogen, causing various pyogenic infections and toxin-mediated illnesses in normal hosts. Other species are collectively termed coagulase-negative staphylococci. These are generally non-pathogenic, apart from *S. epidermidis*, which causes nosocomial bacteraemia and device-related infections, and *S. saprophyticus*, which is a common cause of urinary tract infection (UTI) (Török and Day, 2005). The bacteria belonging to this genus are Gram-positive bacteria, round shaped (cocci) and forming grape-like clusters (Figure I.1.) (Singleton and Sainsbury, 2001).

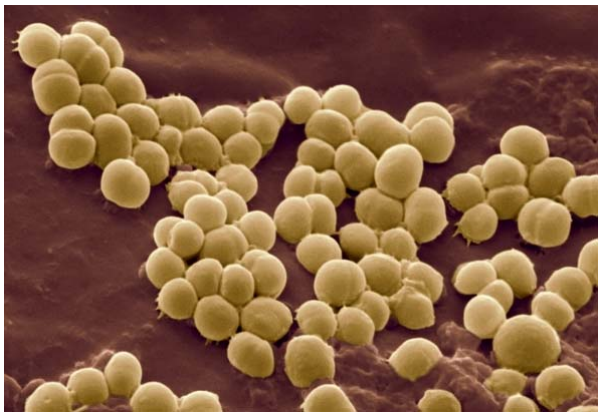


Figure I.1. *Staphylococcus epidermidis* biofilm showing cells organised like a cluster of grapes.
Adapted from: http://c.photoshelter.com/img-get/I0000A2Auhlc04ms/s/600

This genus includes thirty-five species and eight sub-species of which most are harmless and reside normally on the skin and mucous membranes of humans and other organisms (Kloos and Schleifer, 1986). As example, *S. epidermidis* is the most frequently isolated species from human epithelia, and predominantly colonizes the axillae, head and nares (Kloos and Musselwhite, 1975). *Staphylococcus* can cause a wide variety of diseases in humans and other animals through either toxin production or penetration. Staphylococcal toxins are a common cause of food poisoning, as it can grow in improperly-stored food. The main classification of staphylococci is based on their ability to produce coagulase, an enzyme that converts fibrinogen

to fibrin and causes blood clot formation (Prescott *et al.*, 1999). *Staphylococcus epidermidis* is a coagulase-negative staphylococcus species, is a commensal of skin, but can cause severe infections in immune-suppressed patients and those with central venous catheters. Historically, *Staphylococcus epidermidis* has been regarded as an innocuous commensal bacterium of the human skin (Vuong *et al.*, 2003). Nowadays, this bacterium is seen as an important opportunistic pathogen and ranks first among the causative agents of nosocomial infections (Otto, 2009). *Staphylococcus epidermidis* may grow in a biofilm on implants and prosthetic devices thus causing persistent or recurrent infections. This bacterium has only a limited number of exotoxins and degradative exoenzymes. Therefore, infections by *S. epidermidis* are of a less acute and more long-lasting nature. The most important type of disease caused by *S. epidermidis* is the colonization and infection of indwelling medical devices. Infections are associated with intravascular devices (prosthetic heart valves, shunts, etc.) but also commonly occur in prosthetic joints, catheters, and large wounds (Figure I.2.A). Septicemia and endocarditis (Figure I.2.B) are also diseases associated with *S. epidermidis.* Septicemia is especially prevalent resulting from neonatal infections, particularly in very low birth weights. Endocarditis is an infection of the heart valves and parts of the inside lining of the heart muscle. *S. epidermidis* is very likely to contaminate patient-care equipment and environmental surfaces, possibly explaining the high incidence of *S. epidermidis* in the hospital setting. In these infections, the main virulence mechanism of *S. epidermidis* is biofilm formation (Vuong *et al.*, 2003).

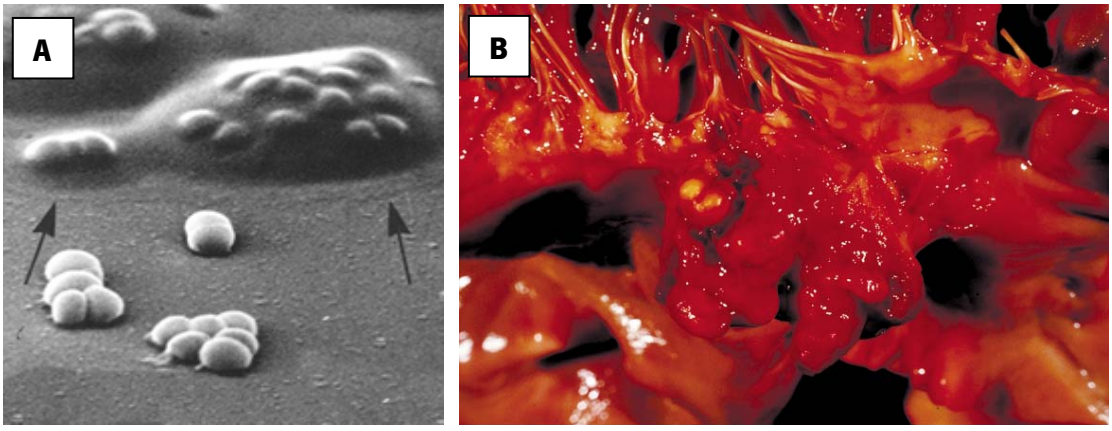


Figure I.2. (A) *Staphylococcus epidermidis* attaching to a catheter inside a blood vessel. Bacteria grow on the catheter and shed into the blood stream to cause infection. (B) Mitral valve vegetations in coagulase-negative staphylococcal native-valve endocarditis (Török and Day, 2005).

Adapted from: (A) http://www.ls.manchester.ac.uk/undergraduate/courses/microbiology/

| BIOFILM FORMATION

A biofilm consists of a structured community of bacterial cells enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton *et al.*, 1999; Hajdu *et al.*, 2009). Biofilms are dynamic and responsive to their environment, that is, they can adapt to changes in their environment. Biofilms are characterized by high concentrations of organisms with little turnover (Saginur *et al.*, 2006). They have a characteristic physiology and architecture that form the basis of biofilm resistance to many antibiotics and mechanisms of host defence (Costerton *et al.*, 1999). Biofilm formation depends on the characterisitics of the surface, the bacterial cells, the growth medium and other environmental conditions (Donlan and Costerton, 2002) and proceeds by initial adhesion of cells to a surface and their subsequent aggregation into multicellular structures (Figure I.3.). Adhesion to abiotic surfaces such as catheters is mainly governed by bacterial cell surface hydrophobicity (Vacheethasanee *et al.*, 1998). Several factors are believed to be involved in the primary attachment step, which is dependent on physico-chemical interactions of the bacterial cell surface with the abiotic surface (Vuong *et al.*, 2003). The best described and most important factor influencing primary attachment in *S. epidermidis* is the autolysin AtlE, in which repetitive sequences presumably interact with the abiotic surface by hydrophobic interaction (Heilmann *et al.*, 1997). The AtlE protein consists of two enzimatic domains: an amidase and an *N*-acetylglucosamidase part (Vuong *et al.*, 2003). AtlE plays an important role in cell-wall processing and recycling, and it is interesting to speculate that its major role in the process of biofilm formation may be to orientate and expose cell-surface adhesins correctly to maximize and ensure effective interaction with either host factors or device surfaces (Stevens *et al.*, 2009). This autolysin and the Bap protein (also Known as Bhp) (Tormo *et al.*, 2005), are likely to contribute to the hydrophobic character of the cell surface. *In vivo*, matrix proteins quickly cover abiotic surfaces such as those of indwelling medical devices. *S. epidermidis* has a vast array of surface proteins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (Table I.1.), having the potential to interact with matrix proteins. Cell-cell adhesion and accumulation requires the polysaccharide intercellular adhesin (PIA), an unbranched homopolymer of partially deacetylated *N*-acetylglucosamine residues linked by β-1-6 glycosidic bonds (Vuong *et al.*, 2003). Synthesis of PIA is linked to the biosynthetic enzyme-coding genes located in the intercellular adhesion operon (*icaADBC*) (Heilmann *et al.*, 1996), and biofilm production involving PIA is known to be *ica*-

dependent and is considered the primary mechanism employed by staphylococci (Stevens *et al.*, 2008). PIA and biofilm formation is regulated by the alternative sigma factor σ^B and is influenced by a variety of environmental conditions including disinfectants and other antimicrobial substances (Cramton *et al.*, 2001; Knobloch *et al.*, 2001; Knobloch *et al.*, 2002a; Knobloch *et al.*, 2002b; Rachid *et al.*, 2000; Rohde *et al.*, 2001). Moreover, the development of a biofilm requires adhesive forces for both the colonization of surfaces and the cell-cell interactions.

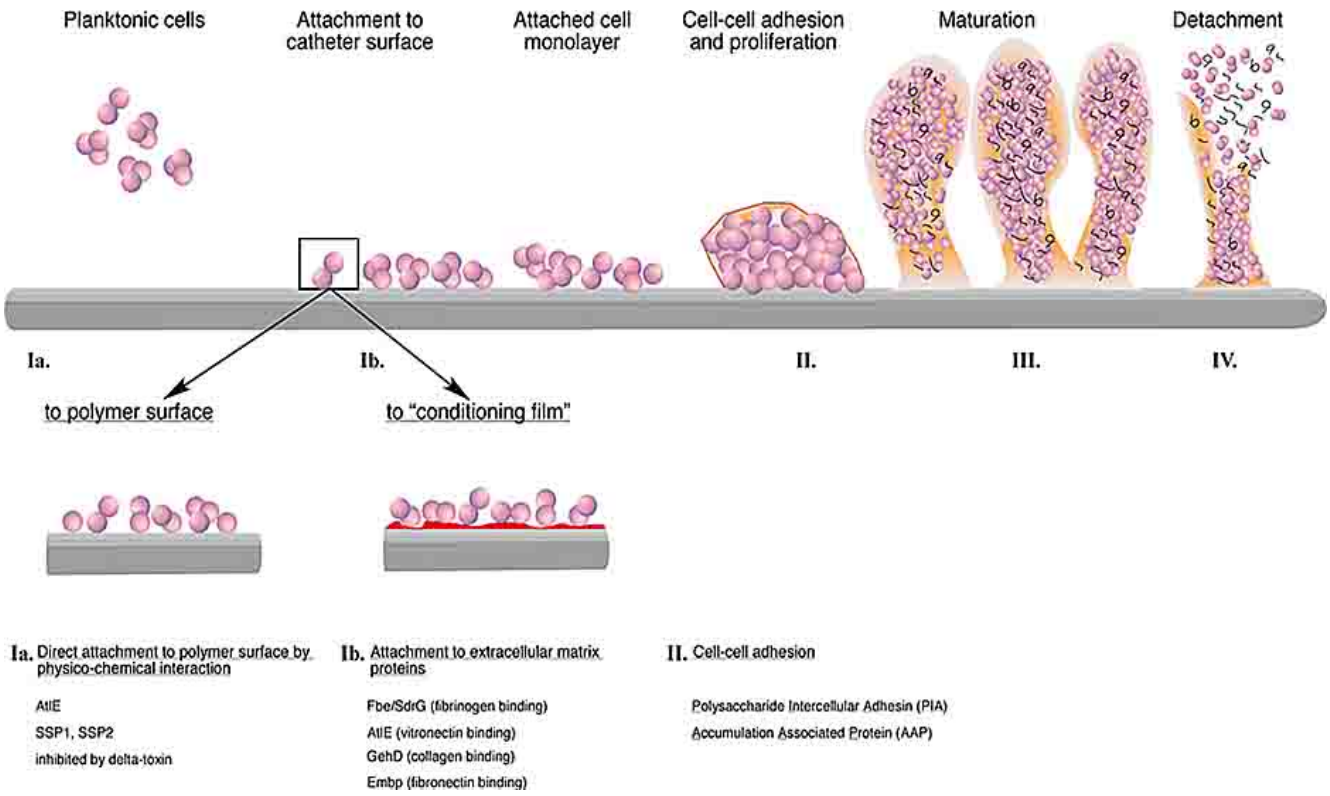


Figure I.3. Biofilm formation. Model of biofilm formation on a catheter surface by *Staphylococcus epidermidis.* Biofilm formation is shown as a four-step process involving initial attachment, accumulation, maturation, and detachment. Several factors involved in the attachment and accumulation phases have been described and are noted on the bottom of the figure. Factors involved in maturation and detachment have not been identified yet. Initial attachment can occur as direct adhesion to the polymer surface or depend on the interaction of dedicated bacterial binding proteins with host matrix proteins that cover the catheter as a "conditioning film". Adapted from: http://www.bioscience.org/2004/v9/af/1295/figures.htm.

Disruptive forces are needed for the formation of fluid-filled channels that are important for nutrient delivery to all biofilm cells and give the mature biofilm its typical three-dimensional

structure. Disruptive forces are also involved in a phenomenon that seems to be common among all biofilms, known as detachment of cell clusters from the biofilm, which limits biofilm expansion and may lead to the dissemination of infection (O'Toole *et al.*, 2000). Bacterial cells can detach from their biofilm colony individually or in clumps. When individual microorganisms detach from a biofilm, these isolated microorganisms are relatively easy to kill with chemicals designed for this purpose. When microorganisms detach from their biofilm colony in clumps, the clumps are pieces of the biofilm that are at the moment not attached to a surface; in this case they maintain the protective properties of the original biofilm and are thus much more difficult to kill. In the right conditions, biofilms can migrate across surfaces over a period of time in a variety of ways, as illustrated below (Figure I.4.).

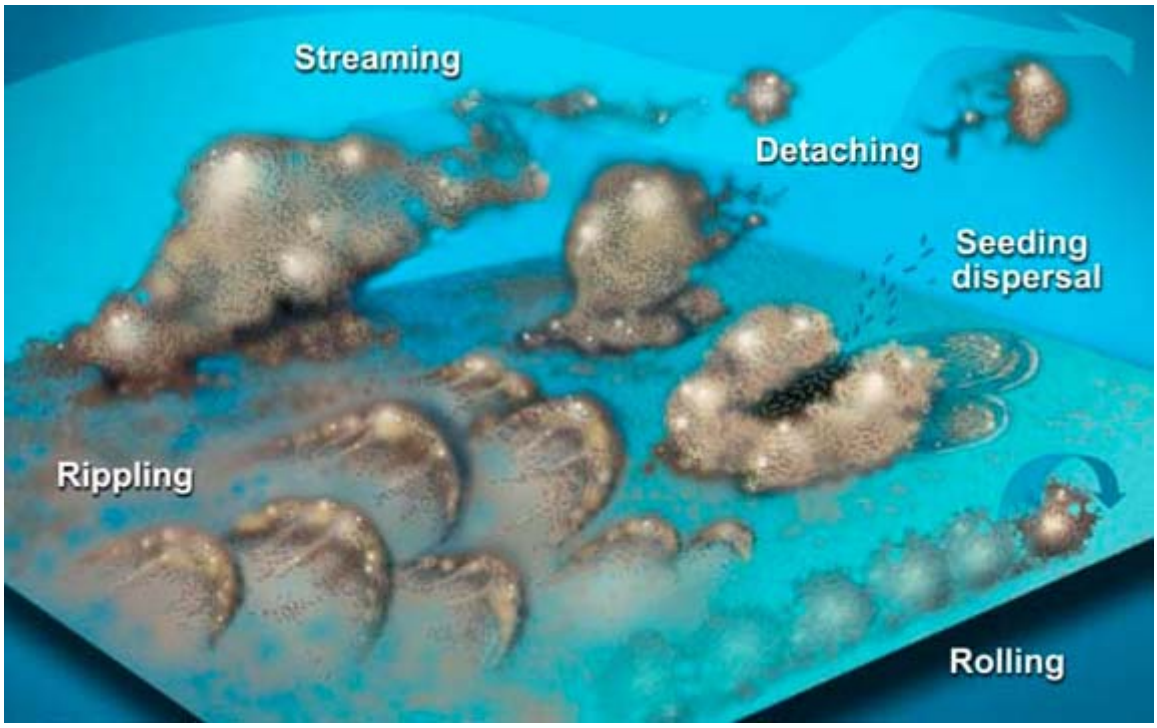


Figure I.4. Biofilm migration. Different ways of biofilm cells dispersion.

Adapted from: http://biofilmbook.hypertextbookshop.com/public_version/contents/chapters/chapter001/section004/green/page001.html

Table I.I. Virulence factors of *Staphylococcus epidermidis.*

Virulence factor	Gene	Function
Biofilm formation through primary attachment to abiotic surfaces		
AtlE	*atlE*	An abundant bifunctional autolysin and adhesin that affects surface hydrophobicity
Aae	*aae*	A bifunctional autolysin and adhesin
Teichoic acids	Multiple biosynthetic genes	In *Staphylococcus aureus*, teichoic

		acids affects attachment (through the binding of autolysins?)
Biofilm formation through primary attachment to matrix proteins		
SdrF	*sdrF*	Binds to collagen
SdrG (also known as Fbe)	*sdrG* (also known as fbe)	Binds to fibrinogen
Sdr H	*sdrH*	Putative binding function only
Ebp	*ebp*	Binds to elastin (in *S.aureus*)
AtlE and Aae	*atlE* and *aae*	Binds to various matrix proteins
Intercellular aggregation		
PNAG (also known as PIA)	*icaA, icaD, icaB* and *icaC*	An intercellular polysaccharide adhesin
Biofilm-associated protein Bap (also known as Bhp)	*bap* (also known as *bhp*)	An intercellular protein adhesin
Accumulation-associated protein Aap	*aap*	An intercellular protein adhesin precursor that requires proteolytic processing for its activation
Teichoic acids	Multiple biosynthetic genes	Components of the biofilm matrix
Protective exopolymers		
PNAG	*icaA, icaD, icaB* and *icaC*	Protect from IgG, AMPs, phagocytosis and complement
PGA	*capA, capB, capC* and *capD*	Protects from AMPs and phagocytosis
Resistance to AMPs		
SepA protease	*sepA*	Involved in AMP degradation
Dlt, MprF, VraF and VraG	*dltA, dltB, dltC, dltD, mprF, vraF* and *vraG*	Analogous to *S. aureus*, these proteins function in the D-alanylation of teichoic acids (Dlt), lysylation of phospholipids (MprF) and putative AMP export (VraF and VraG)
Aps system	*apsR* (also known as *graR*), *apsS* (also known as *graS*) and *apsX*	This system senses AMPs and regulates AMP resistance mechanisms
Toxins		
PSMs	*psmα*, *psmδ*, *psmε*, *hld*, *psmβ1* and *psmβ2*	Pro-inflammatory cytolysins
Exoenzymes		
Cysteine protease (SspB and Ecp); *S. aureus* staphopain homologue	*sspB*	Unknown: tissue damage?
Metalloprotease or elastase (SepA); *S. aureus* aureolysin homologue	*sepA*	Involved in lipase maturation, AMP resistance and, potentially, tissue damage
Glutamylendopeptidase and serine protease (GluSE, SspA and Esp); *S. aureus* V8 protease homologue	*sspA*	Degradation of fibrinogen and complement factor C5
Lipases GehC and GehD	*gehC* and *gehD*	Persistance in fatty acid secretions ?
Other factors		
Staphyloferrins	*sfna* locus (*S.aureus*	Siderophores (iron acquisition)

	staphyloferrin A)	
SitA, SitB and SitC	*sitA, sitB* and *sitC*	An iron importer
FAME	unidentified	Detoxication of bactericidal fatty acids

AMP, antimicrobial protein; FAME, fatty acid modifying enzyme; IgG, immunoglobulin G; PGA, poly-γ-glutamic acid; PNAG, poly-*N*-acetylglucosamine; PSM, phenol-soluble modulin.

| BIOFILM MATRIX

S. epidermidis biofilm matrix comprises several extracellular polymeric substances (EPS) such as polysaccharides (Donlan, 2001a), proteins (Cucarella *et al.*, 2001; Lasa and Penades, 2006b; Rohde *et al.*, 2005), considerable amounts of extracellular teichoic acids (Sadovskaya *et al.*, 2005; Sadovskaya *et al.*, 2004), and also extracellular DNA (Qin *et al.*, 2007). The composition of the matrix varies according to the nature of the organisms present. Matrix polymers of bacterial biofilms are primarily exopolysaccharides, and many are negatively charged due to the presence of carboxil, sulphate or phosphate group. Smaller amounts of proteins nucleic acids and lipids can also be present. *S. epidermidis* produces exopolymers, namely poly-γ-glutamic acid (PGA) and poly-*N*-acetylglucosamine (PNAG) also called PIA, that protect the bacterium from important mechanisms of innate host defence. The PGA, which is synthesized, is crucial for *S. epidermidis* resistance to neutrophil phagocytosis and antimicrobial proteins (AMPs), despite its low levels of production (Kocianova *et al.*, 2005). In addition to its role as part of the extracellular biofilm matrix, PNAG/PIA, a polymer of *N*-acetylglucosamine (Mack *et al.*, 1996; Maira-Litran *et al.*, 2002; McKenney *et al.*, 1998; Sadovskaya *et al.*, 2005) has been found to protect *S. epidermidis* from neutrophil killing, complement deposition, immunoglobulins and AMPs (Kristian *et al.*, 2008; Vuong *et al.*, 2004). Furthermore, PNAG/PIA has been described as crucial for the process of cell-to-cell adhesion and biofilm accumulation (Christensen *et al.*, 1990; Mack *et al.*, 1996; Peters *et al.*, 1987; Tojo *et al.*, 1988) and as an essential component of the extracellular matrix (Vuong *et al.*, 2004). A scheme is illustrated below showing some constituents of the biofilm matrix and their main functions on pathogenesis and colonization of *Staphylococcus epidermidis* (Figure I.5.)

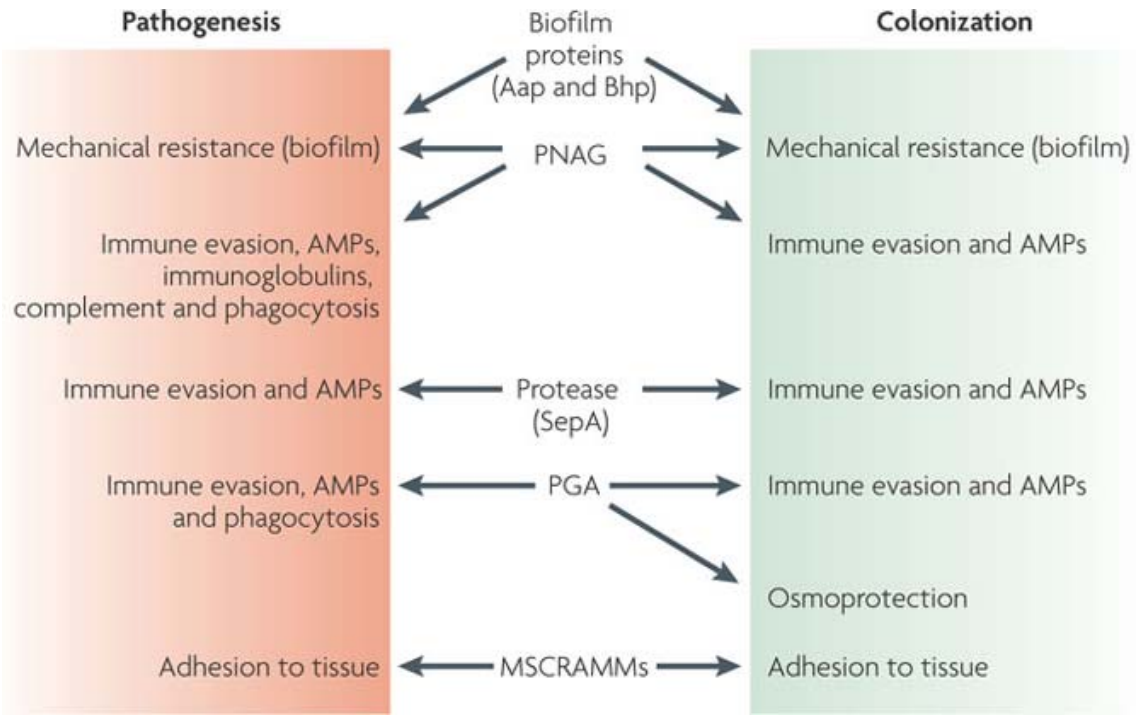


Figure I.5. Main *Staphylococcus epidermidis* biofilm matrix constituents. Determinants that are thought to contribute to both the colonization and the pathogenesis of *S. epidermidis* are shown, along with their functions. Adapted from: http://www.nature.com/nrmicro/journal/v7/n8/fig_tab/nrmicro2182_F6.html

| BIOFILM STRUCTURE

Biofilms are highly structured communities of amazing structural and functional sophistication (Costerton and Stewart, 2001). A mature biofilm is seen as a very heterogeneous arrangement, with a basic community structure consisting of microcolonies of bacterial cells encased in EPS matrix (Donlan and Costerton, 2002; Lewandowsky, 2000). The matrix material can comprise as much as 85% of the volume of the biofilm. The matrix is one of the most distinctive features of a microbial biofilm where in addition to PNAG/PIA and protein, extracellular DNA has also been shown to be important in stabilizing the biofilm structure (Izano *et al.*, 2008). It forms a three-dimensional, gel-like, highly hydrated and locally charged environment in which the microorganisms are largely immobilized (Flemming *et al.*, 2000). Matrix-enclosed microcolonies, sometimes described as “stacks” or “towers” are separated by water channels (Donlan and Costerton, 2002). Liquid flow occurs in these water channels, allowing diffusion of nutrients, oxygen, and even antimicrobial agents. Tolker-Nielsen and Molin (2000) noted that every microbial biofilm community is unique although some structural attributes can generally be considered universal. The organisms composing the biofilm may also have a marked effect on

the biofilm structure. James *et al.* (1995) showed that the biofilm thickness could be affected by the number of component organisms. Biofilm architecture is heterogeneous both in space and time, constantly changing because of external and internal processes. The structure of a biofilm can range from a dense biofilm model (Winpenny and Colasanti, 1997), to a heterogeneous mosaic model (Keevil and Walker, 1992) or to one consisting of a more complex organisation involving mushroom-like aggregates separated by water channels, normally considered the most typical biofilm architecture (Costerton *et al.*, 1994).

| CELL-CELL COMMUNICATION – QUORUM-SENSING

Bacteria have the ability to signal and sense the state of population density in order to changing physiological needs under different growth conditions. This phenomenon is commonly called quorum-sensing (Swift *et al.*, 1996). Therefore, quorum-sensing is a strategy of cell-cell communication benefiting the biofilm community by controlling unnecessary overpopulation and competition for nutrients with important implications for the infectious process (Davey and O'Toole, 2000; Douglas, 2003) (Figure I.6.). In fact, biofilms are not simply amalgamations of randomly dividing cells (Ramage *et al.*, 2002). Biofilms are precisely organised communities that are dependent on the quorum-sensing abilities of microorganisms (Ramage *et al.*, 2002). This phenomenon has been the focus of much research, and quorum-sensing molecules have been recognised as important regulators of virulence and demonstrated to be essential for biofilm formation in many bacteria (Jabra-Rizk *et al.*, 2006a; Rice *et al.*, 2005; Vuong *et al.*, 2003). As example, there is a single quorum-sensing system in *S. epidermidis* encoded by the *agr* operon that can regulate biofilm formation by regulation of AtlE and γ–toxin expression (Vuong *et al.*, 2003).

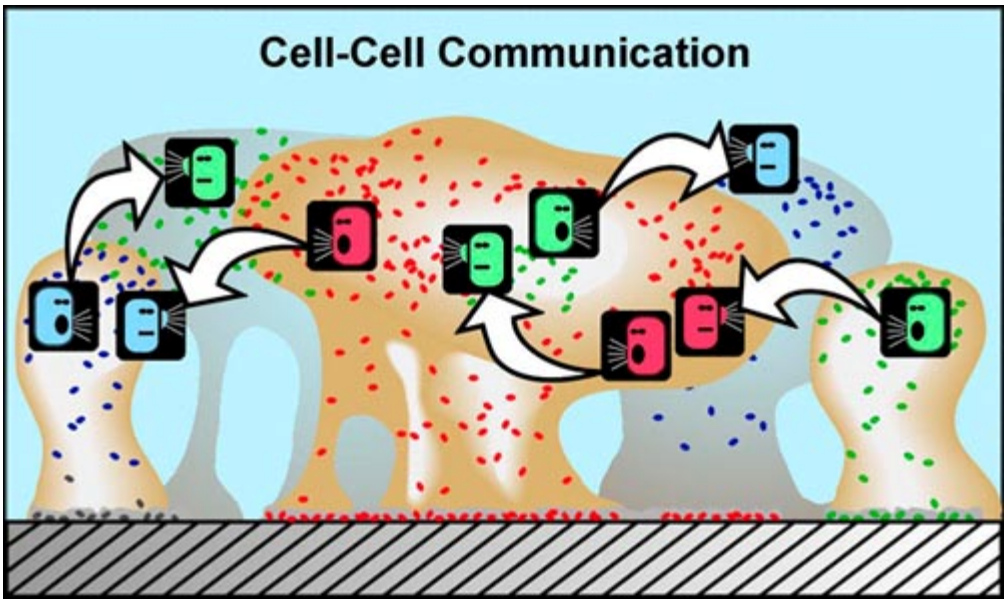


Figure I.6. Quorum-sensing phenomenon. Cell to cell communication in a biofilm.

In the cartoon above, various species of bacteria are represented by different colors. Bacteria can produce chemical signals ("talk") and other bacteria can respond to them ("listen") in a process commonly known as cell-cell communication or cell-cell signaling. This communication can result in coordinated behavior of microbial populations.

Adapted from: http://biofilmbook.hypertextbookshop.com/public_version/contents/chapters/chapter001/section004/green/page001.html

The systems involved in quorum-sensing of Gram-negative and Gram-positive bacteria have been proposed as promising targets for anti-microbial therapy. In pathogenic bacteria, many of the extracellular virulence factors are regulated by such systems.

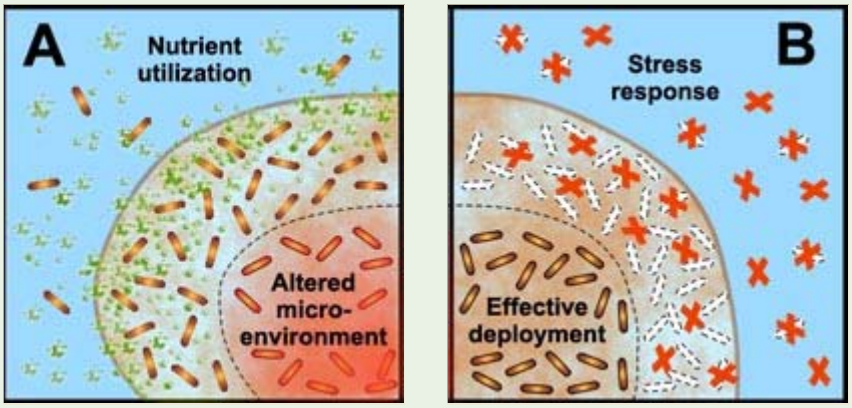
| ANTIBIOTIC RESISTANCE

Nowadays it is established that the natural mode of bacterial life is in multicellular complexes referred to as biofilms, which behave in a quite different way than free-floating cells (Lasa, 2006a) and are physiologically distinct from bacteria growing as a free-swimming planktonic state (Hall-Stoodley *et al.*, 2004). It has been established that the main problem with infections caused by biofilms is the increased chemoresistance compared with bacteria in suspensions (Saginur *et al.*, 2006). When bacteria are growing in a biofilm state, they are resistant to antibiotic levels 10- to- 1,000-fold higher than genetically identical planktonic bacteria (Amorena *et al.*, 1999; Saginur *et al.*, 2006). Consequently, staphylococcal infections involving biofilm formation, can be extremely difficult to treat with antibiotics, are often chronic or relapsing,

and frequently necessitate invasive procedures, such as removal of the infected tissue or device (Costerton *et al.*, 1999; Donlan, 2001b; Jefferson *et al.*, 2005). The properties of biofilms that result in antibiotic resistance may include slow growth, phenotypic heterogeneity, the presence of persister cells, inactivation of antibiotics within the biofilm exopolysaccharide matrix, and limitations on antibiotic penetration imparted by the biofilm matrix (Jefferson *et al.*, 2005; Stewart, 2002). Some of the hypothesized mechanisms of protection from antimicrobial agents are pictured in the diagram below (Figure I.7.).

A. Free-floating cells utilize nutrients, but do not have sufficient metabolic activity to deplete substrates from the neighborhood of the cells.
In contrast, the collective metabolic activity of groups of cells in the biofilm leads to substrate concentration gradients and localized chemical microenvironments. Reduced metabolic activity may result in less susceptibility to antimicrobials.

B. Free-floating cells carry the genetic code for numerous protective stress responses. Planktonic cells, however, are readily overwhelmed by a strong antimicrobial challenge. These cells die before stress responses can be activated.
In contrast, stress responses are effectively implemented in some of the cells in a biofilm at the expense of other cells which are sacrificed.



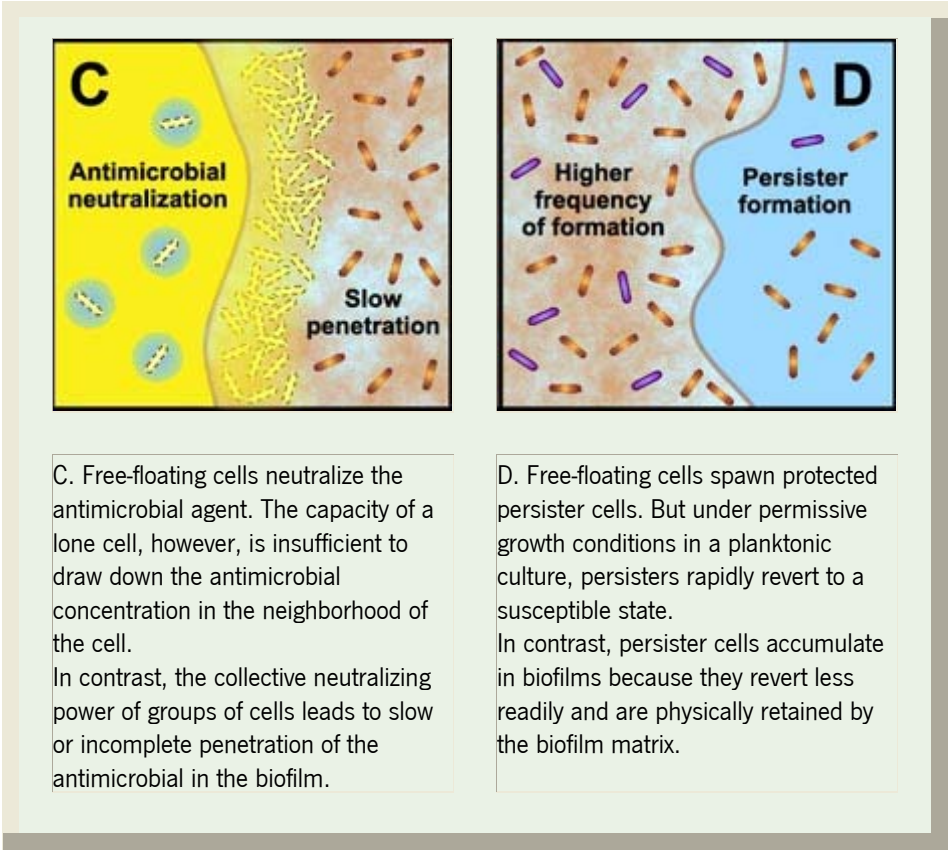


Figure I.7. Antimicrobial agents resistance – comparison between planktonic cells and biofilm cells. Hypothesized mechanisms of protection from antimicrobial agents. Adapted from: http://biofilmbook.hypertextbookshop.com/public_version/contents/chapters/chapter001/section004/green/page001.html

In many countries, 70-80% of all hospital isolates of *S. epidermidis* are resistant to methicillin, a formerly first-choice antibiotic against staphylococcal infections. In addition to methicillin resistance, *S. epidermidis* strains have acquired resistance to several other antibiotics, including rifampicin, fluoroquinolones, gentamycin, tetracycline, chloramphenicol, erytromycin, clindamycin and sulphonamides (Rogers *et al.*, 2009). Resistance to streptogramins, linezolid and tigecycline also occurs, although rarely. Despite, resistance to methicillin and other antibiotics, 80% of catheters infected with *S. epidermidis* can still be treated with antibiotics such as vancomycin without catheter removal (Raad *et al.*, 2007b). However, intermediate resistance to vancomycin has also been described (Schwalbe *et al.*, 1987) and staphylococcal biofilm formation significantly decreases the activity of vancomycin and other antibiotics (Gagnon *et al.*, 1993; Raad *et al.*, 2007a; Richards *et al.*, 1989). The frequency of antibiotic resistance in *S. epidermidis* reflects the overuse of antibiotics. Furthermore, the ubiquity of *S. epidermidis* as a human commensal microorganism renders this bacterium an optimal carrier and reservoir for antibiotic resistance genes, particularly those that do not inflict a major fitness cost to the

bacterium. Vaccination and decolonization do not seem to be appropriate for *S. epidermidis*. First, there is no anti-staphylococcal vaccine and the several lines of evidence indicate that it may be difficult to use traditional active immunization for staphylococci (Deleo and Otto, 2008; Otto, 2008). Second, eradication of *S. epidermidis* as a common part of the human microflora may not only be difficult to achieve, owing to the fact that re-colonization from other individuals is fast, but it may also turn out to be counterproductive, as it may allow potentially more harmful microorganisms to take the place of *S. epidermidis*. Therefore, it is commonly agreed that the best way to deal with *S. epidermidis* infections is by prevention, which includes sterilization of medical equipment and of body parts of patients and those health care personnel who are in contact with indwelling medical devices during surgery (Rogers *et al.*, 2009).

| BIOFILM INFECTION CONTROL

Infection of medical implanted material is associated with considerable morbidity and costs (Hajdu *et al.*, 2009). Biofilm-associated infections are frequently resistant to conventional antimicrobial therapy and difficult to eradicate without the removal of the infected device (Aslam *et al.*, 2007) because the bacterial biofilm on the surfaces serves as a reservoir where bacteria are quasi inacessible to antibiotics and host defences (Hajdu *et al.*, 2009; Kuźma *et al.*, 2007). Bacterial pathogens have evolved numerous defence mechanisms against antimicrobial agents and resistance to old and new produced drugs are on the rise. These are the reasons why many research groups investigate potential strategies, which could be accessory or alternative to antibiotic therapy. Natural plant compounds are on the focus of some biotechnological companies which are looking for new antimicrobial and anti-biofilm drugs (Kuźma *et al.*, 2007). Essential oils are complex mixes of hydrophobic liquids containing volatile aromatic compounds, which are products of plants secondary metabolism (Prabuseenivasan *et al.*, 2006). Of all the claimed properties of essential oils, its antimicrobial activity is the one that receives special attention due to the serious threat that antibiotic resistance has become. Therefore, the study of potential antibiotic compounds found in these oils could be of interest in the development of novel antimicrobial agents (Derengowski *et al.*, 2009).

| FARNESOL

Historically, plant extracts such as essential oils have been used for therapeutic purposes. In recent years, much research has been devoted to investigating such plant extracts: their active components, modes of action and synergistic effects with other antimicrobial compounds (Cowan, 1999). Terpenoids are highly complex compounds based on an isoprene structure that are found in essential oils and used in perfumery, cosmetics, food flavourings, food preservatives and for medical purposes (Loza-tavera, 1999; O'Hara, 1998).

Farnesol, a natural sesquiterpene alcohol (3,7,11-trimethyl-2,6,10-dodecatrien-1-ol) (Figure I.8.) is produced by many organisms and is also found in several essential oils, e.g. from citrus fruits (Derengowski *et al.*, 2009; Koo *et al.*, 2003), from *Pluchea dioscoridis*, *Zea mays* and *Pittosporum undulatum*, possibly protecting these plants from parasitic induced damages (Grace, 2002; Schnee *et al.*, 2002).

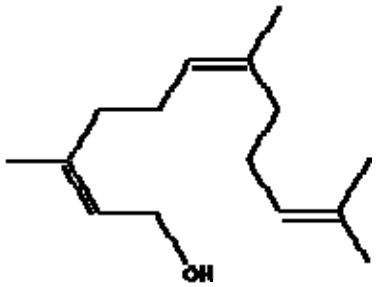


Figure I.8. Structure of farnesol. Adapted from: chemicalland21.com/info/TERPENES.htm

Recently, farnesol was described as a quorum-sensing molecule in *Candida* genus with possible antimicrobial properties (Jabra-Rizk *et al.*, 2006a). Studies revealed that farnesol affects the growth of a number of bacteria and fungi, such as the human pathogens *Staphylococcus aureus* (Inoue *et al.*, 2004; Jabra-Rizk *et al.*, 2006a), and *Streptococcus mutans* (Koo *et al.*, 2002), and the plant pathogenic fungus *Fusarium graminearum* (Semighini *et al.*, 2008), pointing to a potential role as an antimicrobial agent (Derengowski *et al.*, 2009).

In *S. aureus*, farnesol was shown to inhibit biofilm formation and compromise cell membrane integrity (Jabra-Rizk *et al.*, 2006a). In *Streptococcus mutans*, farnesol affected growth and metabolism by disrupting the bacterial membrane (Koo *et al.*, 2002), as well as the accumulation and polysaccharide content of biofilms of the *Streptococci* (Koo *et al.*, 2003). Agents that disrupt the properties of cell membrane can also affect glucan synthesis (Bowen,

2002) and consequently reduce the accumulation and biomass of biofilms (Koo *et al.*, 2003). Accordingly, farnesol significantly affected the rate of glucan synthesis in *S. mutans*, the main polysaccharide in the biofilm matrix, and consequently reduced the accumulation and biomass of the biofilms (Koo *et al.*, 2003). Investigations suggested that the damage to cell membranes might be one of the major antibacterial mechanisms (Inoue *et al.*, 2004). Farnesol also exhibited antimicrobial activity against *Streptomyces tendae* and *Saccharomyces cerevisiae*, but not against *E. coli* (Machida, 1999). Moreover, farnesol acted as a potent antimicrobial agent against *Paracoccidioides brasiliensis*. The fungicide activity of farnesol against this pathogen was probably associated to cytoplasmic organelles degeneration (Derengowski *et al.*, 2009).

Farnesol was identified as a quorum-sensing molecule produced by the dimorphic fungus *Candida albicans*. In this role, farnesol produced extracellularly, inhibits filamentation in *Candida albicans* (Ramage *et al.*, 2002), preventing the germination of yeast cells into mycelia, a phenomenon that may be pertinent to *C. albicans* biofilm formation (Cao *et al.*, 2005; Chen *et al.*, 2004; Enjalbert and Whiteway, 2005; Hornby and Nickerson, 2004; Sato *et al.*, 2004). The capacity to switch from yeast morphology to a hyphal morphology is one of its major virulence determinants (Lo *et al.*, 1997). In another work, farnesol appeared to be employed by *Candida albicans* in order to reduce competition with other microbes, since this compound mediated apoptosis in the filamentous fungus *Aspergillus nidulans* (Semighini *et al.*, 2006), and inhibited biofilm formation in other *Candida* species (Jabra-Rizk *et al.*, 2006b; Rossignol *et al.*, 2007).

Nowadays, it is also known that among *Candida* species, farnesol effects are not restricted to *C. albicans* (Martins *et al.*, 2007). Specifically, farnesol prevents the yeast-to-pseudohyphae transition in *C. dubliniensis* (Henriques *et al.*, 2007) but has no effect on *Candida parapsilosis* morphology (Rossignol *et al.*, 2007), although it reduces biofilm formation in both of these *Candida* species (Jabra-Rizk *et al.*, 2006b, Laffey and Butler, 2005). Another study demonstrated that farnesol showed cariostatic properties in rats without significant effects on the microbial viability in the animals' mouths (Stark *et al.*, 1995). Moreover, farnesol has been shown to be non-mutagenic and non-toxic both *in vitro* and *in vivo* (Burke *et al.*, 1997; Haug *et al.*, 1994; Machida *et al.*, 1999; Voziyan *et al.*, 1995).

Therefore, inhibition of formation and accumulation of biofilm communities by affecting the synthesis of polysaccharides can be an attractive route for preventing biofilm-related infections (Jabra-Rizk *et al.*, 2006a). Farnesol seems to be a potent antimicrobial agent giving effective k^+ leakage from cytoplasm (Inoue *et al.*, 2004). Since membrane damage facilitates

penetration of antibiotics such as macrolides, aminoglycosides and quinolones, farnesol is believed to enhance antimicrobial activity (Brehm-Stecher and Johnson, 2003). In fact, studies have already demonstrated that farnesol is known to intensify the effect of antimicrobial agents (Jabra-Rizk *et al.*, 2006a; Kuroda *et al.*, 2007).

In general, farnesol plays a crucial role in biofilm development and survival. Biomaterial infections are an increasingly alarming problem, and due to their intrinsic recalcitrance to conventional therapy new methods of dealing with these infections must be explored. Specifically, there is a critical need for identifying therapeutic strategies that are directed toward the inhibition of biofilm formation and effective treatment of biofilms once they have been formed. Farnesol may be an interesting prospect as an anti-infective strategy in this setting (Ramage *et al.*, 2002). Furthermore, the fact of farnesol being devoid of toxic effects and nonmutagenic and also able to enhance microbial susceptibility to antibiotics, indicates a putative application as an adjuvant therapeutic agent (Brehm-Stecher and Johnson, 2003; Jabra-Rizk *et al.*, 2006b).

| *N*-ACETYLCYSTEINE

NAC, a mucolytic agent, is used in medical treatment of patients with chronic bronchitis (Olofsson *et al.*, 2003), cancer and paracetamol intoxication (Riise *et al.*, 2000; Stey *et al.*, 2000). This molecule is one of the smallest drug molecules in use (Noszal *et al.*, 2000) (Figure I.9.) and it has antibacterial properties. The molecule is a thiol-containing antioxidant that disrupts disulfide bounds in mucus (Blanco *et al.*, 1997; Sheffner, 1963) and competitively inhibits amino acid (cysteine) utilization (Ventura *et al.*, 1999; Zygmunt and Martin, 1968).

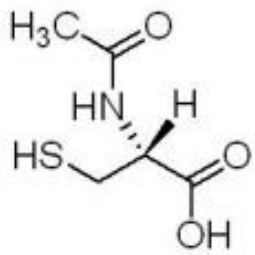


Figure I.9. Structure of *N*-acetylcysteine. Adapted from: http://www.made-in-china.com/showroom/ggbiology/product-detailtMmnbvUVqHkJ/China-N-Acetyl-L-Cysteine-NAC-616-91-1-.html

The positive effects of NAC treatment have primarily been atributed to the mucus-dissolving properties of NAC, as well as its ability to decrease biofilm formation, which reduces bacterial infections (Olofsson *et al.*, 2003). Recently, it was shown that NAC reduced adhesion of *Streptococcus pneumoniae* and *Haemophilus influenzae* to oropharyngeal epithelial cells *in vitro* (Riise *et al.*, 2000). NAC also decreases biofilm formation by a variety of bacteria (Olofsson *et al.*, 2003; Pérez-Giraldo *et al.*, 1997; Schwandt *et al.*, 2004) and reduces the production of extracellular polysaccharide matrix (Olofsson *et al.*, 2003), while promoting the disruption of mature biofilm (Marchese *et al.*, 2003; Schwandt *et al.*, 2004). By degrading the extracellular polysaccharide matrix of biofilm (Marchese *et al.*, 2003; Olofsson *et al.*, 2003), it is possible that NAC may make the biofilm-associated bacteria more susceptible to other antimicrobial agents (Aslam *et al.*, 2007). This was verified by Aslam *et al.* (2007), where NAC potentiated the effect of tigecycline. Besides to inhibits slime synthesis, NAC also demonstrated to promote slime disruption of *E. coli* biofilms (Marchese *et al.*, 2003). However, NAC did not significantly affect the viability of sessile cells (Marchese *et al.*, 2003). While NAC seems not to have a significant effect in cell viability of biofilm cells, it appears to be a possible candidate as a new therapeutic strategy against biofilms being used as antimicrobial adjuvant.

| ANTIBIOTICS - COMBINATION/NOVEL GENERATION OF ANTIBIOTICS

Biofilms are generally insensitive to individual antimicrobials, but they are frequently susceptible to combinations (Saginur *et al.*, 2006). Combinations that are frequently active are ones that should be considered for presumptive therapy of staphylococcal foreign body infections (Saginur *et al.*, 2006). Antibiotic combinations represent a therapeutic option in the treatment of *Staphylococcus epidermidis* infections, as a result of the increasing appearance of multi-resistant microorganisms. In treatments involving antibiotics like rifampicin, that can lead to the rapid selection of resistant mutants (Mick *et al.*, 2010), combination therapy is used to avoid the appearance of antimicrobial resistance in the infectious agent. In other treatments, combinations are used in order to enhance the effect of individual antimicrobials by means of synergic interactions (Monzón *et al.*, 2001). This enhancement has been very useful in clinical practice involving treatment of chronic staphylococcal infections, frequently associated with the formation of biofilms on the biomaterials used in implants or prostheses and the consequent decreased susceptibility (Monzón *et al.*, 2001).

Some recently available antibiotics (antibiotics of novel generation), e.g. daptomycin, tigecycline, linezolid, dalbavancin, may be suitable for treatment of foreign-body infections, caused by sessile and biofilm-producing bacteria such as *S. epidermidis* (Hellmark *et al.*, 2009), and may provide alternatives for monotherapy or combination therapy with rifampicin (Hellmark *et al.*, 2009). Tigecycline, a glycylglycine, is active against a range of multiresistant organisms and is bactericidal against biofilm-associated *Staphylococcus epidermidis* at a lower minimal bactericidal concentration (MBC) than that of vancomycin and daptomycin (Labthavikul *et al.*, 2003). Experimental data regarding the effect of antibiotics on staphylococcal biofilms showed promising results: daptomycin, tigecycline and linezolid reduced the biofilm burden and the number of viable bacteria within the biofilms significantly (Hajdu *et al.*, 2009). Daptomycin (lipopeptide) and tigeclycline are alternatives agents to vancomycin, a standard antimicrobial agent used in the treatment of methicillin-resistant *Staphylococcus aureus* and *S. epidermidis* (Chi *et al.*, 2004; Paterson, 1999). Both present excellent activity against methicillin-resistant *Staphylococcus aureus* and *S. epidermidis*. However, as these agents are in clinical use for a short time only, the extent of toxicity is yet to be experienced (Hajdu *et al.*, 2009).

The chance to treat implant infections is very low once infection involving bacterial biofilms has begun (Hajdu *et al.*, 2009). Up to now, debridement and removal of a heavily infected implant plus antimicrobial treatment are the only options (Trampuz and Zimmerli, 2006). However, more experimental work has to be performed to investigate the level of debridement and the optimal type and dosage of antimicrobial substance needed for the eradication of the bacterial biofilm (Hajdu *et al.*, 2009), specifically of *S. epidermidis*.

| OBJECTIVES AND THESIS OUTLINE

Following the above rationale, the main objective of this thesis was to investigate new strategies to control *S. epidermidis* biofilms, as a means to impair infections related to medical indwelling devices.

The first part of the study focused on the effect of the natural sesquiterpenoid farnesol, recently described as having antibacterial properties, against planktonic and biofilm cells viability of *S. epidermidis*, as reported in Chapter II.

The effect of farnesol on the biofilm matrix was also assessed to get an overall insight on its action on the sessile form of life of this organism (Chapter III). Taking into account the low efficacy of the most common antibiotics against *S. epidermidis* biofilms, the combination of each antibiotic with farnesol was evaluated to search for possible synergistic effects between the two agents, as described in Chapter IV.

Biofilm matrix has been considered a barrier hampering the action of antimicrobial agents, thus a mucolytic and antibacterial drug like *N*-acetylcysteine might be a potential adjuvant in biofilm control by farnesol and this hypothesis was also tested (Chapter V).

Although a new generation of antibiotics is now available their effect on *S. epidermidis* biofilms is still not fully evaluated and they have also the disadvantage of very high costs. Moreover, a new strategy, based in antibiotics combination has been used in clinical practice to minimize resistance development. So, the subject of Chapter VI was the evaluation of double combinations of the most common and traditional antibiotics in the eradication of *in vitro S. epidermidis* biofilms.

Another specific purpose of this study was the evaluation of the expression of biofilm-related genes (virulence and stress regulator genes), by biofilm persister cells after treatment with the antimicrobial agents tested compared with untreated cells to assess their potential hazard for the host. This was performed for cells submitted to two of the most efficient combinations of antibiotics tested and is the subject of Chapter VII.

Chapter VIII finalizes the thesis presenting the main conclusions of the work performed and making some suggestions to get further insights in this field.

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II. EFFECT OF FARNESOL ON PLANKTONIC AND BIOFILM CELLS OF *Staphylococcus epidermidis*

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| ABSTRACT

Staphylococcus epidermidis is now amongst the most important pathogenic agents responsible for bloodstream nosocomial infections and for biofilm formation on indwelling medical devices. Its increasing resistance to common antibiotics is a challenge for the development of new antimicrobial agents. Accordingly, the goal of this study was to evaluate the effect of farnesol, a natural sesquiterpenoid, on *Staphylococcus epidermidis* planktonic and biofilm cells. Farnesol displayed a significant inhibitory effect on planktonic cells. Small concentrations (100 μM) were sufficient to exhibit antibacterial effect on these cells. In biofilm cells the effect of farnesol was not so pronounced and it seems to be strongly dependent on the cells metabolic activity and amount of matrix. Interestingly, the effect of farnesol at 200 μM was similar to the effect of vancomycin at peak serum concentration either in planktonic or biofilm cells. Overall, the results indicate a potential antibacterial effect of farnesol against *S. epidermidis*, and therefore the possible action of this molecule on the prevention of *S. epidermidis* related infections.

Keywords: *Staphylococcus epidermidis*; planktonic cells; biofilm; farnesol.

Staphylococcus epidermidis and related coagulase-negative staphylococci (CoNS) are now well established as major nosocomial pathogens associated with infections of indwelling medical devices (Cerca *et al.*, 2004; Cerca *et al.*, 2005; Vuong *et al.*, 2003). This bacterium has become the primary cause of nosocomial bloodstream infections, also of the eye, ear, nose, and throat as well as cardiovascular infections (Vuong *et al.*, 2003). This is related, in part, to the organism's ability to adhere to surfaces and form biofilms (Cerca *et al.*, 2004; Izano *et al.*, 2007; Oliveira *et al.*, 2007; Wang *et al.*, 2007). In fact, the formation of biofilms has been considered the main virulence mechanism of *S. epidermidis* (Oliveira *et al.*, 2007; Vuong *et al.*, 2003), and is the main cause of chronic infections (Izano *et al.*, 2007). Biofilm formation requires the bacterial attachment to solid surfaces, the development of bacterial multilayers and their enclosing in a large exopolymeric matrix (Oliveira *et al.*, 2007). This structure impairs the action of phagocytic cells from the immune system and of antimicrobial agents (Izano *et al.*, 2007; Oliveira *et al.*, 2007; Wang *et al.*, 2007), and releases planktonic cells from the outer layers, allowing the persistence of bacterial infections (Oliveira *et al.*, 2007). Biofilms are notoriously difficult to eradicate and are a source of many recalcitrant infections (Wang *et al.*, 2007). However, a more important consequence of biofilm formation, with profound clinical implications is the markedly enhanced resistance to antimicrobial agents by biofilm-associated microorganisms, which are estimated to be much more resistant than their planktonic counterparts (Jabra-Rizk *et al.*, 2006). The ability of biofilm-embedded cells to resist to antimicrobial agents points to the importance of a continuous search for novel agents that are effective against bacteria in this mode of growth or that can work in synergy with the currently available myriad of antimicrobial agents (Jabra-Rizk *et al.*, 2006). Several studies have been assessing the antimicrobial potential of natural terpene alcohols. Specifically, farnesol has been reported to have antibacterial properties. For instance, in *Streptococcus mutans*, brief exposure to farnesol affected growth and metabolism by disrupting the bacterial membrane, as well as the accumulation and polysaccharide content of biofilms of the streptococci (Jabra-Rizk *et al.*, 2006). In *Staphylococcus aureus*, farnesol was shown to inhibit biofilm formation and compromise cell membrane integrity (Jabra-Rizk *et al.*, 2006). Inoue *et al.* (2004) also demonstrated that farnesol have antibacterial activity against *S. aureus*. The results of these authors suggested that farnesol might act on cell membranes, where the damage to those membranes might be one of the major

modes of action of this terpene alcohol (Inoue *et al.*, 2004). Accordingly the goal of this study was to investigate the effect of farnesol on planktonic and biofilm cells of *S. epidermidis*.

| MATERIALS AND METHODS

| BACTERIAL STRAINS AND GROWTH CONDITIONS

Four *S. epidermidis* strains were used in this study: two good biofilm-producing (1457 and 9142) and the respective mutants nonbiofilm-producing (1457-M10 and 9142-M10). These strains are clinical isolates and were stored at or below –120°C in a liquid-nitrogen freezer and used as needed. All strains were provided by Dr. G. B. Pier, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, USA. Tryptic soy broth (TSB) and tryptic soy agar (TSA) were prepared according to the manufacturer's instructions. All strains were inoculated into 15 mL of TSB from TSA plates not older than 2 days and grown for 24 (±2) hours at 37°C in an orbital shaker at 130 rpm. Cells were harvested by centrifugation (for 5 minutes at 9500 ×g and 4°C), and resuspended in TSB adjusted to an optical density (OD) (640 nm) equivalent to 1×10^6 cells mL^{-1} and then used in the subsequent assays. Each stock solution of farnesol was prepared in methanol. It was confirmed that methanol, at the concentration used, had no effect on the growth of the *S. epidermidis* strains studied.

| EFFECT OF FARNESOL ON PLANKTONIC CELLS

| FARNESOL SUSCEPTIBILITY OF PLANKTONIC CELLS ASSESSED BY ALAMAR BLUE (AB) ASSAY

Viability assays were performed in six well tissue-culture plates (Sarstedt, Newton, NC, USA) containing 4 mL of a *S. epidermidis* cell suspension (1×10^6 cells mL^{-1}) in the presence of farnesol (0, 30, 100, 150, 200, 250, and 300 μM; 0–66 μg mL^{-1}) (Sigma). The suspensions were incubated at 37°C and at 130 rpm. After 6 and 12 hours of exposure to the different concentrations of farnesol, an aliquot of 200 μL of *S. epidermidis* culture was removed from all experimental and control wells and 20 μL (10% v/v of culture volume) of AB (Biosource), an

oxidation–reduction indicator, was added. Exposure of AB to light was minimized throughout the experiment. The plates were shaken gently and incubated for 1 hour at 37°C. The growth was indicated by a change in colour from dark blue to pink. The tolerance threshold was defined as the lowest concentration of farnesol that prevented the development of a pink colour. This experiment was repeated twice, in triplicates.

| FARNESOL SUSCEPTIBILITY OF PLANKTONIC CELLS ASSESSED BY CFU PLATING

Colony forming units (CFU) were obtained from the same wells used to obtain AB fluorescence as follows: 100 µL of culture were removed from all experimental and control wells and the viable cells were determined by performing 10-fold serial dilutions in saline blanks and plated in TSA. Colonies were counted after 24 hours at 37°C. This experiment was repeated twice, in triplicates.

| EFFECT OF FARNESOL ON BIOFILM CELLS

| FARNESOL SUSCEPTIBILITY OF BIOFILM CELLS ASSESSED BY CRYSTAL VIOLET (CV) AND XTT ASSAYS

In those assays only two *S. epidermidis* strains were used: *S. epidermidis* 1457 and *S. epidermidis* 9142, both good biofilm producers. Biofilms were formed in 96 well tissue culture plates (Sarstedt, Newton, NC, USA) containing 200 µL of a *S. epidermidis* cell suspension (1×10^6 cells mL^{-1}) in TSB supplemented with 0.25% of glucose per well to promote biofilm formation. Plates were incubated at 37°C with orbital shaking at 130 rpm for 24 hours. At the end, planktonic cells were removed carefully, and the biofilm was washed twice with 200 µL of ultrapure water. The biofilms were incubated in fresh nutrient medium containing farnesol (0, 30, 100, 200, and 300 µM) (Sigma) and CV and XTT assays were performed at time 4, 8, and 24 hours. A similar assay was carried out to assess the effect of vancomycin at peak serum concentration (PS = 40 mg L^{-1}) on 24 hours biofilm cells. The aim of this assay was to compare the effect of farnesol with the effect of the most common ‘‘last resort’’ antibiotic used to control nosocomial infections. Crystal violet was used as indicator of total biofilm biomass. After exposure to farnesol and vancomycin, biofilms were washed with 200 µL of 0.9% NaCl, then 250

µL of methanol was added and allowed to act for 15 minutes. Afterwards, methanol was removed and crystal violet was added (5 minutes). The wells were washed with water and finally, acetic acid 33% (v/v) was added. The absorbance was measured at 570 nm. Another colorimetric method based on the reduction of XTT ({2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide}) was applied to determine farnesol and vancomycin susceptibility (XTT is converted to a coloured formazan salt in the presence of metabolic activity) (Kuhn *et al.*, 1999). After exposure to antimicrobial agents, biofilms were washed with 200 µL of 0.9% NaCl, then 250 µL of a solution containing 200 mg L^{-1} of XTT and 20 mg L^{-1} of phenazine methosulphate (PMS) (Sigma) was added to each well. The microtiter plates were incubated for 3 hours at 37°C in the dark. The absorbance was measured at 490 nm. Controls were biofilms not exposed to any antimicrobial agent tested. All experiments were carried out in triplicate and repeated three times.

| STATISTICAL ANALYSIS

The data from all assays were compared using one-way analysis of variance (ANOVA) by applying Tukey's and Bonferroni tests with all calculations carried out using SPSS software (Statistical Package for the Social Sciences). Differences achieving a confidence level of 95% were considered significant.

| RESULTS AND DISCUSSION

Four *S. epidermidis* strains were selected for this study: strain 1457 is a producer of the PNAG/PIA, the major surface polysaccharide promoting coagulase-negative staphylococci intercellular adherence and biofilm formation, and its isogenic biofilm-negative mutant *icaA*::Tn917 transductant 1457-M10 (Rupp *et al.*, 1999); *S. epidermidis* 9142 another well-known producer of PNAG/PIA and *S. epidermidis* 9142-M10 an isogenic strain to 9142, containing a transposon inserted into the *ica* locus which encodes the biosynthetic enzymes for producing PNAG/PIA and thus does not produce this molecule (Cerca *et al.*, 2004). The growth inhibition effect of farnesol on planktonic cells of *S. epidermidis* was assessed using a rapid colorimetric assay that measures cellular metabolic activity and is based on the reduction of Alamar Blue. The viability assays showed, for all *S. epidermidis* strains used, that incubation of

planktonic cells in the presence of 100 μM (22 μg mL^{-1}) farnesol resulted in no change in colour from blue to pink, indicating that this concentration inhibited oxidation-reduction reactions in the suspensions (data not shown). These data indicated that low concentrations of farnesol were sufficient to exhibit antibacterial effect, as was demonstrated by viability assays. For *Staphylococcus aureus* this was observed in the presence of 200 μM (44 μg mL^{-1}) of farnesol (Jabra-Rizk *et al.*, 2006). This inhibitory effect can be due to the hydrophobic nature of farnesol that favours its accumulation in the membrane, possibly causing membrane disruption (Jabra-Rizk *et al.*, 2006). In fact, exposure to terpene alcohols has been recently shown to affect the cell membranes of *S. aureus*, *Escherichia coli* and *Listeria monocytogenes*, resulting in leakage of K^+ ions from cells (Jabra-Rizk *et al.*, 2006).

Figure II.1. depicts the effect of different farnesol concentrations on planktonic cells of the four strains studied. Generally, there was a steep decrease in CFUs at 100 μM of farnesol ($p < 0.05$), levelling off for higher concentrations. A farnesol concentration of 100 μM seems to be sufficient to promote a very significant reduction in *S. epidermidis* planktonic cells viability. It should be stressed that for concentrations above 100 μM, the cellular viability remains almost the same independently of farnesol concentration and exposure time. In fact, it seems that there is a threshold of farnesol uptake acting as limiting factor rather than farnesol concentration. Besides, bacterial populations produce persister cells that neither grow nor die in the presence of microbicidal agents. Persisters are largely responsible for high levels of biofilm tolerance to antimicrobials (Keren *et al.*, 2003). This was also confirmed in experiments with *E. coli*, where a small sub-population of cells remained alive irrespective of the concentration of the antibiotic (persisters) (Lewis, 2007).

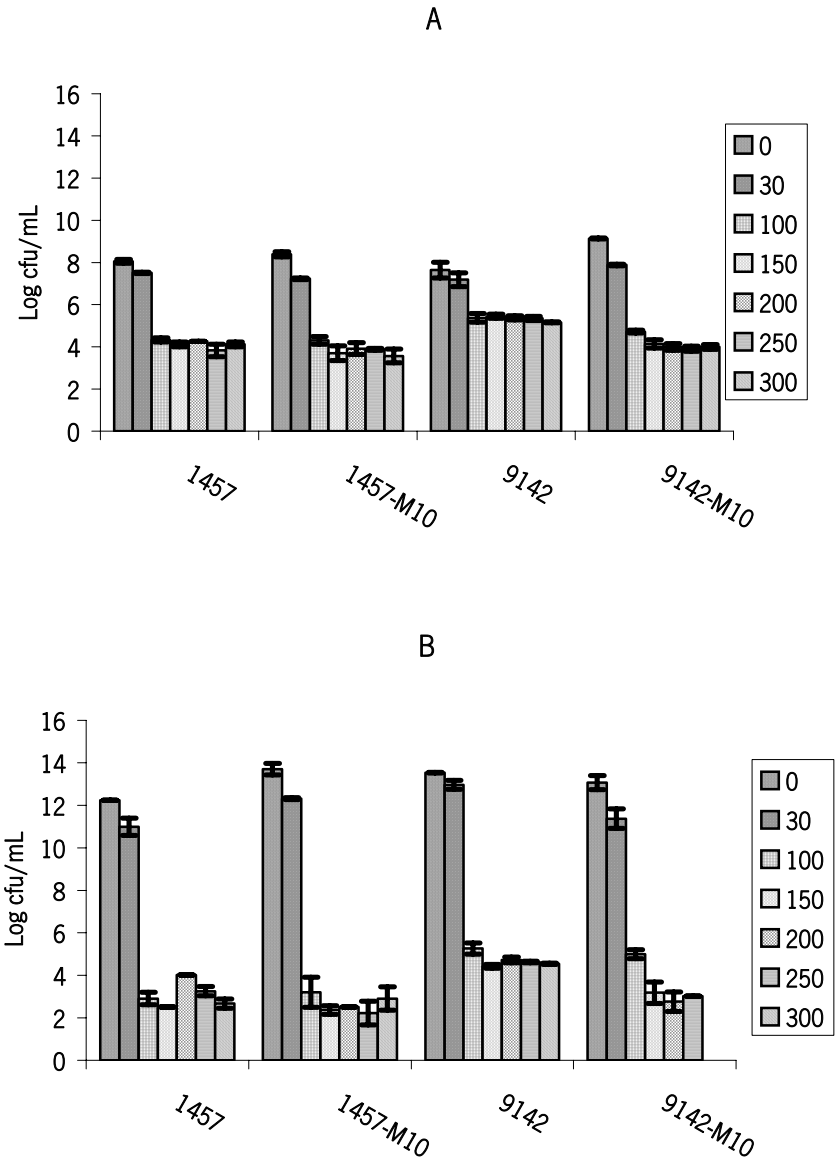


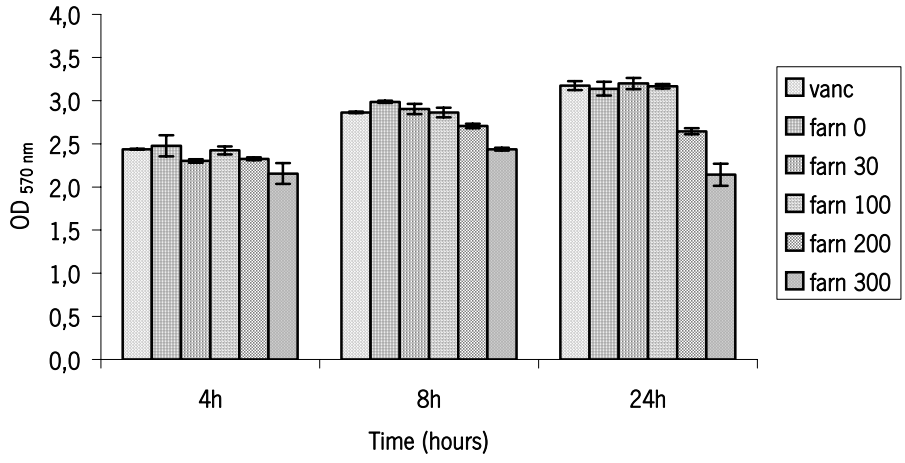
Figure II.1. Effect of farnesol (0–300 μM) on *S. epidermidis* planktonic cells assessed by CFU enumeration, after 6 (A) and 12 hours (B) of exposure to farnesol. Bars represent standard deviation of the mean.

According to the other goal of this work, farnesol was added to 24 hours biofilms of *S. epidermidis* 1457 and 9142 strains and biofilm biomass and activity were evaluated. These two strains were selected because they are both good biofilm producers. As could be expected, biofilm cells were much less sensitive to farnesol than their planktonic counterparts. Noticeably, strain 9142 biofilm was more sensitive to farnesol than the sessile cells of strain 1457 (Figures II.2. and II.3.). This corresponds to an inversion of the observed behaviour for planktonic cells (Figure II.1.). Those facts prove that the biofilm structure along with the specific physiology of the

sessile mode of life determine a response to antimicrobial agents, which cannot be extrapolated from tests performed with planktonic cells.

To have some comparison of the response of biofilm cells it was decided to assess the effectiveness of vancomycin, one of the most frequently used antibiotics to treat resistant nosocomial infections, which is primarily effective against coagulase-negative Staphylococcus species (Rybak, 2006). Furthermore, both tested agents act at the cell wall level, vancomycin inhibits the biosynthesis of bacterial cell wall, while farnesol is considered to disrupt the normal barrier function of the cell membrane (Brehm-Stecher and Johnson, 2003). Interestingly, farnesol at a concentration higher than 200 μM displayed the same or higher effectiveness of vancomycin at peak serum concentration (Figures II.2. and II.3.). In fact, the response of the strains tested was very similar for both farnesol (> 200 μM) and vancomycin. Accordingly, the biofilm of strain 1457 showed higher tolerance to vancomycin than the biofilm of strain 9142. In a previous study, Sousa *et al.* (2008) have shown that planktonic cells of strain 1457 displayed a higher glucose uptake, compared to 9142 planktonic cells. Conversely, in biofilm form the glucose uptake of strain 9142 was 3.5 times higher than that of sessile cells of strain 1457. This suggests that cell susceptibility increases with cell metabolic activity. Despite the effect of specific changes in cell physiology triggered by the sessile form of life, the higher amount of exopolysaccharides present in the biofilm matrix of strain 1457 (Sousa *et al.*, 2008), can also play a role in the increased tolerance to the antimicrobials tested. This is another point in favour of the virulence character of the matrix.

A



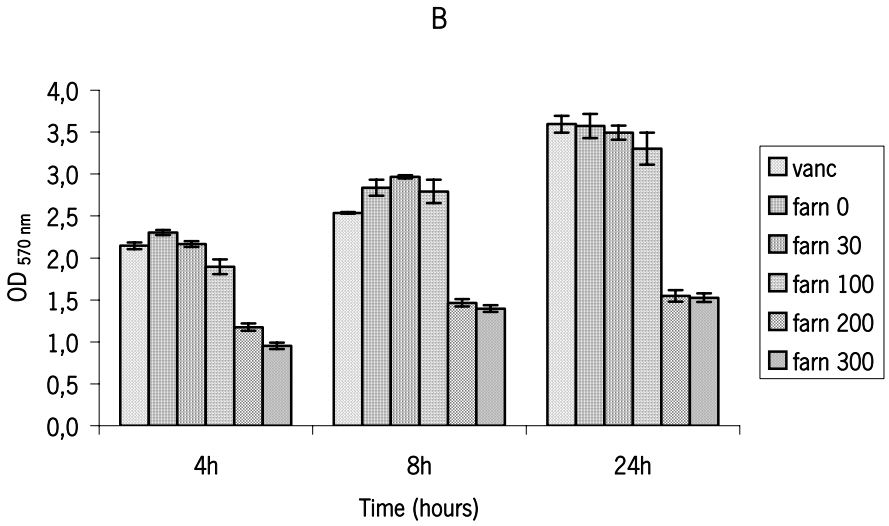
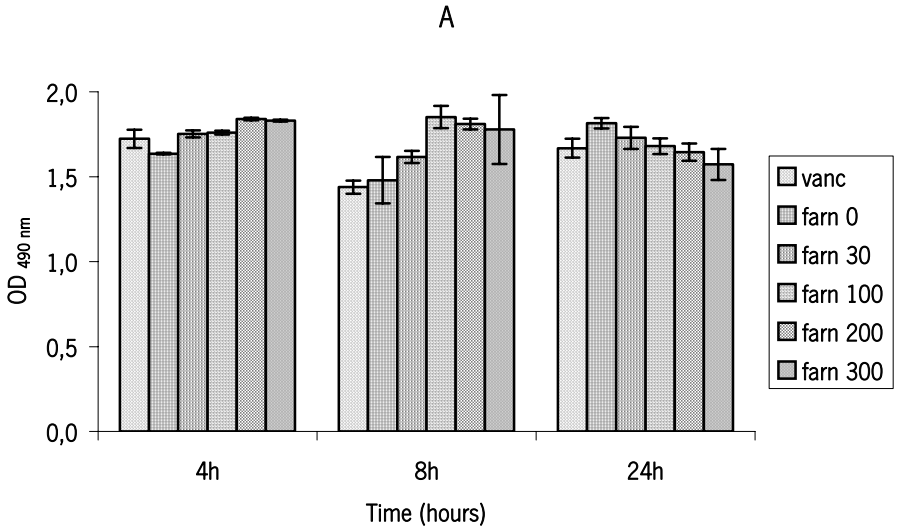


Figure II.2. Effect of farnesol (farn) (0–300 μM) and vancomycin (vanc) (40 mg L^{-1}) on biofilm cells of *S. epidermidis* 1457 (A) and 9142 (B), expressed as CV absorbance (total biofilm biomass)



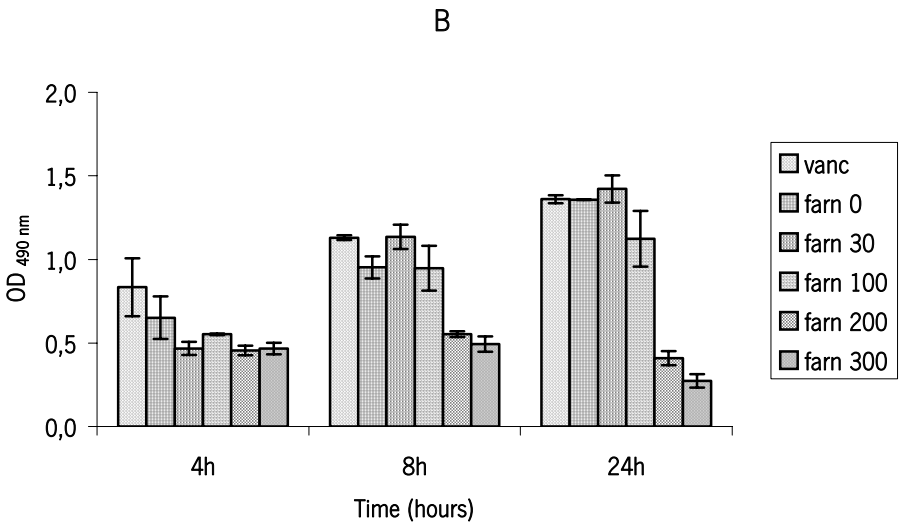


Figure II.3. Effect of farnesol (farn) (0–300 μM) and vancomycin (vanc) (40 mg L^{-1}) on biofilm cells of *S. epidermidis* 1457 (A) and 9142 (B), expressed as XTT absorbance (cellular activity) methodologies to assess sessile cells susceptibility to antimicrobial agents.

As conclusion, the results show the significant effect of farnesol in the reduction of the viability of planktonic cells of the four *S. epidermidis* strains assayed and less pronounced on mature biofilm cells. In fact, on planktonic cells, small concentrations of this sesquiterpenoid (100 μM) are sufficient to exhibit antibacterial effect, as was shown by viability assays. Additionally, the loss of cellular viability and consequently the loss of biofilm formation ability (main virulence factor) induced by farnesol suggest a potential use of this molecule in the prevention of *S. epidermidis* infection.

It was once again demonstrated that the response of planktonic cells cannot be extrapolated for biofilm cells and this must be a driving force for the development of new methodologies to assess sessile cells susceptibility to antimicrobial agents.

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III. EFFECT OF FARNESOL ON STRUCTURE AND COMPOSITION OF *Staphylococcus epidermidis* BIOFILM MATRIX

Submitted for publication

| ABSTRACT

Staphylococcus epidermidis is the most frequent cause of nosocomial sepsis and catheter-related infections in which biofilm formation is considered to be one of the main virulence mechanisms. Moreover, their increased resistance to conventional antibiotic therapy enhances the need to develop new therapeutical agents. Farnesol, a natural sesquiterpenoid present in many essential oils, has been described as impairing bacterial growth. The goal of this study was to evaluate the effect of farnesol on the structure and composition of biofilm matrix of *S. epidermidis*. Biofilms formed in the presence of farnesol (300 μM) contained less biomass, and displayed notable changes in the composition of the biofilm matrix. Changes in the spatial structure were also verified by confocal scanning laser microscopy (CSLM). The results obtained by the quantification of extracellular polymers and by wheat germ agglutinin (WGA) fluorescent detection of glycoproteins containing β(1→4)-*N*-acetyl-D-glucosamine support the hypothesis that farnesol causes disruption of the cytoplasmic membrane and consequently release of cellular content.

Keywords: Staphylococci; farnesol; nosocomial infection; biofilm.

| INTRODUCTION

Staphylococcus epidermidis is a coagulase-negative staphylococcus that has emerged in the last years as one of the most important nosocomial and opportunistic pathogens (Sousa *et al.*, 2009). Due to its ability to attach to polymeric surfaces *S. epidermidis* is a common pathogen in chronic, medical device-associated infections (Izano *et al.*, 2007; Knobloch *et al.*, 2002). Nowadays, it is established that the natural mode of bacterial life is in multicellular complexes referred to as biofilms, which behave in a quite different way than free-floating cells (Sandberg *et al.*, 2008). A biofilm can be defined as a surface-attached agglomeration of cells that are embedded in a heterogeneous matrix (Vuong *et al.*, 2004; Ziebuhr *et al.*, 2006).

The matrix is one of the most distinctive features of a microbial biofilm. It forms a three-dimensional, gel-like, highly hydrated and locally charged environment in which the micro-organisms are largely immobilized. Matrix-enclosed microcolonies, sometimes described as "stacks" or "towers", are separated by water channels which provide a mechanism for nutrient circulation within the biofilm. The composition of the matrix varies according to the nature of the organisms present. Matrix polymers of bacterial biofilms are primarily exopolysaccharides, and many are negatively charged due to the presence of carboxyl, sulphate or phosphate groups. Smaller amounts of proteins, nucleic acids and lipids can also be present. Generally, the *S. epidermidis* biofilm matrix comprises several extracellular polymeric substances such as polysaccharides, proteins, considerable amounts of extracellular teichoic acids and also extracellular DNA (Sousa *et al.*, 2009). Two of the best characterized matrix polysaccharides in bacteria are alginate produced by *Pseudomonas aeruginosa*, and poly β-1,6-linked *N*-acetylglucosamine secreted by *Staphylococcus epidermidis*. Synthesis of both polysaccharides has been related to bacterial virulence (Al-Fattani and Douglas, 2006). Essential for *S. epidermidis* cell accumulation is the expression of PNAG/PIA which mediates cell-to-cell adhesion (Knobloch *et al.*, 2002; Sousa *et al.*, 2009).

Bacterial pathogens have evolved numerous defence mechanisms against antimicrobial agents, and resistance to older and newly produced drugs are on the rise. Moreover, microbial biofilms being responsible for a number of diseases of chronic nature demonstrate extremely high resistance to antibiotics and host defence systems (Kuźma *et al.*, 2007; Teixeira *et al.*, 2007). These are the reasons why many research groups investigate potential strategies, which could be accessory or alternative to antibiotic therapy (Kuźma *et al.*, 2007).

Farnesol, a natural sesquiterpenoid present in many essential oils, has been described to have antibacterial effect. In fact, we have published previously that farnesol is very effective against planktonic cells and also in reducing total biofilm mass of *S. epidermidis* (Gomes *et al.*, 2009). Other authors have also shown the antimicrobial effect of farnesol on *S. aureus* (Jabra-Rizk *et al.*, 2006) and it has been hypothesized that its mode of action is by compromising cell membrane integrity.

Because we are generally concerned with *S. epidermidis* biofilm control, the main goal of this study was to evaluate the effect of farnesol on biofilm structure and matrix composition.

| MATERIALS AND METHODS

| BACTERIAL STRAINS AND GROWTH CONDITIONS

In this study, a good biofilm-producing strain was used, *S. epidermidis* 1457. This strain is a clinical isolate and has been previously used in mutagenesis studies to determine the basis of biofilm formation (Mack *et al.*, 1996). Tryptic soy broth and tryptic soy agar were prepared according to the manufacturer's instructions. Strains were grown as previously described (Cerca *et al.*, 2004). Briefly the strain was inoculated into 15 mL of TSB from TSA plates not older than 2 days and grown for 18 (± 2) hours at 37°C in an orbital shaker at 130 rpm. Cells were harvested by centrifugation (for 10 minutes at 9500 ×g and 4°C), and resuspended in TSB adjusted to an OD (640 nm) equivalent to 1×10^9 cells mL^{-1} and then used in the subsequent assays. Each stock solution of farnesol was prepared in methanol. It was confirmed that methanol, at the concentration used, had no effect on the growth of the *S. epidermidis* strain studied.

| BIOFILM MATRIX EXTRACTION

Biofilms were formed in 6 well tissue culture plates containing 4 mL of *S. epidermidis* cell suspension (1×10^6 cells mL^{-1}) in TSB supplemented with 0.25% glucose per well to promote biofilm formation. Plates were incubated at 37°C with orbital shaking at 130 rpm for 24 hours. At the end, planktonic cells were removed carefully, and the biofilm was washed twice with 4 mL of

0.9% NaCl. The biofilms were incubated in fresh nutrient medium containing farnesol (0, 30 and 300 μM) (Sigma) for 24 hours.

The extraction of the biofilm extracellular material was performed using the cation exchange Dowex resin (50 × 8, Na^+ form, 20-50 mesh Aldrich-fluka 44445), according to the procedure described by Frølund *et al.* (1996). Prior to extraction, the Dowex resin was washed with the extraction buffer [2 mM Na_3PO_4 (Merck); 4 mM NaH_2PO_4 (Merck); 9 mM NaCl and 1mM KCl (Merck); pH 7.0]. Then, the biofilms previously scrapped off the 6 well plates were washed with phosphate buffer (0.01 M; pH 7.0) and centrifuged for 5 minutes, at 9000 ×g. The extraction was performed using 2 g of washed Dowex resin and 10 mL of extraction buffer per g of biofilm and stirring for 2 hours at 400 rpm and -4°C. The extracellular polymers (supernatant) were obtained by centrifugation at 9000 ×g for 20 minutes.

| PROTEINS AND POLYSACCHARIDES QUANTIFICATION

The total protein content extracted from the matrix was determined by the colorimetric bicinchoninic acid (BCA) assay (Bicinchoninic Acid Kit for Protein Determination, Sigma, USA) using bovine serum albumin (BSA) as standard. The extracted polysaccharides were quantified by the phenol-sulphuric acid method of Dubois *et al.* (1956), using glucose as standard.

| BIOFILM DRY-WEIGHT MEASUREMENTS

After 24 hours of farnesol exposure, biofilm dry-weight was assessed. The biofilm cells were filtered through preweighed filters (0.22 μm) and washed three times with ultrapure sterilized water. Filters were dried at 80°C until constant weight and cell dry weight were determined. This step was repeated at least four times. Biofilm dry-weights were assessed by the difference between the weight of the membrane with and without biomass.

| CONFOCAL SCANNING LASER MICROSCOPY

CSLM was performed as described before (Cerca *et al.*, 2005). Briefly, a 24 hours biofilm was formed on tissue culture plates by dispensing 4 mL of cell suspension into each well of a 6 well microtiter plate. Thereafter, farnesol at concentrations of 0 and 300 μM was added to

the already formed biofilms. Plates were incubated 24 hours at 37°C and at 130 rpm. Following incubation, the biofilms were washed twice with 0.9% NaCl and stained with 4',6-diamidino-2-phenylindole (DAPI) and WGA (conjugated with Alexa Fluor 488 - Molecular Probes) for the fluorescent detection of glycoproteins containing β(1→4)-*N*-acetyl-D-glucosamine, or with Live/Dead (L/D) staining, to determine cell viability. For L/D staining, a negative control was used, to determine the baseline threshold for dead cells, by killing the biofilm with 96% ethanol for 4 hours. The plates were incubated for 20 minutes at room temperature in the dark. After staining, the biofilms were gently rinsed with 0.9% NaCl. The biofilm images (1024 × 1024) were acquired in an Olympus™ FluoView FV1000 confocal scanning laser microscope. Biofilms were observed using a 60× water-immersion objective (60×/1.2W). For each condition, three independent biofilms were used, and in each biofilm four different regions of the surface were analyzed. For biofilm maximum thickness determination, twenty different regions per surface were analyzed, by determining the first and last layer of the biofilm, and calculating the maximum thickness of each region.

| ACQUISITION OF RESISTANCE/TOLERANCE DETERMINATION

S. epidermidis were grown planktonically in TSB with sub-inhibitory concentrations of farnesol (30 μM) during 12 hours. Then the cells were harvested by centrifugation and adjusted to a cellular concentration of ≈ 1×10^6 cells mL^{-1} in fresh medium with and without farnesol (at an inhibitory concentration of 100 μM) for 24 hours at 37°C at 130 rpm. After that, cellular activity was assessed by CFU. The initial cells, harvested after being in contact with a sub-inhibitory concentration of farnesol (30 μM) were again subjected to that sub-inhibitory concentration of farnesol and this process was repeated for five consecutive days. Controls were cells not exposed to farnesol.

All experiments were carried out in triplicate and repeated three times.

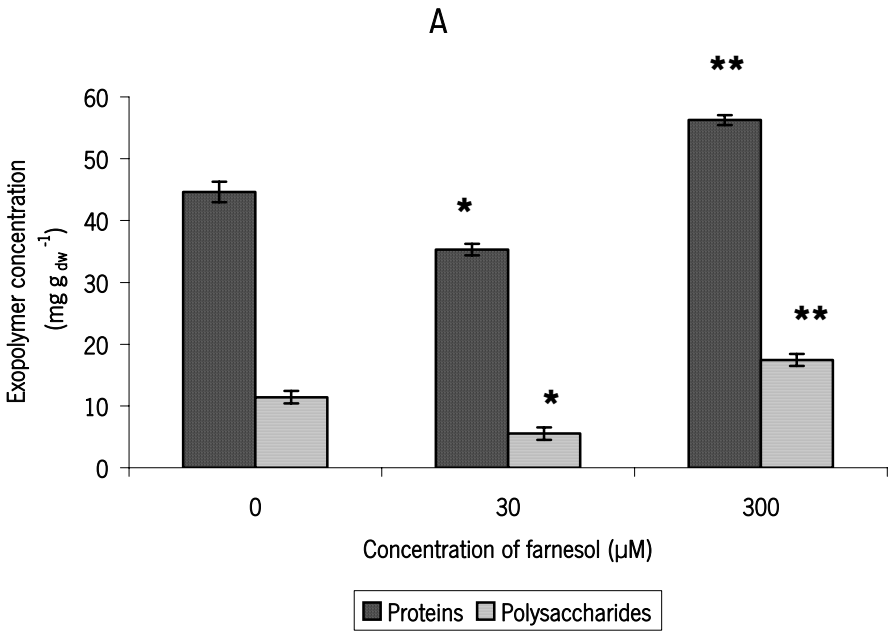
| STATISTICAL ANALYSIS

The data from all assays were compared using one-way analysis of variance by applying Tukey's and Bonferroni tests with all calculations carried out using SPSS software (Statistical

Package for the Social Sciences). Differences achieving a confidence level of 95% were considered significant.

| RESULTS

Figure III.1.A presents the quantification of polysaccharides and proteins in the biofilm matrix of *S. epidermidis* 1457 strain. According to the results, after 24 hours of farnesol (300 μM) exposure there was an increase in the exopolymers concentration present in the matrix of the biofilm ($p < 0.05$). On the other hand, the sub-inhibitory concentration of farnesol tested (30 μM) appears to inhibit the formation of biofilm matrix as there was a reduction in the amount of existing exopolymers in the matrix ($p < 0.05$). Farnesol at 300 μM caused a slight reduction on total biomass of biofilms of *S. epidermidis* 1457 strain (Figure III.1.B).



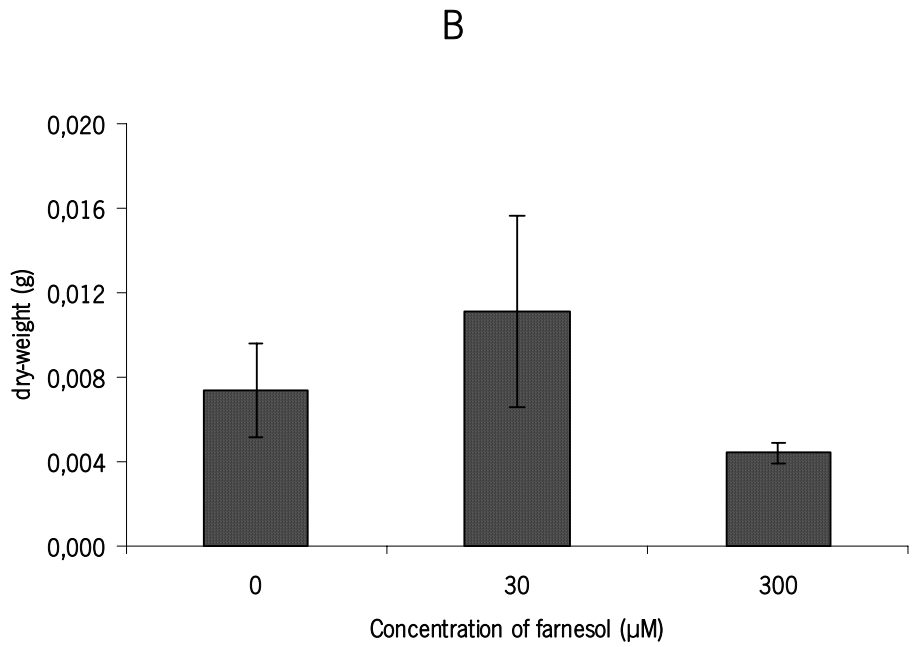


Figure III.1. Concentration of polysaccharides (glucose as standard) and proteins (BSA as standard) extracted (mg g_{dw}^{-1}) by Dowex resin method from *S. epidermidis* strain 1457 biofilm matrix (A) and biofilm biomass expressed by dry weight (B). Error bars represent standard deviation. * Exopolymers concentration decreased significantly after treatment with 30 µM farnesol compared with non-treated biofilms ($p < 0.05$); ** Concentration of exopolymers after treatment with 300 µM of farnesol significantly increased compared with non-treated biofilms ($p < 0.05$).

WGA was used to detect the presence of PNAG/PIA (Cerca *et al.*, 2005) because this lectin binds to the biofilm matrix of *S. epidermidis* (Neu *et al.*, 2001), due to its ability to recognize the *N*-acetylglucosamine component of PNAG/PIA antigen, although it may recognize other components, such as peptidoglycan and teichoic acid, which also contain glucosamine and form part of biofilm matrix (Cerca *et al.*, 2006).

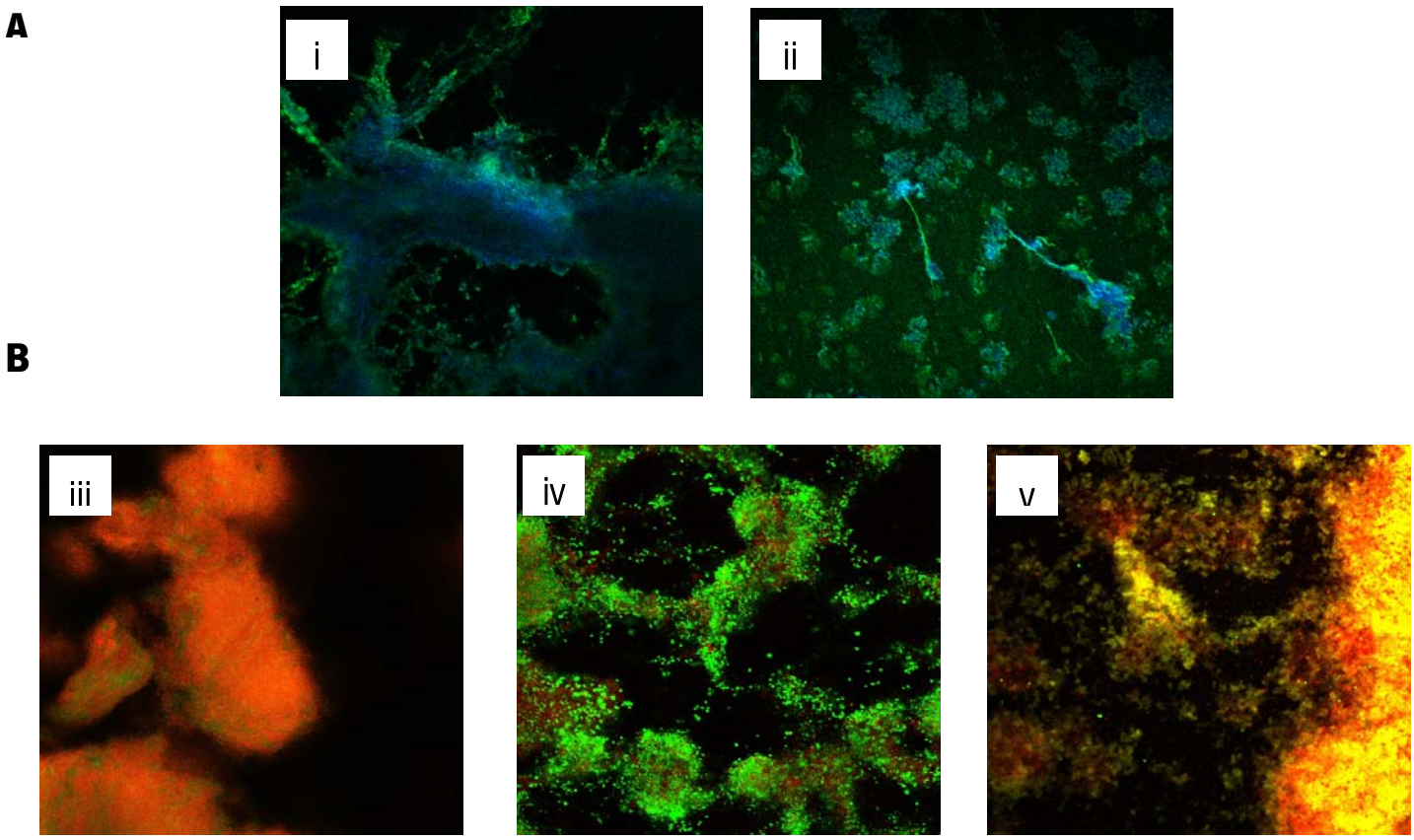


Figure III.2. (A) CSLM images of 24 hours biofilm following 24 hours exposure to 0 μM (i) and 300 μM (ii) farnesol, stained with DAPI (bacterial cells in blue) and WGA (green represents PNAG/PIA). (B) Biofilms stained with Live/Dead: (iii) left image is a negative control (biofilm bacteria killed with 96% ethanol), (iv) biofilm following 24 hours exposure to 0 μM farnesol and (v) biofilm exposed 24 hours to 300 μM farnesol.

CSLM images showed that *S. epidermidis* 1457 strain formed a thick biofilm when grown in the absence of farnesol having a noticeable amount of PNAG/PIA. After addition of farnesol, a significant destruction of biofilm structure (Figure III.2.A) and a clear reduction of biofilm thickness (Figure III.3.) were observed. Furthermore, most cells inside the biofilm where either dead or with damaged cell wall, as determined by Live/Dead staining (Figure III.2.B).

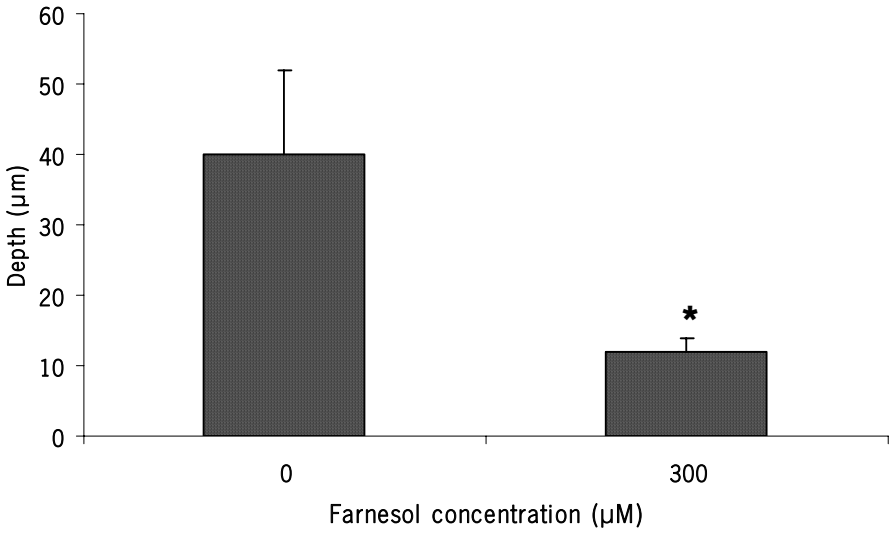


Figure III.3. Biofilm maximum depth average obtained by CSLM for biofilms without exposure to farnesol and after 24 hours exposure to 300 µM of farnesol. * Statistically different from control (untreated cells) ($p < 0.05$).

Although the mechanism of action of farnesol is not yet understood, it appears to have an antimicrobial effect against *S. epidermidis* cells (Gomes *et al.*, 2009). It was previously showed that sometimes biofilm bacteria can be induced to increase their resistance to antimicrobials by continuing exposure to sub-inhibitory concentrations of antibiotics (Cerca *et al.*, 2005). Therefore, it is important to study the ability of cells to acquire tolerance/resistance to this agent. For that, planktonic cells were treated with a sub-inhibitory concentration of farnesol over several generations to determine if after contact with this compound the cells acquire tolerance/resistance. The results obtained are shown in figure III.4.

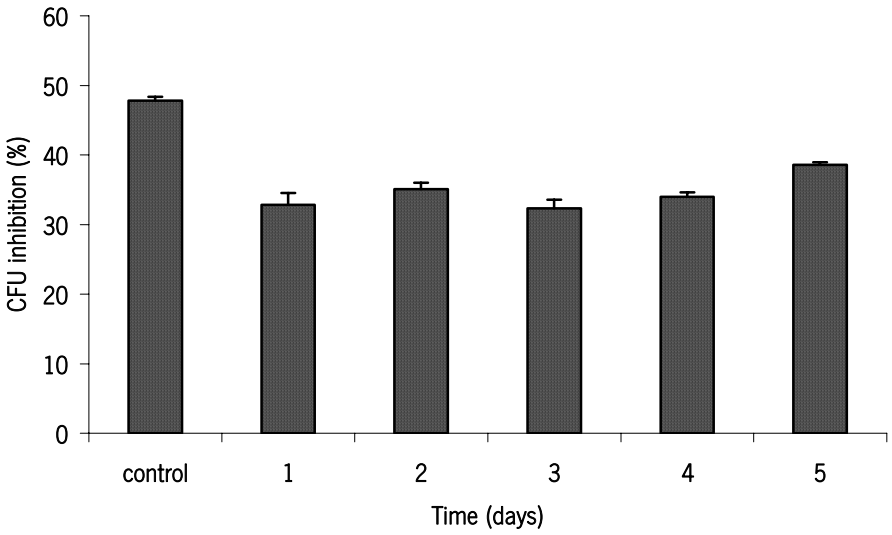


Figure III.4. Percentage of CFU inhibition by farnesol against planktonic *S. epidermidis* cells (strain 1457). Control corresponds to cells not exposed to sub-inhibitory concentrations of farnesol. Error bars represent standard deviation.

The results of CFU determination evidenced an acquisition of tolerance to farnesol by the cells after being exposed to farnesol at a concentration of 30 μM. However, over time it was not observed resistance to farnesol.

| DISCUSSION

Nowadays, *S. epidermidis* ranks first among the causative agents of nosocomial infections and represents the most common source of infections on indwelling medical devices (Otto, 2009). Simultaneously, the resistance to antibiotics has become an important problem in *S. epidermidis* infections. In this context, the interest in studying the antimicrobial activity of potential alternatives to antibiotics has increased in recent years. Considering our previous studies on the effect of farnesol against *S. epidermidis* biofilms (Gomes *et al.*, 2009), in this work we evaluated the role of farnesol in *S. epidermidis* biofilm structure and matrix composition.

First, the polysaccharide and protein biofilm matrix content, as well as the total biomass of biofilm were quantified. The results showed an increase of proteins and polysaccharides per gram dry weight of biofilm after treatment with farnesol at 300 μM. This may be derived from the bursting of the cells and consequent release of cellular content. This fact can also be due to an

overexpression of some *S. epidermidis* virulence genes responsible for the production of PNAG/PIA and other exopolymers, which can be a protective mechanism triggered by cells under stress. On the other hand, there is a slight decrease in the amount of polysaccharides and proteins in the extracellular matrix after exposure to 30 μM of farnesol. This sub-inhibitory concentration has no effect either on cell metabolic activity and consequently in cell replication or in the total biofilm biomass (Figure III.1.B) ($p > 0.05$) (Gomes *et al.*, 2009). Thus, although this concentration was not significantly inhibitory against *S. epidermidis* biofilm, it can possibly decrease the matrix development and therefore the biofilm formation over time.

Many *S. epidermidis* strains produce exopolymers, namely poly-gama-glutamic acid and a poly-*N*-acetylglucosamine homopolymer, that surrounds and connects *S. epidermidis* cells inside biofilms (Otto, 2009). In general, *S. epidermidis* exopolymers protect the cells from antibody recognition and consequently, protect the bacterium from important mechanisms of innate host defence. Relatively to PNAG/PIA, in addition to its role as part of the extracellular biofilm matrix, it has been found to protect *S. epidermidis* from neutrophil killing, complement deposition, immunoglobulins and antimicrobial peptides (Cerca *et al.*, 2006; Otto, 2009). Moreover, our immune system may have evolved to react less strongly to prevalent colonizing bacteria, hampering *S. epidermidis* biofilm eradication.

The presence of PNAG/PIA in *S. epidermidis* biofilm was detected by WGA binding (Cerca *et al.*, 2005). WGA is a carbohydrate-binding protein of approx. 36 kDa that selectively recognizes sialic acid and *N*-acetylglucosaminyl sugar residues which are predominantly found in biofilm matrix and namely in PNAG/PIA molecules. Farnesol at 300 μM promoted a modification in biofilm structure and a decrease in biofilm thickness. The decrease of the biofilm thickness could either be a result of biofilm bacteria death or biofilm bacteria dispersion. To clarify this point, biofilms were stained with L/D and as indicated by the results the observed biofilm reduction was in fact mainly due to cell death (Figure III.2.B). As we have previously shown *S. epidermidis* planktonic cells after 12 hours exposure to 300 μM farnesol have an average reduction of about 4 log. So, any cells released from 24 hours treated biofilms are most likely killed when assuming a planktonic lifestyle.

To test the potential antimicrobial action of farnesol against *S. epidermidis*, it is crucial to determine the cells abilitity to acquire tolerance/resistance. For that *S. epidermidis* cells were exposed to sub-inhibitory and inhibitory concentrations of farnesol.

S. epidermidis 1457 seems to be capable of rapid adaptation after an initial contact with farnesol but this effect was not a progressive mechanism. So, it can be concluded that this is a reversible mechanism and therefore a case of tolerance and not resistance. Moreover, resistance is translated by an increased survival of individuals which are immune to the effects of the antibacterial agent, whose offspring then inherit the resistance creating a new population of resistant bacteria. Our results demonstrated that over cell generations there is no decrease of the susceptibility to farnesol.

In conclusion, the results obtained by the quantification of extracellular polymers and by WGA fluorescent detection of PNAG/PIA support the hypothesis that farnesol causes disruption of the cytoplasmic membrane and consequently release of cellular content. In addition to cell death, farnesol seems to destroy the biofilm structure reducing its biomass. This general promotion of biofilm weakness may be a potential help to the human immune system to eradicate focus of *Staphylococcus epidermidis* infection.

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IV. FARNESOL AS ANTIBIOTICS ADJUVANT IN *Staphylococcus epidermidis* CONTROL *IN VITRO*

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| ABSTRACT

Farnesol is a sesquiterpenoid that has been described as impairing bacterial growth. Therefore, the goal of this study was to compare the *in vitro* Post-Antimicrobial Effect (PAE) of farnesol against *S. epidermidis* with the corresponding values of most common practice antibiotics and also to evaluate the combined effect of farnesol with these antibiotics against planktonic and biofilm cells. After exposure of *S. epidermidis* cells to farnesol and antibiotics at minimum inhibitory concentration (MIC) for 1 hour, the cells were regrown in medium without any antimicrobial agent. Cellular viability was assessed by colony forming units, every hour for 12 hours and then the PAE was determined. The combined effect of farnesol (0, 30, 100 and 300 μM) with vancomycin, tetracycline and rifampicin was also evaluated, using these antibiotics at peak serum concentration. As PAE is concerned it was found that cells grown in 100 μM of farnesol behaved similarly to cells that had never been in contact with farnesol, while a clear difference was obtained with cells exposed to 300 μM of farnesol, displaying a longer PAE. Farnesol showed a combined effect with the tested antibiotics against planktonic cells although this was not so evident against biofilm cells. Despite the reduced efficacy against biofilm cells, farnesol seems to be a potential adjuvant therapeutic agent to antibiotics against *S. epidermidis* planktonic cells. Moreover its long PAE makes farnesol a potential candidate in the prevention of biofilm formation since alone it also showed to be very effective against planktonic cells.

Keywords: Biofilm; planktonic cells; *Staphylococcus epidermidis*; farnesol; antibiotics.

| INTRODUCTION

Staphylococcus epidermidis has been regarded as an innocuous commensal bacterium of the human skin (Cerca *et al.*, 2004; Vuong *et al.*, 2003). In recent decades, however, it has emerged as a common cause of numerous nosocomial infections (Ziebuhr *et al.*, 2006). Medical implants, such as catheters, prosthetic heart valves and joint replacements, can be colonised by microorganisms that form an adherent biofilm on the surface of the device (Adam *et al.*, 2002). *S. epidermidis* does not produce many toxins and tissue-damaging exoenzymes. Generally, the success of this bacterium as a pathogen is attributed to its ability to adhere to surfaces and remain there, under the protection of an extracellular matrix. Biofilm formation is therefore one of the major virulence factors of these organisms, often leading to persistent infections (Cerca *et al.*, 2005a). A biofilm represents a surface-attached agglomeration of cells that are usually embedded in the heterogeneous matrix (Vuong *et al.*, 2004). These structures impair the action of phagocytic cells from the host immune system and of antimicrobial compounds, and release planktonic cells outer layers, allowing the persistence of bacterial infections (Cerca *et al.*, 2006; Oliveira *et al.*, 2007). When growing and surviving in biofilms, coagulase-negative staphylococci are more resistant to antibiotics agents when compared to planktonic cells, and often the antibiotic concentrations needed to eradicate the biofilm are above the peak serum concentration of the antibiotic, rendering it ineffective in treating biofilm infections (Cerca *et al.*, 2005a). Although it is not yet clear how biofilms resist to antimicrobial agents, several possible mechanisms have been proposed, namely: biofilms present a diffusional barrier to antibiotics; slow growth of cells within the biofilm; activation of the general stress response; emergence of a biofilm-specific phenotype and persister cells. These mechanisms normally only partially explain the increased resistance phenotype and, probably, this one is the result of more than one specific mechanism (Cerca *et al.*, 2005a).

Because of the increasing resistance to antibiotics, much effort is being exerted to identify novel compounds with antibacterial activity and to analyse their mechanism of action. Furthermore, there is a critical need for identifying therapeutic strategies that are directed towards the inhibition of biofilm formation and effective treatment of biofilms once they have been formed. Recently, farnesol was described as a molecule with antimicrobial properties (Jabra-Rizk *et al.*, 2006), and we have recently demonstrated its potential against planktonic cultures of *S. epidermidis* (Gomes *et al.*, 2009). Farnesol ($C_{15}H_{26}O$; molecular weight, 222.37) is a natural

sesquiterpenoid present in several fruits aroma and also secreted by *Candida albicans* as a quorum-sensing molecule. In this role, farnesol prevents the transition from yeast to hyphal growth in *Candida albicans* and greatly compromises biofilm formation by this fungus (Jabra-Rizk *et al.*, 2006).

The purpose of this study was to investigate the post-antimicrobial effect of farnesol on *S. epidermidis* planktonic cells and the antimicrobial action of farnesol alone and in combination with vancomycin, tetracycline and rifampicin in the eradication of *S. epidermidis* planktonic and biofilm cells.

| MATERIALS AND METHODS

| BACTERIAL STRAINS AND GROWTH CONDITIONS

In this study, previously well characterized biofilm-producing *S. epidermidis* strains were used: 1457, 9142, IE186, IE75, IE214 and LE7 (Cerca *et al.*, 2005b). These strains are clinical isolates and were stored at –80°C. TSB and TSA were prepared according to the manufacturer's instructions. All strains were inoculated into 15 mL of TSB from TSA plates not older than two days and grown for 18 (± 2) h at 37°C in an orbital shaker at 130 rpm. Cells were harvested by centrifugation (for 10 minutes at 9500 ×g and 4°C), resuspended in TSB and the suspension was adjusted to an optical density (640 nm) equivalent to 1×10^9 cells mL^{-1} before being used in the subsequent assays. Each stock solution of farnesol was prepared in methanol. It was confirmed that methanol, at the concentration used, had no effect on the growth of the *S. epidermidis* strains studied.

| PLANKTONIC CELLS

| POST ANTIMICROBIAL EFFECT OF FARNESOL, VANCOMYCIN, TETRACYCLINE AND RIFAMPICIN

S. epidermidis (1457 and 9142 strains) were grown planktonically in 30 mL of TSB medium with farnesol at 0, 100 and 300 μM (Sigma) and antibiotics at minimum inhibitory

concentration, for 1 hour at 37°C in a shaking incubator. Each assay was performed with an inoculum size of approximately 10^6 cells mL^{-1}.

After that, the cells were harvested by centrifugation and placed in fresh medium at 37°C and 130 rpm (time 0). Cellular viability was assessed by colony forming units, every hour until hour 12.

CFU were obtained as follows: a 1000 μL aliquot was removed from each assay Erlenmeyer. Serial ten-fold dilutions were made in saline solution and plated in TSA. Colonies were counted after 24 hours incubation at 37°C. The PAE was defined as the time difference (in hours) for the antimicrobial-treated organism to increase in number by 1 $\log_{10}$ minus the same determination for non-treated cultures of the same test organism (Aldridge, 2002).

All experiments were carried out in triplicate and repeated four times.

| COMBINED EFFECT OF FARNESOL AND ANTIBIOTICS

Viability assays were performed in 100 mL Erlenmeyer's containing a *S. epidermidis* cell suspension (2×10^8 cells mL^{-1}) (1457 and 9142 strains) in the presence of farnesol (0, 100, 200 and 300 μM) and each tested antibiotic at the corresponding peak serum concentration (vancomycin: 40 mg L^{-1}; tetracycline: 16 mg L^{-1} and rifampicin: 10 mg L^{-1}) (Sigma). The peak serum concentrations were obtained according to National Committee for Clinical Laboratory Standards (NCCLS) (Cerca *et al.*, 2005a), now Clinical and Laboratory Standards Institute (CLSI). The suspensions were incubated at 37°C and at 130 rpm. The assays made with tetracycline and rifampicin were protected from light because these antibiotics are light-sensitive. A 24 hour kinetic study was performed sampling after 2, 4, 6, 8 and 24 hours of *S. epidermidis* exposure to farnesol and/or antibiotics. Cellular viability was assessed by CFU, and obtained as follows: 1000 μL of each cellular suspension after being washed with 0.9% NaCl, were resuspended in 0.9% NaCl, followed by 20 s of sonication at 22 W to homogenize the suspension. This procedure disrupted the cell clumps without impairing cell viability (Cerca *et al.*, 2005a). Viable cells were determined by performing 10-fold serial dilutions in saline solution and plating in TSA. Colonies were counted after 24 hours incubation at 37°C.

Controls included cells not exposed to farnesol or antibiotics and also cells exposed either to farnesol or to each antibiotic alone. All experiments were carried out in triplicate and repeated four times.

| BIOFILM CELLS

| COMBINED EFFECT OF FARNESOL AND ANTIBIOTICS

Biofilms were formed in 96 well tissue culture plates containing 200 μL of *S. epidermidis* cell suspension (1×10^6 cells mL^{-1}) (1457, 9142, IE186, IE75, IE214 and LE7 strains) in TSB supplemented with 0.25% glucose per well to promote biofilm formation. Plates were incubated at 37°C with orbital shaking at 130 rpm for 24 hours. At the end, planktonic cells were removed carefully, and the biofilm was washed twice with 200 μL of 0.9% NaCl. The biofilms were incubated in fresh nutrient medium (TSB) containing farnesol (0, 100, 200 and 300 μM) (Sigma) and/or antibiotics at the peak serum concentration. Crystal violet and CFU assays were performed after 2, 4, 6, 8 and 24 hours of exposure to farnesol and the antibiotics tested. At time 0 (before exposure to farnesol and antibiotics) the initial cellular concentration of biofilm (≈ 2×10^8 cells mL^{-1}) was determined.

CV was used as indicator of total biofilm biomass. For that, biofilms were washed with 250 μL of 0.9% NaCl, then 250 μL of methanol were added and left to act during 15 minutes. Afterwards, methanol was removed and 250 μL of crystal violet 1% (v/v) was added (5 minutes). The wells were washed with distilled water and finally, acetic acid 33% was added. The absorbance was measured at 570 nm.

CFU were obtained as follows: the planktonic cells were removed carefully and the biofilm was washed twice with 200 μL of 0.9% NaCl. The wells were thoroughly scraped and resuspended in 1 mL of 0.9% NaCl, followed by centrifugation for 10 minutes at 9500 ×g. The pellet was resuspended in 0.9% NaCl and washed twice, followed by 20 seconds of sonication at 22 W to homogenize the suspension. Viable cells were determined by performing 10-fold serial dilutions in saline solution and plating in TSA. Colonies were counted after 24 hours incubation at 37°C.

Controls were cells not exposed to farnesol or antibiotics, and cells exposed either to farnesol or to each antibiotic alone. All experiments were carried out in triplicate and repeated four times.

| STATISTICAL ANALYSIS

The data from all assays were compared using one-way analysis of variance by applying Tukey's and Bonferroni tests with all calculations carried out using SPSS software. Differences at a confidence level of 95% were considered significant.

| RESULTS

In this work, the PAE of farnesol on *S. epidermidis* planktonic cells was studied. In previous work we have demonstrated that for 100 μM and higher concentrations (200 and 300 μM) and to an exposure time of 6 and 12 hours, the reduction in cellular viability of different strains of *Staphylococcus epidermidis* remained almost the same, independently of farnesol concentration and exposure time (Gomes *et al.*, 2009). Accordingly, it was investigated if the cells subjected to different farnesol concentrations (100 and 300 μM) resume the same ability to grow as cells grown in medium without farnesol. The same was done to all antibiotics tested with the aim to compare the PAE of farnesol and antibiotics. The PAE values are given in Table IV.I. The PAE of farnesol at 100 μM was the lowest (1 hour) among all the antimicrobial agents tested. All the antibiotics exhibited a PAE of 2 hours, except rifampicin for the strain 9142, whose PAE was 3 hours. The PAE of farnesol at 300 μM (> 8 h) was higher than the PAE of all the antibiotics studied.

Table IV.I. Comparison of the PAE of the various antimicrobial agents tested against *S. epidermidis*.

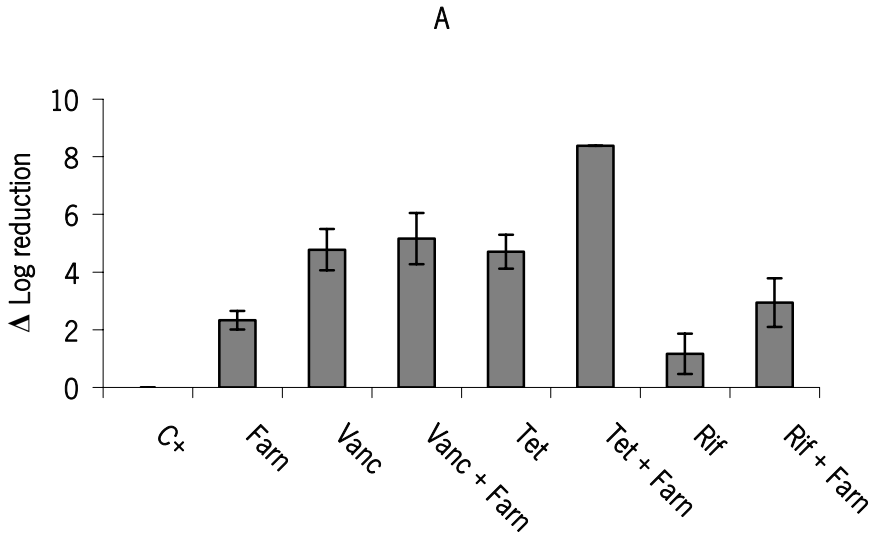
Antimicrobial agent (Concentration)	**Post Antimicrobial Effect (time)***	
	***S. epidermidis* 1457**	***S. epidermidis* 9142**
Farnesol (100 μM)	1 h	1 h
Farnesol (300 μM)	> 8 h	> 8 h
Vancomycin (1× MIC)	2 h	2 h
Tetracycline (1× MIC)	2 h	2 h
Rifampicin (1× MIC)	2 h	3 h

* Expressed in hours (h).

Another aim of the work was to evaluate the possible combined effect of farnesol and antibiotics on planktonic and biofilm cells of *S. epidermidis* strains. For that three antibiotics with

different known mechanisms of action were tested: vancomycin (cell wall synthesis inhibitor), tetracycline (protein synthesis inhibitor) and rifampicin (RNA synthesis inhibitor). A 24 hours kinetic study was performed using these antibiotics at the peak serum concentration along with farnesol at concentrations of 0, 100, 200 and 300 μM.

In planktonic cells, the inhibitory effect of the antibiotics tested was expressed in terms of reduction in log cell number (Δ log) to establish a comparison among their effectiveness alone or in combination with farnesol. Although their effect was strain dependent, it was possible to observe a general increasing tendency of inhibitory effect in the following order: rifampicin; farnesol 300 μM; tetracycline and vancomycin. Moreover, the results also showed a synergistic effect of farnesol with tetracycline and rifampicin (Figure IV.1.). For example, for strain 1457 treatment with tetracycline alone was able to reduce bacterial population from 8 log to 3.5, a nearly 4.5 log reduction. Farnesol alone was only able to reduce bacterial numbers by 2 log, at a concentration of 300 μM. However, the combined effect of tetracycline and farnesol was able to reduce bacterial population by 8 log units, demonstrating synergy between the two antimicrobial agents. In fact, farnesol in combination with tetracycline and irrespective of its concentration (100, 200 and 300 μM) promoted reductions above 5.6 log. Notably, for strain 1457 almost 100% log reduction (8 log) was attained. The combined effect with vancomycin cannot be considered synergistic or additive; nevertheless there was an increase in the antibiotic inhibitory effect ($p < 0.05$).



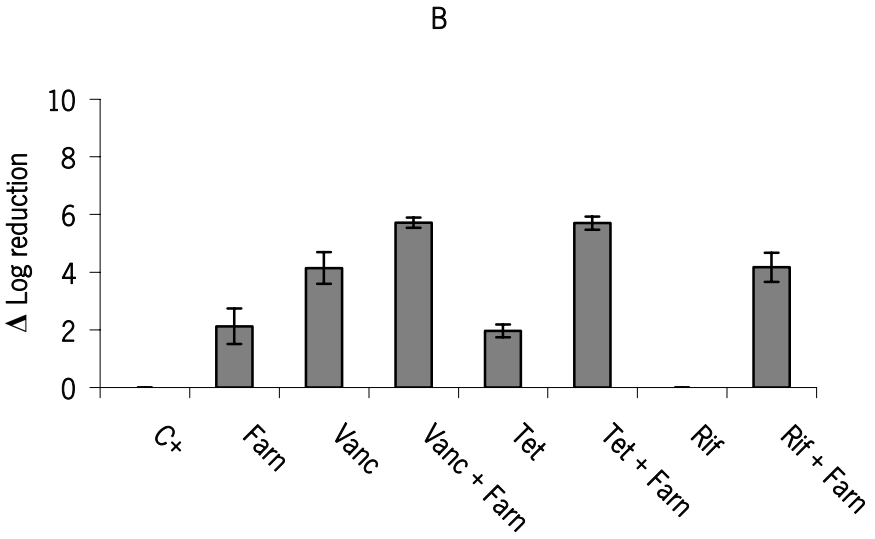


Figure IV.1. Effect of farnesol (Farn) (300 μM) and antibiotics [Vancomycin (Vanc), tetracycline (Tet), rifampicin (Rif)] alone and in combination on planktonic cells of *S. epidermidis* 1457 (A) and 9142 (B), after 24 hours of treatment. Δ Log reduction - differences between positive control (C+) (untreated control) and treated samples in $\log_{10}$ CFU/mL. Error bars represent standard deviation.

In contrast to planktonic cells, biofilm cells were much less susceptible to farnesol, vancomycin and tetracycline, showing higher susceptibility to rifampicin (Figure IV.2.), which is in accordance with previous results, as each antibiotic's effect alone is concerned (Cerca *et al.*, 2005a).

Furthermore, in biofilms, and for all strains tested, no synergistic effect of farnesol with the antibiotics assayed was detected. However, in some cases there was an additive effect of farnesol and antibiotics, such as for strain 9142 where farnesol at 300 μM seems to have an additive effect with vancomycin (Figure IV.2.) and for strain IE186 where farnesol at 100 μM seems to slightly potentiate the effect of tetracycline. Also for strain IE214 farnesol seemed to increase the inhibitory effect of vancomycin and tetracycline, expressed by the reduction of biomass and cellular activity of biofilm cells ($p < 0.05$) (data not shown).

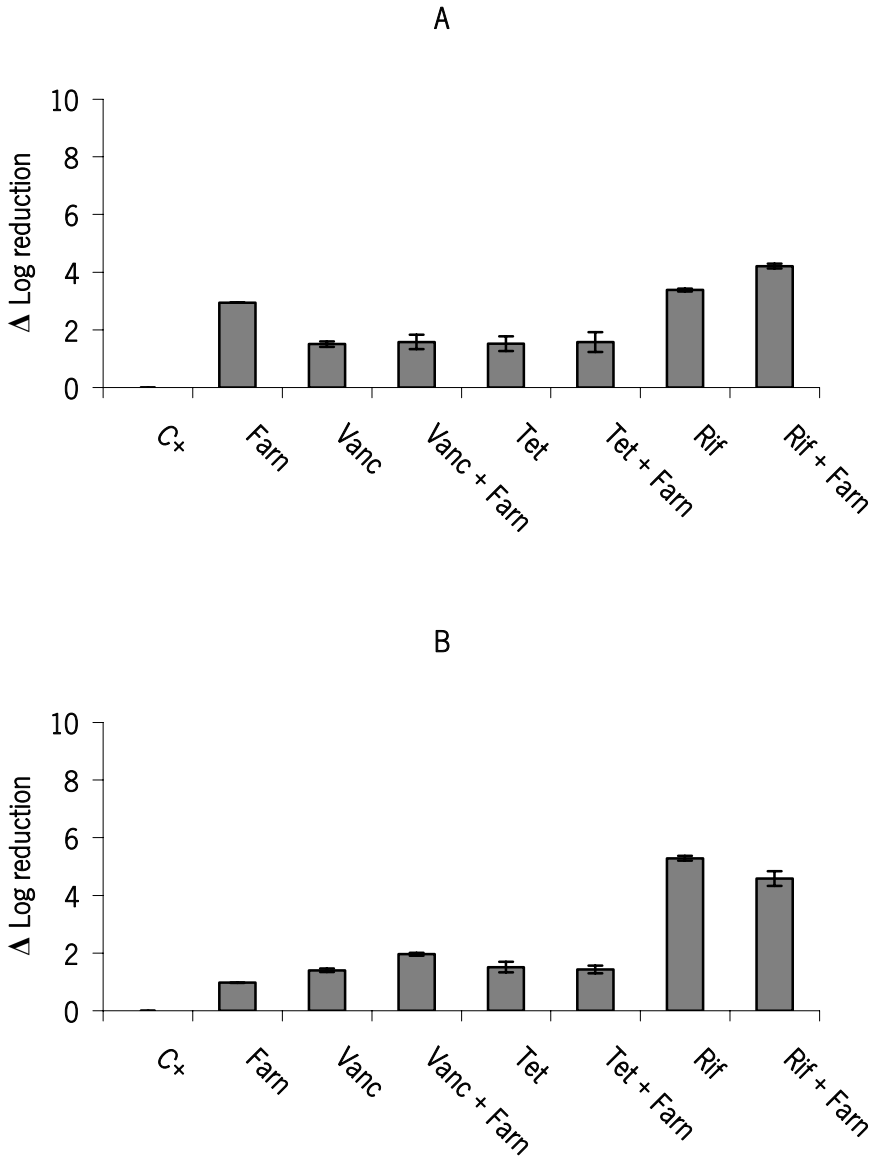


Figure IV.2. Effect of farnesol (Farn) (300 μM) and antibiotics [vancomycin (Vanc), tetracycline (Tet), rifampicin (Rif)] alone and in combination on biofilm cells of *S. epidermidis* 1457 (A) and 9142 (B), after 24 hours of treatment. Δ Log reduction - differences between positive control (C+) (untreated control) and treated samples in $\log_{10}$CFU/mL. Error bars represent standard deviation.

| DISCUSSION

The rapid evolution of antibiotic resistance in pathogenic bacteria, probably due to overuse and misuse of antibiotics, is today a major public health problem. Therefore, there is a

critical need for the development of novel antimicrobial compounds to treat the growing number of infections where antibiotic resistance is a serious threat.

Bacteria that survive exposure to an antimicrobial agent do not resume growth immediately after the drug is removed (Suller and Lloyd, 1998). Rather, there is a period of recovery from the toxic effects (PAE), the duration of which depends on the bacterial strain, the type and concentration of the antibiotic and the exposure time (Nagl *et al.*, 1999; Suller and Lloyd, 1998). PAE is then defined as the delayed re-growth of the bacteria after complete removal of an antimicrobial agent. Lag of re-growth of bacteria after sub-lethal treatment has been considered to be a valuable parameter for the evaluation of antimicrobial agents (Suller and Lloyd, 1998). Compounds inducing an extended lag will inhibit bacterial replication for longer than the contact time, and hence, application intervals may be extended (Nagl *et al.*, 1999). There is an increasing interest in the PAE as an important parameter for the dosage (Suller and Lloyd, 1998) and frequency of administration of a drug. The clinical implication of long PAE lies in the possibility of increasing the intervals between drug administrations, thus allowing for fewer daily doses and thereby potentially reducing treatment costs, increasing patient compliance and decreasing drug exposure. Two proposed mechanisms of PAE are: limited persistence of drug at a bacterial binding site and drug-induced nonlethal damage (Suller and Lloyd, 1998).

In this work, the PAE of farnesol on *S. epidermidis* planktonic cells was evaluated. The results showed that the PAE of farnesol was concentration dependent. Cells grown in 100 μM of farnesol behaved similarly to cells that had never been in contact with farnesol (PAE = 1 h), while cells exposed to 300 μM of farnesol lost the ability to grow and had a very long PAE (Table IV.I.). A short time in contact with farnesol (1 hour in farnesol at 300 μM) was enough to reduce the ability of multiplication by the cells. The end of PAE was marked by an increase in cell number and cell activity as the cells recover their integrity and activity and resume multiplication (Suller and Lloyd, 1998). Our results appear to reflect that the cells have been exposed to nonlethal damage, from which they may have the potential to recover and resume multiplication.

Farnesol at 300 μM shows not only significant microbicidal activity (Gomes *et al.*, 2009), but also a pronounced PAE when compared with the antibiotics tested (vancomycin, tetracycline and rifampicin) (Table IV.I.). Although 100 and 300 μM of farnesol have the same antibacterial effect on *S. epidermidis* planktonic cells (Gomes *et al.*, 2009), 300 μM seems to be more effective than 100 μM due to an associated longer PAE. It should be noted that farnesol is lipophilic and it has been reported that drugs with lipophilic nature are able to accumulate intracellularly (Jabra-

Rizk *et al.*, 2006), which may enhance the post-antibiotic effect and therefore their killing efficacy. Moreover, at this concentration farnesol is expected to be devoid of toxic effects (Jabra-Rizk *et al.*, 2006; Navarathna *et al.*, 2007). It was shown that its LD_{50} for mice was 2.95 g/kg of body weight, which corresponds to 75 mg for a 25 g mouse (Navarathna *et al.*, 2007). For comparison, 1 mL of 300 μM farnesol contains only 66 x 10^{-3} mg of farnesol.

Since the principal interaction of farnesol appears to be with the cytoplasmic membrane, it is likely that farnesol can non-specifically enhance the permeability of bacterial cells to certain exogenous chemical compounds, including antimicrobials (Jabra-Rizk *et al.*, 2006). In order to test this hypothesis, we studied the ability of farnesol to act in combination with some antibiotics by disrupting the membrane of biofilm-embedded bacterial cells, thereby facilitating antibiotic entry and aiding in the clearance of staphylococcal biofilms.

In planktonic cells, there was an increased effect of vancomycin and tetracycline when combined with farnesol which was probably due to the lipophilic nature of farnesol that favors their penetration through the cell wall envelope. Although this synergistic effect was not observed in biofilm cells they were indeed more susceptible to the most lipophilic antibiotic tested (rifampicin). Comparing the results obtained for planktonic and biofilms cells it was observed that planktonic cells are more susceptible to vancomycin than biofilm cells. The change in vancomycin efficacy from the highest inhibitory effect in planktonic cells (Figure IV.1.) to the lowest in biofilms (Figure IV.2.) can be due in part to diffusional limitations imposed by the biofilm matrix. In fact, glycopeptides (vancomycin) are high-molecular-weight antibiotics (ca. 1500 Da) that bind to the terminal D-alanine–D-alanine component of the stem peptide while the subunits are external to the cell membrane but still linked to the lipid carrier and this sterically binding is probably less prone to occur when an extracellular matrix is fully developed. Moreover, vancomycin is known to be higly protein bound and this is another possible mechanism to reduce vancomycin availability in a matrix with some protein content. On the contrary, in planktonic cells the external binding favors its efficacy, because the other antibiotics do not have such facilitated transport inwards. It should be noted that despite the above reasoning on some of the possible mechanisms acting against the susceptibility of *S. epidermidis* biofilm cells to vancomycin, previous results strongly suggested that the phenotypic resistance of cells in biofilms to antibiotics is affected primarily by the mechanism of action of the antibiotic, with a much significant decrease in susceptibility when the antibiotic targets cell wall synthesis (Cerca *et al.*, 2005a).

Therefore, farnesol seems to be a potential adjuvant therapeutic agent to antibiotics for the treatment of *S. epidermidis* systemic related infections. Moreover, the antimicrobial activity and long PAE against planktonic *S. epidermidis* cells make farnesol a promising candidate to be also used as an alternative to antibiotics for the prevention of biofilm formation.

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V. EFFECT OF FARNESOL IN COMBINATION WITH *N*-ACETYLCYSTEINE AGAINST *Staphylococcus epidermidis* PLANKTONIC AND BIOFILM CELLS

Submitted for publication

| ABSTRACT

Staphylococcus epidermidis is the most frequent cause of nosocomial sepsis and catheter-related infections, in which biofilm formation is considered to be the main virulence mechanism. In biofilm environment, microbes exhibit enhanced resistance to antimicrobial agents. This fact boosted the search of possible alternatives to antibiotics. Farnesol and *N*-acetylcysteine (NAC) are non-antibiotic drugs that have demonstrated antibacterial properties. In this study, the effect of farnesol and NAC isolated or in combination (farnesol-NAC) was evaluated. The results demonstrated that there was a higher CFU log reduction of *S. epidermidis* planktonic cells when farnesol was combined with NAC at 1 × MIC relatively to each agent alone. However, these results were not relevant because NAC alone at 10 × MIC was always the condition which gave the best results, having a very high killing effect on planktonic cells and a significant bactericidal effect on biofilm cells.

Keywords: Nosocomial infection; biofilm; Staphylococci; farnesol; *N*-acetylcysteine.

| INTRODUCTION

Staphylococcus epidermidis is a commensal of the human skin flora (Hellmark *et al.*, 2009) and as a natural human inhabitant bacterium it as a low pathogenic potential (Ziebuhr *et al.*, 2006). However, in recent decades, this bacterium has emerged as a common cause of numerous infections on indwelling medical devices (Ziebuhr *et al.*, 2006) and actually *S. epidermidis* ranks first among the causative agents of nosocomial infections (Otto, 2009). These bacteria form biofilms on implanted medical devices such as central venous catheters (CVCs), urinary catheters, prosthetic heart valves, orthopedic devices, contacts lenses, etc, and cause persistent infections (Wang *et al.*, 2007) and diseases such as septicemia and endocarditis (Cargill and Upton, 2009). The ability of *Staphylococcus epidermidis* to adhere to and form multilayered biofilms on host tissue and other surfaces is one of the important mechanisms by which they are able to persist in these infections/diseases (Jabra-Rizk *et al.*, 2006). Infection of medical implanted material sometimes requires the removal of the implants, causing considerable suffering for the patient, with pain and disability and even increased mortality (Hajdu *et al.*, 2009; Hellmark *et al.*, 2009). Moreover, the costs are significantly increased due to prolonged hospitalization, revision surgery and long-term antimicrobial treatment.

The major virulence factor associated with *S. epidermidis* infections is the ability of the organisms to adhere to medical devices and subsequently form biofilms (Cerca *et al.*, 2005b; Vuong *et al.*, 2004). This characteristic is a major clinical problem, mainly due to high level of resistance to antibiotics (Cerca *et al.*, 2005b). Antibiotic combination represents a therapeutic option in the treatment of *S. epidermidis* infections (Monzón *et al.*, 2001). However, increasing multiple resistance to antibiotics has made the development of new treatment options for serious infections a matter of urgent concern. In recent years, much research has been devoted to investigating possible alternatives to antibiotics, studying their mode of action and synergistic effects with other antimicrobial compounds. Farnesol is a sesquiterpene alcohol that has demonstrated to inhibit the growth of some microorganisms, signaling its potential use as antimicrobial agent (Derengowski *et al.*, 2009; Jabra-Rizk *et al.*, 2006). The mechanism of action of this sesquiterpenoid probably involves cell membrane damages (Derengowski *et al.*, 2009; Jabra-Rizk *et al.*, 2006; Kuroda *et al.*, 2007).

N-acetylcysteine is another non-antibiotic drug that has antibacterial properties (Pérez-Giraldo *et al.*, 1997). NAC is one of the smallest drug molecules in use and it is generally used in the

medical treatment of chronic bronchitis, cancer and paracetamol intoxication (Olofsson *et al.*, 2003). The prevention of biofilm formation and adherence to biomaterials devices is another possible role of NAC (Pérez-Giraldo *et al.*, 1997).

Considering the results previously obtained with these compounds, the purpose of this work was to investigate the possible synergistic effect of farnesol with *N*-acetylcysteine against *S. epidermidis* planktonic and biofilm cells.

| MATERIALS AND METHODS

|BACTERIAL STRAINS AND CULTURE CONDITIONS

Two clinical isolates of *S. epidermidis,* known for their ability to form biofilms, were used in this work: strain 1457 (isolated from an infected central venous catheter) and strain 9142, a known producer of the polysaccharide intracellular adhesin. All strains were gently provided by Dr. G. B. Pier, Channing Laboratory, Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston. Both strains were grown for 18 ± 2 hours, at 37°C and 120 rpm in 30 mL of TSB. Then the cells were centrifuged (9500 ×g, 5 minutes, 4°C), washed twice with a saline solution (0.9% NaCl in distilled water) and sonicated (22% amplitude, 10 seconds). The cellular suspensions were adjusted to a final concentration of approximately 1×10^9 cells mL^{-1}, determined by optical density at 640 nm, prior to be used in biofilm assays.

| PLANKTONIC ASSAYS

Viability assays were performed in 100 mL Erlenmeyers containing a *S. epidermidis* cell suspension (2×10^8 cells mL^{-1}) in the presence of farnesol (300 μM), NAC (NAC 1 × MIC = 4 mg mL^{-1} and 10 × MIC = 40 mg mL^{-1}) and farnesol-NAC. It should be noted that 300 μM farnesol was previously shown to be highly effective against planktonic cells of *S. epidermidis* (Gomes *et al.*, 2009). The suspensions were incubated for 24 hours, at 37°C and at 130 rpm. Afterwards, cellular viability was assessed by colony forming units, while cell activity was determined by the XTT ({2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide}) reduction assay (Kuhn *et al.*, 2003). CFU were obtained as follows: 1000 μL of each cellular suspension after being washed with 0.9% NaCl, were resuspended in 0.9% NaCl, followed

by 20 seconds of sonication at 22 W to homogenize the suspension. This procedure disrupted the cell clumps without impairing cell viability (Cerca *et al.*, 2005a). Viable cells were determined by performing 10-fold serial dilutions in saline solution and plating in TSA. Colonies were counted after 24 hours incubation at 37°C.

For XTT assay, aliquots of 200 μL of each cell suspension were collected. The cells were washed with 0.9% NaCl by centrifugation for 10 minutes at 9500 ×g and 4°C. The pellet was resuspended in 200 μL of 0.9% NaCl and dispensed in a well of a microtiter plate. Then, 50 μL of a solution containing 200 mg L^{-1} of XTT and 20 mg L^{-1} of PMS (Sigma) were added. The microtiter plates were incubated for 3 hours at 37°C in the dark. The absorbance was measured at 490 nm.

Controls included cells not exposed to farnesol or NAC (positive control) and also cells exposed either to farnesol or to NAC alone. All experiments were carried out in triplicate and repeated three times.

| BIOFILM ASSAYS

Biofilms were formed in 96 well tissue culture plates containing 200 μL of *S. epidermidis* cell suspension (1×10^6 cells mL^{-1}) (1457 and 9142 strains) in TSB supplemented with 0.25% glucose per well to promote biofilm formation. Plates were incubated for 24 hours at 37°C on an orbital shaker (130 rpm). At the end, planktonic cells were removed carefully, and the biofilm was washed twice with 200 μL of 0.9% NaCl. The biofilms were incubated in fresh nutrient medium containing farnesol (300 μM), NAC (1 × MIC and 10 × MIC) and combination of both. XTT, CFU and CV assays were performed after 24 hours of exposure to antimicrobial agents (alone and in combination) tested. At time 0 (before exposure to antimicrobial agents) the initial cellular concentration of biofilm (≈ 2×10^8 cells mL^{-1}) was determined.

The quantification of biofilm cellular activity was assessed through the XTT reduction assay. After exposure to farnesol and NAC, biofilms were washed with 0.9% NaCl. Then, 250 μL of a solution containing 200 mg L^{-1} of XTT and 20 mg L^{-1} of PMS were added to each well. The microtiter plates were incubated for 3 hours at 37°C in the dark. The absorbance was measured at 490 nm.

CFU were obtained as follows: the planktonic cells were removed carefully and the biofilm was washed twice with 200 mL of 0.9% NaCl. The wells were thoroughly scraped and

resuspended in 1 mL of 0.9% NaCl, followed by centrifugation for 10 minutes at 9500 ×g. The pellet was resuspended in 0.9% NaCl and washed twice, followed by 20 seconds of sonication at 22 W to homogenize the suspension. Viable cells were determined by performing 10-fold serial dilutions in saline solution and plating in TSA. Colonies were counted after 24 hours incubation at 37°C.

CV was used as indicator of total biofilm biomass. For the measurement of this parameter, biofilms were washed with 250 μL of 0.9% NaCl, then 250 μL of methanol were added and left to act during 15 minutes. Afterwards, methanol was removed and 250 μL of crystal violet 1% (v/v) were added (5 minutes). The wells were washed with distilled water and finally, acetic acid 33% (v/v) was added. The absorbance was measured at 570 nm.

Controls were cells not exposed to farnesol or NAC (positive control), and cells exposed either to farnesol or NAC alone. All experiments were carried out in triplicate and repeated three times.

| SCANNING ELECTRON MICROSCOPY (SEM)

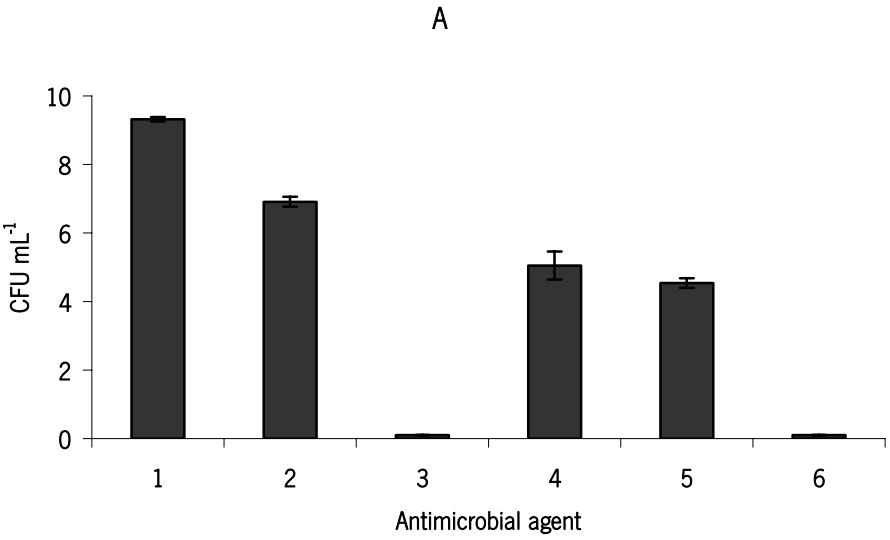
Biofilms were dehydrated by immersion in increasing ethanol concentration solutions: 70 (10 minutes), 95 (10 minutes) and 100% (20 minutes) (v/v), having then been placed in a sealed desiccator. Samples were mounted on aluminium strubs with carbon tape, sputter coated with gold and observed with a Field Emission Gun - Scanning Electron Microscope (FEG/ESEM) - Nova Nano SEM 200 from FEI Company.

Three fields were used for image analysis. All photographs were taken at a magnification of × 40 000.

| STATISTICAL ANALYSIS

The results from all assays were compared by the one-way analysis of variance by applying the Bonferroni and Tukey multiple comparison tests, using the SPSS software. All tests were performed with 95% confidence level.

Figure V.1. presents the effect of farnesol, NAC and the association farnesol-NAC on *Staphylococcus epidermidis* planktonic cells. NAC at 1 × MIC concentration is less effective than farnesol at 300 μM ($p < 0.05$) (Figure V.1.). The combination of farnesol at 300 μM with NAC at 1 × MIC caused a higher cfu log reduction when compared to each one alone ($p < 0.05$). This combination resulted into an additional log reduction of 0.5 and 1 for strains 1457 and 9142, respectively ($p < 0.05$) and relatively to the most effective of both antimicrobial agents tested, ie farnesol at 300 μM. However, NAC at 10 × MIC was more effective than farnesol alone and farnesol and NAC 1 × MIC. After 24 hours, NAC 10 × MIC caused an 8 log reduction resulting in total cell death (Figure V.1.).



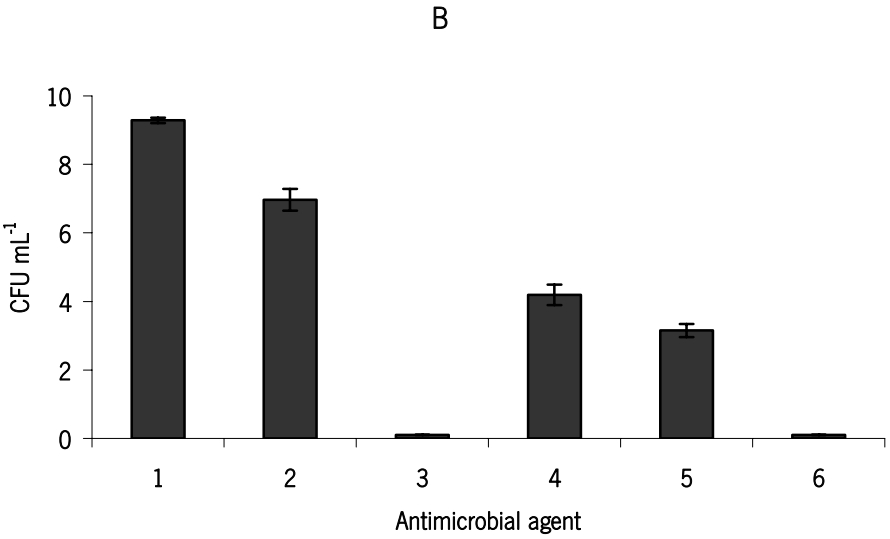


Figure V.1. Effect of farnesol and/or NAC on planktonic cells of *S. epidermidis* 1457 (A) and 9142 (B), after 24 hours of contact with farnesol (300 μM), NAC (4 mg mL^{-1} and 40 mg mL^{-1}) and farnesol-NAC. Error bars represent standard deviation. Legend: 1- Positive control; 2- NAC 1 × MIC; 3- NAC 10 × MIC; 4- Farnesol 300 μM; 5- Farnesol 300 μM + NAC 1 × MIC; 6- Farnesol 300 μM + NAC 10 × MIC.

Relatively to biofilm cells, although NAC 10 × MIC did not cause total cell death it was the most efficient against *S. epidermidis* biofilm cells causing a reduction of approximately 4 log (Figure V.2.). Conversely to planktonic cells, farnesol and NAC 1 × MIC had a similar effect in biofilms. For strain 1457, NAC 1 × MIC and farnesol worked better together than alone ($p < 0.05$) (Figure V.2.A). There was no synergistic or additional effect when NAC 10 × MIC was combined with farnesol at 300 μM ($p < 0.05$).

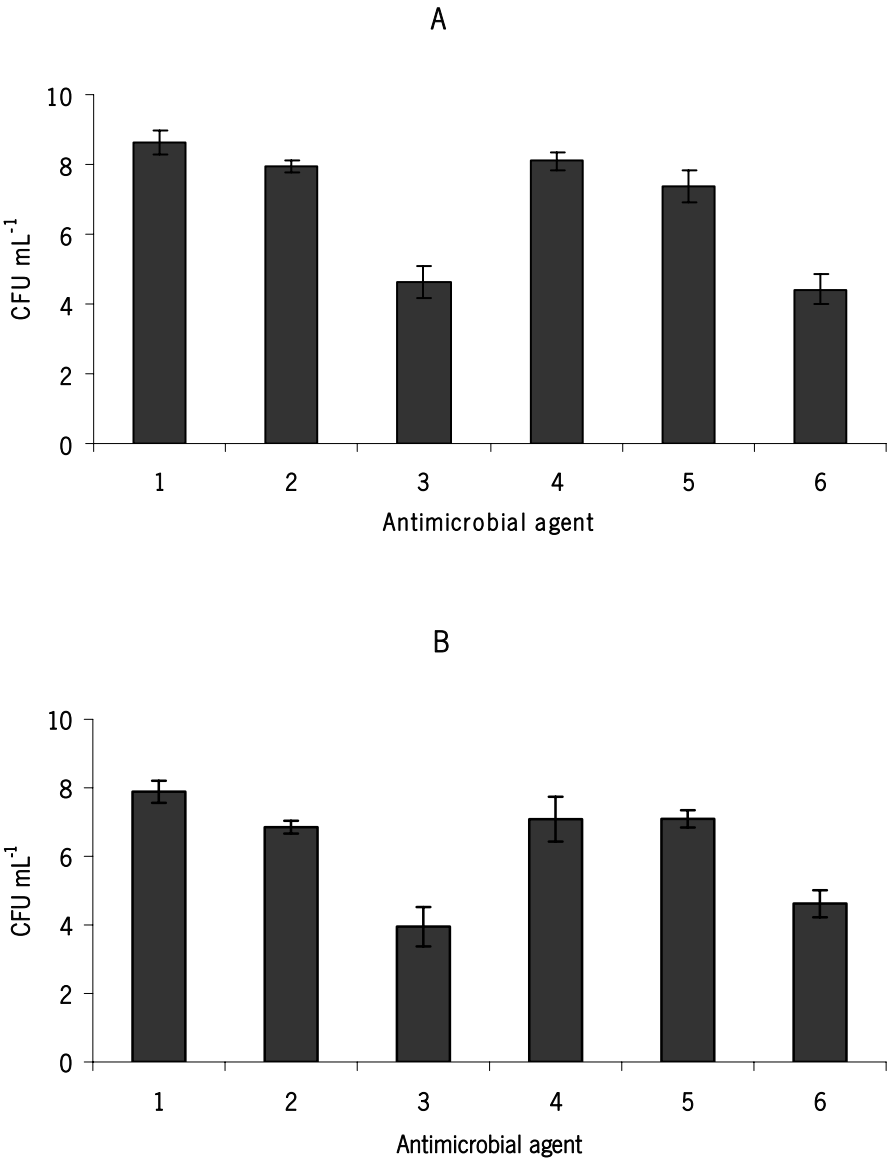


Figure V.2. Effect of farnesol and/or NAC on biofilm cells of *S. epidermidis* 1457 (A) and 9142 (B), after 24 hours of contact with farnesol (300 μM), NAC (4 mg mL^{-1} and 40 mg mL^{-1}) and farnesol-NAC. Error bars represent standard deviation. Legend: 1- Positive control; 2- NAC 1 × MIC; 3- NAC 10 × MIC; 4- Farnesol 300 μM; 5- Farnesol 300 μM + NAC 1 × MIC; 6- Farnesol 300 μM + NAC 10 × MIC.

Representative scanning electron microscopy images of 1457 *S. epidermidis* biofilms after being exposed to farnesol, NAC and farnesol-NAC are presented on figure V.3. These images specifically show the effect on the biofilm matrix and biofilm cell viability, and are in agreement with the results presented above. All biofilms treated with NAC revealed a desintegration of the

matrix which is more noticeable for NAC at 40 mg mL^{-1} (10 × MIC). Farnesol seems to have also an effect on biofilm matrix but not as pronounced as NAC.

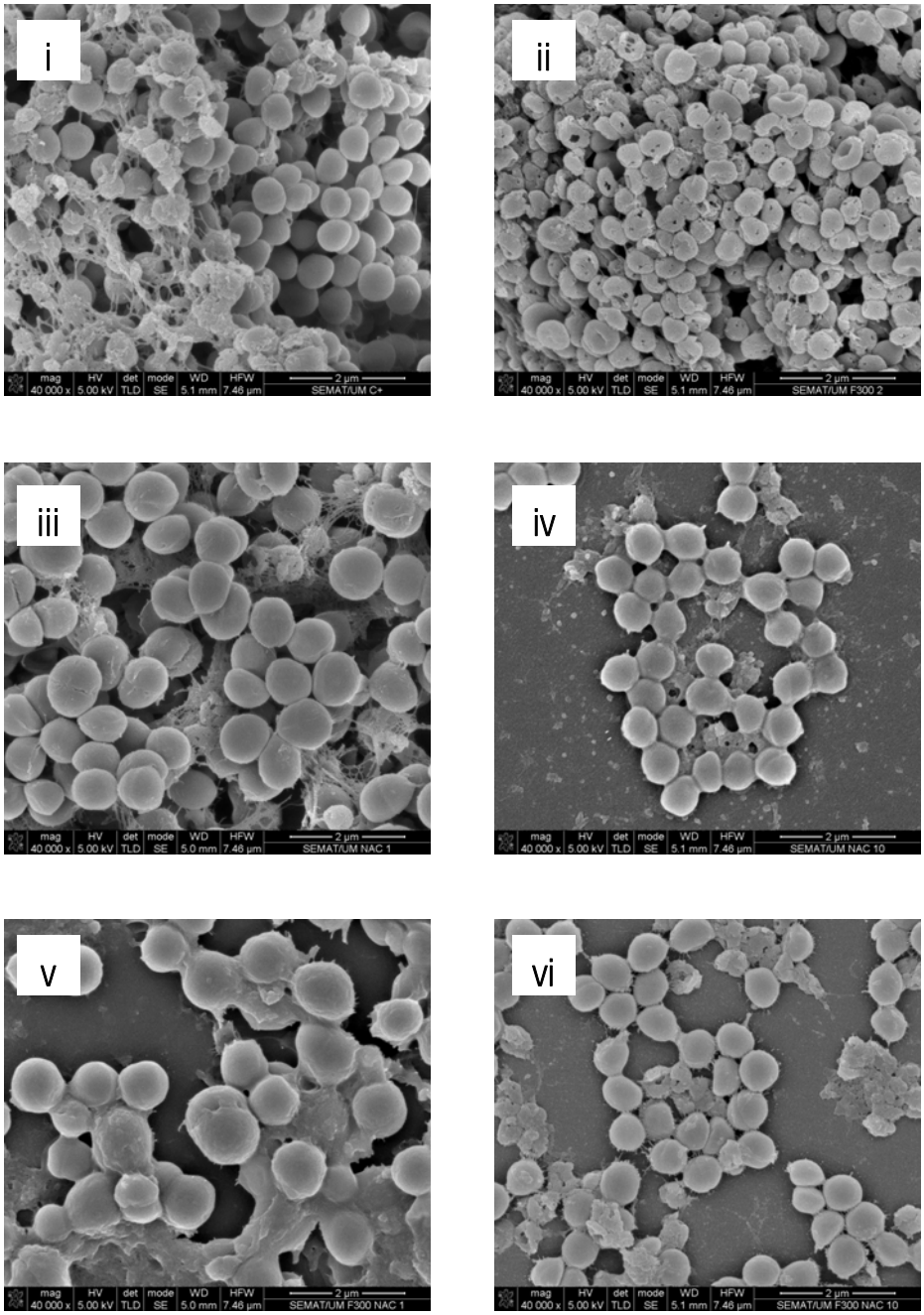


Figure V.3. Scanning electron micrographs of 24 hours-biofilm of *S. epidermidis* 1457 after exposure to farnesol, NAC, and the combination of both for 24 hours. (i) Positive control; (ii) 300 µM farnesol; (iii) NAC 1 × MIC; (iv) NAC 10 × MIC; (v) Farnesol 300 µM + NAC 1 × MIC; (vi) Farnesol 300 µM + NAC 10 × MIC. Magnification × 40 000.

| DISCUSSION

In this work, the effect of farnesol, NAC and farnesol-NAC combination against *S. epidermidis* planktonic and biofilm cells was studied. For that, two good biofilm-forming strains were selected, strains 1457 and 9142 (Sousa *et al.*, 2009). Comparing these two strains, 1457 produces slightly more biofilm than 9142 (Sousa *et al.*, 2009). The biofilm formation ability is due to the formation of PNAG/PIA homopolymer, which surrounds and connects *S. epidermidis* cells in biofilm form (Otto, 2009). The extracellular matrix is extremely important for intercellular connection during surface colonization (Hussain *et al.*, 1991) and protection against the host immune system and resistance to antibiotics (An and Friedman, 1997). Figure V.3.A represents a 48 hours biofilm of *S. epidermidis* 1457 and shows the thickness of biofilm and the presence of a noticeable amount of biofilm matrix.

N-acetylcysteine, a potent antioxidant that reduces disulphide bonds linking mucin oligomers, has been widely used as a mucolytic agent for inhalation therapy in patients with chronic bronchitis. NAC has been shown not only to reduce adhesion but also to detach bacterial cells adhered to surfaces and to inhibit bacterial growth *in vitro* (Olofsson *et al.*, 2003). NAC decreases biofilm formation by a variety of bacteria and reduces the production of extracellular polysaccharide matrix, while promoting the disruption of mature biofilm (Aslam *et al.*, 2007).

On the other hand, the principal interaction of farnesol appears to be with the cytoplasmatic membrane (Jabra-Rizk *et al.*, 2006). Farnesol is a sesquiterpenoid that already demonstrated synergistic effect with another antimicrobial agent (gentamicin) indicating a potential application as an adjuvant therapeutic agent (Jabra-Rizk *et al.*, 2006). According to previous studies, where farnesol was tested at concentrations ranging from 30 to 300 μM, the last concentration demonstrated to have an antimicrobial effect against *S. epidermidis* as well as against other bacteria (Gomes *et al.*, 2009; Jabra-Rizk *et al.*, 2006).

We hypothesized that the combination of NAC with farnesol could be synergistic in the treatment of *S. epidermidis* infections as they both act on different components of the biofilm. Our results revealed that additionally to be bactericidal NAC seems also to act against the matrix. In fact, NAC seems to destroy the biofilm matrix resulting in the detachment of cells and thus the biofilm cells become more exposed and susceptible. This high effect against biofilm cells of *S. epidermidis* must be due in part to the small molecular size of NAC (Molecular Weight = 163.19), which easily penetrates into the biofilm. NAC at 1 × MIC in combination with 300 μM farnesol

resulted in a higher antimicrobial effect against planktonic cells of *S. epidermidis* 1457 and 9142 than both antimicrobial agents alone. Nevertheless NAC alone at 10 x MIC, similarly to biofilms, showed a very high bactericidal effect. Although its very high effect on plancktonic cells promoting CFU reductions above 8 log, it is probably more impressive its bactericidal effect on biofilms, which are always very tolerant to the most common antibiotics (Gomes *et al.*, 2009). However, unlike it was expected it did not work in synergy with farnesol at 300 μM against biofilm cells.

Comparatively to planktonic cells, biofilm cells were much more tolerant to the inhibitory effect of farnesol, NAC and farnesol-NAC. As mentioned above, this fact must be due to the protective effect of the matrix. The effect of NAC was concentration dependent. While with NAC at 1 × MIC an average reduction of 2.5 log was observed, NAC 10 × MIC was enough to kill all planktonic cells. However, for biofilm cells this concentration (10 × MIC) only promoted an approximately 4 log reduction in the number of viable cells within the biofilm, while only 1 log was attained with 1 × MIC.

The peak serum concentration of NAC after a 600 mg oral dose was estimated to be 0.465 mg mL^{-1} (Rehman *et al.*, 2008). The concentration of NAC tested in our study (1 × MIC and 10 × MIC, 4 and 40 mg mL^{-1}, respectively) are rather higher than those reached in serum when applied by the intravenous or oral route. Nevertheless, it may be possible by local application to obtain useful concentrations to avoid the formation of biofilms and consequently the adherence of CoNS (Pérez-Giraldo *et al.*, 1997).

In another study, a concentration of 80 mg mL^{-1} of NAC was tested *in vitro* based on preliminary data that showed a dose-response relashionship on planktonic bacteria (Aslam *et al.*, 2007). Based in these results it seems to be feasible the use of 40 mg mL^{-1} *in vivo*.

In conclusion, NAC at 40 mg mL^{-1} was the only of the tested treatments that was bactericidal against *S. epidermidis* cells both in planktonic or in biofilm form. Moreover, although NAC and farnesol have different modes of action, the combination of both has no significant synergistic effect.

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VI. COMBINATION OF ANTIBIOTICS AGAINST *IN VITRO* *Staphylococcus epidermidis* BIOFILMS

Submitted for publication

| ABSTRACT

S. epidermidis is the most common pathogen associated with infections of surgical implants and other prosthetic devices owing to its adhesion and biofilm-forming ability on biomaterials surfaces. The objective of this work was to compare susceptibilities of biofilm-grown cells to single antibiotics and in combination in order to identify those that were effective against *Staphylococcus epidermidis* biofilms. For that it was used the MBEC™ assay system what allowed us a rapid testing of an array of antibiotics alone (eight) and in combination (twenty-five double combinations).

The MBEC™ assay system was seen to produce multiple and reproducible biofilms of *S. epidermidis* and to be a useful tool for antibiotic susceptibility studies. Although none of the antibiotics tested have demonstrated an antimicrobial effect (log reduction ≥ 3) against all *S. epidermidis* strains biofilms, combinations containing rifampicin showed a general broader spectrum namely rifampicin-gentamicin and rifampicin-clindamycin. Levofloxacin in combination with rifampicin showed a killing effect against three strains but failed to attain a bactericidal action against the other two. Undoubtedly rifampicin should be part of any antibiotic therapy directed against *S. epidermidis* biofilms. Nevertheless the most efficient antibiotics combination is undeniably strain dependent.

Keywords: Staphylococci; antibiotics susceptibility; MBEC™ assay; biofilm.

| INTRODUCTION

Previously regarded as an innocuous commensal microorganism on the human skin, *Staphylococcus epidermidis* is now seen as an important opportunistic pathogen (Hellmark *et al.*, 2009; Otto, 2009; Wang *et al.*, 2009). This bacterium has become the leading cause of infections related to indwelling medical devices such as vascular catheters, prosthetic joints and artificial heart valves mainly due to its capacity to form biofilms on such materials thus causing persistent or recurrent infections (Hajdu *et al.*, 2009; Knobloch *et al.*, 2002). Infections of medical implants material are associated with considerable morbidity and costs (Hajdu *et al.*, 2009). These infections are very difficult to eradicate since bacteria in biofilms can be up to 1000-fold more resistant to antibiotic treatment than the same organism growing planktonically (Cargill and Upton, 2009; Gilbert *et al.*, 1997; Mah and O'Toole, 2001). Another problem to be considered is the ability of bacteria to acquire resistance to antibiotics therapy. This arises from the frequent use of antibiotics and mainly those of broad-spectrum. Only a few antibiotics are relatively active against *S. epidermidis* biofilms, and rifampicin, a transcription inhibitor, is among the most effective molecules for treating biofilm-related infections. However, in a study where the prevalence of drug resistance among clinically significant blood isolates of *Staphylococcus epidermidis* (n = 464) and consumption of antibiotics at a tertiary care teaching hospital (Meilahti Hospital, Helsinki) were analysed for the period 1983-1994, it was found a remarkable increase in resistance to rifampin (from 0 to 23%) despite the low usage of this agent (Lyytikfiinen *et al.*, 1996). Accordingly, since rifampicin demonstrated a high rizk of rapid development of resistance, it should not be used as monotherapy (Hellmark *et al.*, 2009).

Taking this fact into account, antibiotic combinations are often necessary in the treatment of *S. epidermidis* infections and these combinations are used in treatments involving antibiotics like rifampicin to avoid the appearance of antimicrobial resistance (Hellmark *et al.*, 2009; Monzón *et al.*, 2001). Moreover, the combinations can also enhance the effects of individual antimicrobial agents by synergic action.

Another alternative to overcome the resistance problem in staphylococci is the use of novel antibiotics such as linezolid, daptomycin, tigecycline and quinupristin/dalfopristin that have been developed and claimed to be 100% efficient (Piette and Verschraegen *et al.*, 2009). Some of the newer antimicrobial agents may provide alternatives for monotherapy or combination therapy with rifampicin (Hellmark *et al.*, 2009). However, this new antibiotic generation is too expensive,

so the use of conventional antibiotics or antibiotic combinations represents nowadays a very valid therapeutic option.

The aim of the present work was to investigate the antimicrobial activity of some of the most common antibiotics alone and in combination against *in vitro S. epidermidis* biofilms.

| MATERIALS AND METHODS

| BACTERIAL STRAINS AND GROWTH CONDITIONS

In this study, previously well characterized biofilm-producing *S. epidermidis* strains were used: 117977, 132034, 150271, 1457 and 9142. These strains are clinical isolates and were stored at –80°C. All the assays were performed using TSB and TSA, prepared according to the manufacturer's instructions.

| ANTIBIOTICS

Antibiotics tested were vancomycin (Sigma), tetracycline (Sigma), rifampicin (Sigma), gentamicin (Sigma), cefazolin (Sigma), cephalothin (Sigma), levofloxacin (Sigma) and clindamycin (Sigma). Interpretation criteria for susceptibility testing were based on NCCLS, now CLSI guidelines (Table VI.I.).

Table VI.I. Antibiotics' break points.

Antibiotic	Break point (µg/mL)			Reference
	Sensitive	Intermediate	Resistant*	
Vancomycin	4	8-16	32	NCCLS
Tetracycline	4	8	16	NCCLS
Rifampicin	1	2	4	NCCLS
Gentamicin	4	8	16	NCCLS
Cefazolin	8	16	32	NCCLS
Cephalothin	8	16	32	NCCLS
Levofloxacin	1	2	4	NCCLS
Clindamycin	0.5	-	2	NCCLS

***** Concentration used in bactericidal antibiotic testing.

| BIOFILM FORMATION

Several colonies of the isolates grown on TSA plates were suspended in saline (0.9% NaCl) to a density of 1.0 on the McFarland scale, as indicated by the manufacturer. Then the bacterial suspension was resuspended in medium to obtain a cellular concentration of 1×10^7 CFU mL^{-1}. This solution was used as inoculum for the MBEC™ device (MBEC™ Biofilm Technologies Ltd. Calgary, Alberta, Canada). The biofilms were grown during 48 hours, at 37°C at 150 rpm and on a rocking platform where the shear force was created against the pegs forming 96 equivalent biofilms. To enumerate the biofilm CFU on individual control pegs, pegs were broken off the MBEC peg lid using sterile forceps, placed into 200 µL of sterile saline and sonicated for 8 minutes. Bacteria were then enumerated by serial dilution plating. CFU/peg counts were determined from at least three independent experiments.

This protocol was performed with three different biofilm growth media: TSB, TSB + 0.25% glucose and brain heart infusion (BHI) medium. After selecting the medium that allowed the highest biofilm formation (CFU per peg ≥ 6 log), the previous procedure was repeated with the selected medium.

| BIOFILM CHALLENGE AND RECOVERY

The challenge plates were prepared using the antibiotics at break point concentration (Table VI.I.) alone and in all possible double combinations (Table VI.II.). The biofilms formed on the lid of the MBEC™ were rinsed twice with 0.9% saline and placed into the challenge plate overnight at 37°C, at 150 rpm, on a rocking platform and 95% relative humidity. After that the challenged biofilms were rinsed twice in saline and were tranferred to a recovery plate that consisted of TSB medium plus tween 1%. A sonication of 8 minutes was performed to release the biofilm cells. The vibration disrupted biofilms from the surface of the 96 pegs into the recovery plate. Then, colony forming units were determined as follows: the recovery medium (containing the sonicated biofilms) was serially diluted. The biofilm cultures (ten-fold diluted) were spotted on TSA plates. Then the plates were incubated for 48 hours at 37°C to ensure maximum recovery of the surviving microorganisms and after that the CFU were counted.

| RESULTS AND DISCUSSION

Standard antibiotic therapy is only able to eliminate planktonic cells, leaving the sessile forms to propagate within the biofilm and to continue to disseminate when therapy is terminated. In biofilms, microbes are protected from antimicrobial agents and the host immune system (Wang *et al.*, 2009). In fact, increasingly microorganisms have the ability to withstanding the effect of antibiotics and individual antibiotics are generally ineffective against bacteria biofilms. In order to overcome these problems, combination of antibiotics is a possible alternative to threat staphylococcal biofilm infections.

In this study, eight antibiotics, usually used in the treatment of Gram-positive infections, were tested at their break point concentrations. The effect of these antibiotics combined in pairs (Table VI.II.) was also assessed. For that, we have used the Calgary Biofilm Device (CBD) which allowed us to study a wide range of antibiotics and combinations.

Table VI.II. Antibiotics used and all combinations studied.

VANC	VANC + RIF	TET + CEPH	GENT + CEPH
TET	VANC + GENT	TET + LEVO	GENT + LEVO
RIF	VANC + CEF	TET + CLIND	GENT + CLIND
GENT	VANC + CEPH	RIF + GENT	CEF + CEPH
CEF	VANC + LEVO	RIF + CEF	CEF + LEVO
CEPH	VANC + CLIND	RIF + CEPH	CEF + CLIND
LEVO	TET + RIF	RIF + LEVO	CEPH + LEVO
CLIND	TET + GENT	RIF + CLIND	CEPH + CLIND
VANC + TET	TET + CEF	GENT + CEF	LEVO + CLIND

Legend: VANC, vancomycin; TET, tetracycline; RIF, rifampicin; GENT, gentamicin; CEF, cefazolin; CEPH, cephalothin; LEVO, levofloxacin; CLIND, clindamycin.

First, and to obtain an appropriate amount of biofilm, it was necessary to select the culture medium that allows the highest production of *S. epidermidis* biofilm in CBD. TSB without glucose was the medium that stimulated more biofilm formation (data not shown). In fact, the amount of glucose (0.25% w/v) usually used to form *S. epidermidis* biofilms in traditional 96-well plates (Cargill and Upton, 2009; Cerca *et al.*, 2005) was not favorable to biofilm formation in CBD.

Then, the effect of the tested antibiotics alone was evaluated against the biofilms of the five clinical isolates of *S. epidermidis* assayed. The results obtained are presented in table VI.III.

and expressed as reduction in treated biofilms compared to untreated controls. In general none of the antibiotics tested was effective against all *S. epidermidis* strains biofilm. In fact, only rifampicin can be considered to be effective against *S. epidermidis* strains 132034 and 150271 as well as levofloxacin against strain 9142 (Table VI.III.) because the $\log_{10}$ CFU reduction observed was higher than 3 log. Although the reduction caused by rifampicin and levofloxacin is mostly inferior to 3 log, those are the antibiotics having the broadest and highest antimicrobial effect against all *S. epidermidis* strains tested.

Table VI.III. Log reduction of antibiotics alone.

Strain	VANC	TET	RIF	GENT	CEF	CEPH	LEVO	CLIND
117977	0.00 ± 0.24	1.20 ± 0.24	2.37 ± 0.68	0.00 ± 0.29	0.00 ± 0.23	0.00 ± 0.29	2.86 ± 0.48	1.68 ± 0.35
132034	0.26 ± 0.73	2.70 ± 0.48	4.01 ± 0.47	0.56 ± 0.30	0.29 ± 0.46	2.39 ± 0.38	0.32 ± 0.19	0.15 ± 0.32
150271	0.36 ± 0.44	0.31 ± 0.40	3.15 ± 0.18	0.14 ± 0.42	0.46 ± 0.36	1.84 ± 0.51	1.87 ± 0.36	2.20 ± 0.32
1457	0.00 ± 0.51	2.02 ± 0.20	1.58 ± 0.22	0.00 ± 0.19	1.63 ± 0.47	2.40 ± 0.31	2.18 ± 0.35	1.72 ± 0.20
9142	0.57 ± 0.53	1.54 ± 0.42	2.42 ± 0.44	0.19 ± 0.38	0.14 ± 0.40	0.26 ± 0.54	3.63 ± 0.12	0.28 ± 0.51

Legend: VANC, vancomycin; TET, tetracycline; RIF, rifampicin; GENT, gentamicin; CEF, cefazolin; CEPH, cephalothin; LEVO, levofloxacin; CLIND, clindamycin. Values are a mean of n = 4 ± standard deviation.

Previous studies have also demonstrated impressive results with rifampicin, however, the rizk of rapid development of resistance is a major problem, and rifampicin should not be used as monotherapy (Hellmark *et al.*, 2009; Mick *et al.*, 2010; Zavasky and Sande, 1998).

Since antibiotics alone were generally not effective against *S. epidermidis* biofilms and taking into consideration the strategy of combined therapy to avoid resistance, the double combinations of the antibiotics were tested against the same biofilms. In a previous study (Saginur *et al.*, 2006), where some double and triple combinations of antibiotics were studied, several triple combinations, all containing rifampicin were active against *S. epidermidis* and only one double combination vancomycin-rifampicin was reported to be active. In that study seventeen *S. epidermidis* strains were assessed and the susceptibility to antibiotics was tested in terms of MIC and MBC. However, a triple combination may be an overload of antibiotics and more prone to the development of secondary effects.

Monzón *et al.* (2001) also tested some double combinations of antibiotics against four *S. epidermidis* strains and the highest reduction they observed was 2.19 log obtained with the

combination vancomycin-rifampicin and only against one specific strain, using both antibiotics at 4 x MIC.

The results presented in table VI.IV. show the reduction in biofilms $\log_{10}$CFU for all combinations of antibiotics tested. Most combinations tested did not promote a 3 log reduction in bacterial counts. Nevertheless, and as it could be expected, most of those containing rifampicin were able to reach very good levels of bactericidal effect with a relatively broad spectrum. Examples are rifampicin-clindamycin and rifampicin-gentamicin, the former promoting reductions above 2.5 log in biofilm cell counts for all strains tested. Notably, the combination rifampicin-levofloxacin displayed a high killing effect specifically against three strains but against strain 9142 the log reduction was below 2.0. It has been considered that combinations of rifampicin with other anti-staphylococcal agents such as quinolones or fusidic acid could prevent the emergence of rifampicin resistance during therapy (Mick *et al.*, 2010; Moellering, 2008).

Table VI.IV. Log reduction of combination of two antibiotics.

Strain	VANC + TET	VANC + RIF	VANC + GENT	VANC + CEF
117977	1,33 ± 0.30	2.32 ± 0.36	0.00 ± 0.38	0.00 ± 0.18
132034	2.89 ± 0.49	4.22 ± 0.42	0.58 ± 0.24	0.25 ± 0.36
150271	0.11 ± 0.24	2.91 ± 0.12	0.28 ± 0.28	0.93 ± 0.78
1457	1.89 ± 0.63	2.47 ± 0.60	1.45 ± 0.59	2.21 ± 0.57
9142	1.72 ± 0.17	2.75 ± 0.43	0.47 ± 0.43	0.40 ± 0.37
Strain	**VANC + CEPH**	**VANC + LEVO**	**VANC + CLIND**	**TET + RIF**
117977	0.00 ± 0.15	2.56 ± 0.38	1.75 ± 0.37	2.49 ± 0.49
132034	1.02 ± 0.40	0.53 ± 0.23	0.26 ± 0.50	3.37 ± 0.43
150271	1.75 + 0.39	2.60 ± 0.35	2.16 ± 0.20	2.03 ± 0.24
1457	2.35 ± 0.44	2.13 ± 0.42	1.24 ± 0.35	2.09 ± 0.49
9142	0.39 ± 0.49	2.50 ± 0.37	0.68 ± 0.29	2.28 ± 0.40
Strain	**TET + GENT**	**TET + CEF**	**TET + CEPH**	**TET + LEVO**
117977	1.43 ± 0.41	1.29 ± 0.48	1.07 ± 0.31	2.62 ± 0.43
132034	2.77 ± 0.35	2.41 ± 0.26	1.92 ± 0.48	3.14 ± 0.17
150271	0.23 ± 0.35	1.12 ± 0.45	1.78 ± 0.13	2.63 ± 0.15
1457	2.21 ± 0.48	1.84 ± 0.46	1.71 ± 0.83	2.07 ± 0.44
9142	2.01 ± 0.27	1.58 ± 0.49	1.51 ± 0.59	1.81 ± 0.29
Strain	**TET + CLIND**	**RIF + GENT**	**RIF + CEF**	**RIF + CEPH**
117977	1.72 ± 0.26	2.46 ± 0.63	1.82 ± 0.24	1.76 ± 0.43
132034	2.86 ± 0.51	3.11 ± 0.56	3.45 ± 0.28	3.08 ± 0.32
150271	2.23 ± 0.52	2.49 ± 0.22	2.69 ± 0.45	2.73 ± 0.24
1457	1.96 ± 0.46	2.06 ± 0.16	1.85 ± 0.37	2.11 ± 0.55
9142	1.84 ± 0.18	3.14 ± 0.56	1.90 ± 0.20	1.41 ± 0.69
Strain	**RIF + LEVO**	**RIF + CLIND**	**GENT + CEF**	**GENT + CEPH**
117977	3.49 ± 0.44	2.65 ± 0.33	0.00 ± 0.44	0.00 ± 0.39
132034	3.18 ± 0.56	3.68 ± 0.23	0.22 ± 0.13	0.79 ± 0.65
150271	2.83 ± 0.21	2.73 ± 0.49	0.06 ± 0.51	1.04 ± 0.42
1457	3.24 ± 0.42	2.46 ± 0.31	2.19 ± 0.35	2.49 ± 0.67
9142	1.71 ± 0.58	2.64 ± 0.27	0.39 ± 0.18	1.03 ± 0.44
Strain	**GENT + LEVO**	**GENT + CLIND**	**CEF + CEPH**	**CEF + LEVO**
117977	3.76 ± 0.57	1.48 ± 0.31	0.00 ± 0.28	3.90 ± 0.30
132034	0.31 ± 0.37	0.14 ± 0.23	1.03 ± 0.43	0.11 ± 0.42
150271	2.91 ± 0.43	2.15 ± 0.25	1.82 ± 0.24	3.06 ± 0.55
1457	2.28 ± 0.43	1.98 ± 0.49	2.40 ± 0.47	2.55 ± 0.26
9142	1.83 ± 0.24	0.16 ± 0.38	1.92 ± 0.65	2.14 ± 0.58
Strain	**CEF + CLIND**	**CEPH + LEVO**	**CEPH + CLIND**	**LEVO + CLIND**
117977	1.11 ± 0.25	3.38 ± 0.42	0.86 ± 0.32	2.68 ± 0.28
132034	0.29 ± 0.16	0.64 ± 0.19	0.87 ± 0.61	0.07 ± 0.30
150271	1.50 ± 0.25	2.74 ± 0.16	1.48 ± 0.27	2.54 ± 0.32
1457	1.44 ± 0.36	2.28 ± 0.50	1.75 ± 0.36	2.25 ± 0.42
9142	0.31 ± 0.43	2.72 ± 0.51	1.13 ± 0.45	2.33 ± 0.61

Legend: VANC, vancomycin; TET, tetracycline; RIF, rifampicin; GENT, gentamicin; CEF, cefazolin; CEPH, cephalothin; LEVO, levofloxacin; CLIND, clindamycin. Values are a mean of n = 4 ± standard deviation.

Therefore, it could be advantageous the use of these combinations in the treatment of *Staphylococcus epidermidis* infections. However, the effect of these combinations is highly strain-dependent and the rate of success will be strongly dependent on the infectious *S. epidermidis* strain.

Alternative agents are novel antibiotics such as linezolid, tigecycline and daptomycin claimed to be highly effective against biofilms, but these agents have some disadvantages. Apart from their very high cost, they have been in clinical use for a short time only and so the extent of their toxicity is yet to be experienced (Hajdu *et al.*, 2009). Moreover, Hajdu *et al.* (2009) observed that no significant reduction in *S. epidermidis* biofilms CFU was achieved with daptomycin and tigecycline, not even at the highest concentrations tested (128 × MIC). Generally these concentrations are far beyond any concentration that can be achieved after administration of standard therapeutic doses (Hajdu *et al.*, 2009). Moreover, Aslam *et al.* (2007) also tested the effect of tigecycline and after 12 hours of treatment only a mean reduction of the bacterial growth by 2 $\log_{10}$ counts was obtained, notably using a concentration of 1mg/mL (1,000 fold higher than its MIC for the organisms tested in the planktonic phase). In this case, the concentration of tigecycline expected to be in human serum after standard dosing is 2 mg/L (Hajdu *et al.*, 2009). Utilizing high doses of antimicrobials to eradicate biofilm has had limited success in the clinical setting (Aslam *et al.*, 2007). Based on these results, the use of such new antibiotics to combat infections caused by *S. epidermidis* does not seem very encouraging.

In conclusion, there are some combinations of more traditional antibiotics that can be strongly considered as therapeutic strategies for an efficient control of *S. epidermidis* biofilms associated infections. Rifampicin is present in all such combinations. Actually, rifampicin combined with clindamycin or with gentamicin showed to have the broadest range of action, considering the strains tested, although rifampicin in combination with levofloxacin displayed a higher killing effect against three out of the five strains. Moreover as an alternative to monotherapy, these combinations can be advantageous avoiding the likehood of resistance development. A point to have in mind is that *S. epidermidis* biofilm control is strongly strain dependent.

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VII. VIRULENCE GENE EXPRESSION BY *Staphylococcus epidermidis* BIOFILM CELLS EXPOSED TO ANTIBIOTICS

| ABSTRACT

Staphylococcus epidermidis have become important causes of nosocomial infections being its pathogenesis correlated with the ability to form biofilms on polymeric surfaces; cells are much more resistant to antimicrobial substances in the biofilm form of life. Production of PNAG/PIA is crucial for *S. epidermidis* biofilm formation and is synthesized by the gene products of the *icaADBC* gene cluster. Production of PNAG/PIA and biofilm formation are regulated by the alternative sigma factor, σ^B, and is influenced by a variety of environmental conditions including disinfectants and other antimicrobial substances. The susceptibility of five *S. epidermidis* strains to individual antibiotics and in double combination was previously tested. Our results demonstrated that some combinations are active and present a general broad spectrum against *S. epidermidis* biofilms, namely rifampicin-clindamycin and rifampicin-gentamicin. Relatively to individual antibiotics, rifampicin was the only that exhibited a significant inhibitory effect against *S. epidermidis* biofilms. In the present study, it was investigated whether the combination of rifampicin with clindamycin and gentamicin and these antibiotics alone influence the expression of specific genes (*icaA* and *rsbU*) of *S. epidermidis* within biofilms using real-time polymerase chain reaction (RT-PCR).

The data showed that the expression of both genes tested significantly increased after exposure to antimicrobial agents alone and in combination in most cases. Besides having a similar antimicrobial effect, rifampicin combined with clindamycin and gentamicin induced a lower expression of biofilm-related genes relatively to rifampicin alone. Associated to the advantage of avoiding the emergence of antibiotic resistance, this study demonstrated that this combinatorial therapy can cause a lower genetic expression of *icaA* and *rsbU* genes, responsible for PNAG/PIA production and consequently reduce biofilm formation recidivism, relatively to rifampicin alone.

Keywords: Staphylococci; antibiotics; combinatorial therapy; biofilm; gene expression.

| INTRODUCTION

Staphylococcus epidermidis normally is a commensal inhabitant of the healthy human skin and mucosa, but also a common nosocomial pathogen in immunocompromised patients. Living at the edge between commensalism and pathogenicity, *S. epidermidis* has developed interesting strategies to conquer the hospital environment as a novel ecological niche and to transform into a notorious pathogen (Schoenfelder *et al.*, 2010). *S. epidermidis* causes a great number of infections, being the most frequent causative agent of infections of indwelling medical devices, such as peripheral or central intravenous catheters (Rogers *et al.*, 2009). As example, *S. epidermidis* may be involved in several device infections, e.g. prosthetic joint, vascular graft, surgical site, central venous system shunt and cardiac device infections (Rogers *et al.*, 2009). These infections usually commence with the introduction of bacteria from the skin of the patient or that of health care personnel during device insertion and have increased in number, probably owing to the increased use of such devices (CDC, 2004; O'Grady, 2002).

The tight pathogenic association is essentially linked to the species ability to form adherent biofilms on artificial surfaces (Rohde *et al.*, 2010) being this feature the main virulence mechanism of *Staphylococcus epidermidis*. Unlike other *Staphylococcus* species, such as *Staphylococcus aureus* that present an aggressive virulence, *Staphylococcus epidermidis* present low virulence potential (Otto, 2009). This is due to the fact of this bacterium be devoid of toxin production, molecules that agressively attack the host. However, *S. epidermidis* is well equipped with determinants that promote persistence, such as immune evasion molecules. Many *S. epidermidis* strains produce PNAG/PIA homopolymer, crucial for biofilm formation, which surrounds and connects *S. epidermidis* cells in a biofilm (Mack *et al.*, 1996). In addition to its role as part of the extracellular biofilm matrix, PNAG/PIA has been found to protect the bacterium from important mechanisms of innate host defence namely from neutrophil killing, complement deposition, immunoglobulins and AMPs (Kristian *et al.*, 2008; Vuong *et al.*, 2004b). The correlation of the presence of PNAG/PIA with the invasiveness of the bacterium may be due to the roles of this exopolymer in biofilm formation and immune evasion (Otto, 2009). Two main putative determinants of *S. epidermidis* invasiveness were identified: the *ica* genes, which regulate the production of PNAG/PIA, and the insertion element *IS*256. The latter is thought to contribute to the genetic adaptation that may have a role during infection (Ziebuhr *et al.*, 1999). The biosynthesis of PNAG/PIA is accomplished by the gene products of the *ica* (intercellular

adhesion) locus (Gerke *et al.*, 1998; Heilmann *et al.*, 1996; Mack *et al.*, 1996), which comprises four intercellular adhesion genes: *icaA*, *icaB*, *icaC*, and *icaD* (Nuryastuti *et al.*, 2009). *IcaA* and *icaD* produce a chain from activated *N*-acetylglucosamine (GlcNac) monomers, the elongation of which is dependent on the *icaC* protein, probably owing to the predicted exporter function of *icaC* (Gerke *et al.*, 1998). Partial deacetylation of the GlcNac residues is accomplished by the cell surface-located enzyme *icaB* after export (Vuong *et al.*, 2004a). The expression of the *ica* operon and biofilm formation depends on a variety of environmental conditions. In addition to *icaADBC* operon, the expression of at least four unlinked genes [*icaR* (Fitzpatrick *et al.*, 2005), σ^B, the *pur*R locus and *sar*A] is known to control PNAG/PIA synthesis at the level of transcription (Conlon *et al.*, 2002; Mack *et al.*, 2000; Mack *et al.*, 2007). Production of PNAG/PIA is subject to a range of regulatory influences (O'Gara, 2007), including many global virulence regulators. PNAG/PIA and biofilm formation is regulated by the alternative sigma factor σ^B, being presumed to play a crucial role in the global regulation of gene expression (Kies *et al.*, 2001). The σ^B operon in staphylococci contains four genes: *rsbU, rsbV, rsbW* and *sigB*. The *rsbW* gene product is key negative regulator, responsible for an inactive *sigB*. The *rsbU* and *rsbV* gene products stimulate *sigB* activity. *sigB* increases its own transcription as a consequence of its activation, further inducing the entire σ^B regulon (Fouet *et al.*, 2000). *RsbV* acts as a positive regulator; in its dephosphorylated form, *rsbV* can bind competitively to *rsbW*, resulting in the release of free and active *sigB*. The binding of *rsbW* to *sigB* or to *rsbV* depends on the phosphorylation status of *rsbV*, which is modulated by the phosphatase *rsbU* (Wise and Price, 1995). *RsbU* is a positive regulator of the activity of σ^B, the general stress-response-factor of Gram$^+$ microorganisms (Delumeau *et al.*, 2004). σ^B may act only indirectly via an additional, unknown factor or *rsbU* may, by itself, be a regulator of *icaADBC* transcription.

Activation of PNAG/PIA expression by different stress stimuli apparently uses different pathways. This implies that a number of environmental conditions and regulatory systems can influence the expression of staphylococcal biofilms, reflecting the magnitude of the complexity associated with biofilm formation (Pintens *et al.*, 2008). The current work, was undertaken to investigate the effect of antibiotics alone and in combination (chosen from a previous study) on the expression of some genes of *Staphylococcus epidermidis* namely *icaA* and *rsbU*, both responsible by PNAG/PIA production and consequently biofilm formation. This will enable to get insights into the response of persister cells after antibiotic treatment.

| MATERIALS AND METHODS

| BACTERIAL STRAINS, ANTIMICROBIAL AGENTS

In this study, five biofilm-producing *S. epidermidis* strains were used: 117977, 132034, 150271, 1457 and 9142. These strains are clinical isolates and were stored at -80°C. Antibiotics tested were rifampicin, gentamicin and clindamycin (Sigma) and used at break point concentration (Table VI.I.).

| BIOFILM FORMATION

Several colonies of the isolates grown on TSA plates were suspended in saline (0.9% NaCl) to a density of 1.0 on the McFarland scale, as indicated by the manufacturer. Then the bacterial suspension was resuspended in TSB to obtain a cellular concentration of 1×10^7 CFU mL^{-1}. This solution was used as inoculum for the MBEC™ device (MBEC™ Biofilm Technologies Ltd. Calgary, Alberta, Canada). The biofilms were grown during 48 hours, at 37°C at 150 rpm and on a rocking platform where the shear force was created against the pegs forming 96 equivalent biofilms.

| BIOFILM CHALLENGE AND RECOVERY

The challenge plates were prepared using the antibiotics at break point concentration (Table VI.I.) alone and in combination (rifampicin + clindamycin and rifampicin + gentamicin). The biofilms formed on the lid of the MBEC™ were rinsed twice with 0.9% saline and placed into the challenge plate overnight at 37°C, at 150 rpm, on a rocking platform and 95% relative humidity. After that the challenged biofilms were rinsed twice in saline and were transferred to a recovery plate that consisted of TSB medium plus tween 1%. Biofilms were removed from all pegs at once, by sonication for 8 minutes on high with Aquasonic sonicator (model 250HT, VWR Scientific) (Ceri *et al.*, 1999). The vibration disrupted biofilms from the surface of the 96 pegs into the recovery plate. Then all samples were collected, preserved in RNA later (Ambion-Applied Biosystems) and kept at -80°C for further study of gene expression.

| GENETIC EXPRESSION ANALYSIS

| PRIMER DESIGN

Primers used for *Staphylococcus epidermidis icaA* and *rsbU* genes analysis by real-time PCR were designed using Primer3 web-based software (http://fokker.wi.mit.edu\cgi-bin\primer3\primer3_www.cgi) (Rozen and Skaletsky, 2000) and are listed in table VII.I. In order to verify the specificity of each primer pair for its corresponding target gene, PCR products were first amplified from 1457 strain genomic DNA (data not shown).

Table VII.I. List of the primers used for the RT-PCR experiments.

Primer name	Sequence	Target
*16S*RNAFW	gggctacacacgtgctacaa	*16S*
*16S*RNAREV	gtacaagacccgggaacgta	*16S*
*rsbU*FW	taacgtgttttgggactcacac	*rsbU*
*rsbU*REV	tgttgaaaagaacgttaaccaaa	*rsbU*
*icaA*FW	gcactcaatgagggaatca	*icaA*
*icaA*REV	taactgcgcctaattttggatt	*icaA*

| RNA EXTRACTION

Total RNA of each sample was extracted using the PureLink™ RNA Mini Kit (Invitrogen) according to manufacturer's recommended protocol. Potential DNA contamination was removed during RNA purification procedure by On-column PureLink™ DNase treatment (Invitrogen). RNA concentration (ng/µl) and purity (OD_{260nm}/OD_{280nm}) were assessed by spectrophotometric measurement using a NanoDrop device (NanoDrop 1000 Spectrophotometer, V3.6.0, Thermo Fisher Scientific, Inc.).

| cDNA SYNTHESIS

To ensure equivalent starting amounts of RNA from control and respective treated samples to be converted into cDNA, appropriate dilutions in RNase-free water were performed in

order to obtain a final concentration of 2 μg of RNA. cDNA of each sample was synthesized using the iScript™cDNA Synthesis Kit (BioRad). Each reaction contained 2.5 μL of iScript Reaction Mix + iScript Reverse Transcriptase and 7.5 μL of RNA template, respecting the proportions recommended by the kit manufacturer to a final reaction volume of 10 μL. Complete reaction mix was incubated in a termocycler (MyCycler, BioRad, USA) with the following reaction protocol: 5 minutes at 25°C, 30 minutes at 42°C and 5 minutes at 85°C.

| QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (QPCR)

Real-time PCR reactions were performed on a CFX96TM real-time PCR Detection System Bio-Rad system (Bio-Rad Laboratories, Inc., USA). Each 20 μL of reaction mixture contained 2 μL of cDNA, 1 μL of each primer (Forward and Reverse primers), 10 μl of 1x SsoFast™ EvaGreen® Supermix (Bio-Rad Laboratories, Inc.), and 6 μL of nuclease-free water. Thermal cycling conditions were as follows: 3 minutes initial denaturation at 95°C, followed by 40 cycles of 5 seconds denaturation at 95°C, 10 seconds annealing/extension at 60°C (this step was performed at 60°C, concerning primers efficiency previously determined - data not shown). A melt curve was inserted at the end of each run, with readings from 65°C to 95°C every 1°C for 5 seconds, in order to confirm that only the desired product was amplified.

| GENE ANALYSIS AND EXPRESSION

Samples for real-time PCR reactions were run in triplicate. Data were analyzed using the Bio-Rad CFX Manager™ version 1.6 (Bio-Rad Laboratories, Inc., USA) and the relative quantification method ($2^{-\Delta\Delta_{CT}}$; Livak and Schmittgen, 2001), which describes the change in expression of the target genes relative to the *16S* rRNA reference genes from untreated control samples (Tscherne *et al.*, 1999; Kundinger *et al.*, 2007). Data were analysed by averaging the cycle threshold values (CT, cycle at which each sample amplification curve crosses a specific threshold) for triplicate samples. The ΔCT values of the target genes were determined by normalizing to the endogenous control genes *16S* rRNA. These samples were subsequently subtracted from the *16S* rRNA genes from the untreated control samples. The ΔΔCT was used to calculate relative expression using the formula $2^{-\Delta\Delta_{CT}}$ (Giulietti *et al.*, 2001; Livak and Schmittgen, 2001; Lehman and Kreipe, 2001).

The cDNA was validated by comparing with a RNA sample not subjected to reverse transcriptase (RT) reaction, in order to determine possible DNA contamination. All these no-RT controls showed an $\Delta\Delta C_T$ above ten cycles, indicating that the cDNA present resulted from the conversion of the RNA.

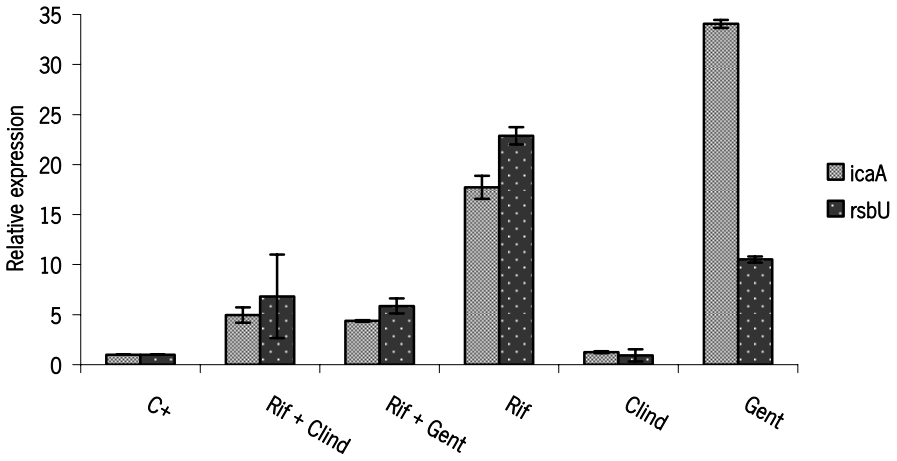
| STATISTICAL ANALYSIS

The data were analysed by applying two-tailed independent samples *t* tests. SPSS software was used to perform the analysis. All tests were performed with a confidence level of 95%.

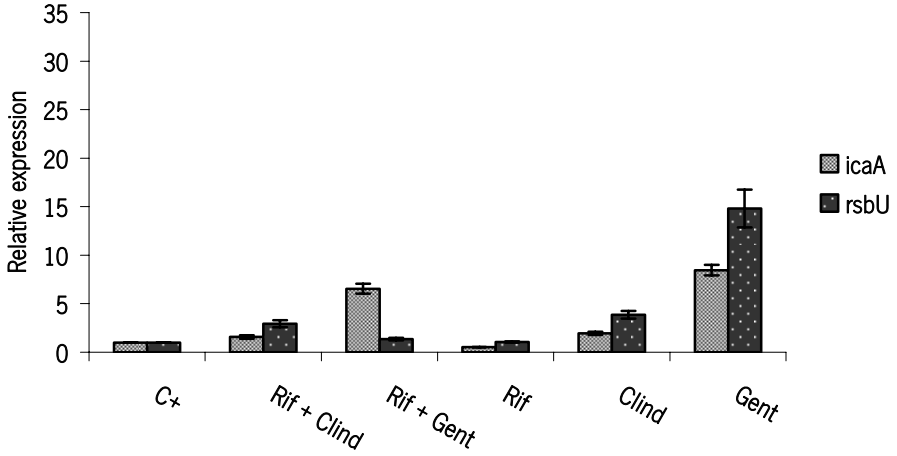
| RESULTS AND DISCUSSION

We have previously demonstrated that some combinations of antibiotics are significantly effective against *S. epidermidis* biofilm cells. Rifampicin alone was generally as or more effective than when used in combination with other antibiotic. However, this kind of combinatorial therapy aims to prevent the emergence of resistance usually associated with monotherapy. Two of the most efficient combinations tested, which induced a greater reduction of bacterial biofilm population and which demonstrated a broader spectrum range being active against all strains tested, were rifampicin+clindamycin and rifampicin+gentamicin. Both combinations were able to reduce bacterial cells number by approximately 3 log. In order to determine the effect of both combinations and of each antibiotic individually on the expression of *S. epidermidis icaA* and *rsbU* genes, real-time PCR analysis was performed. The results obtained for the different strains tested are represented in figure VII.1.

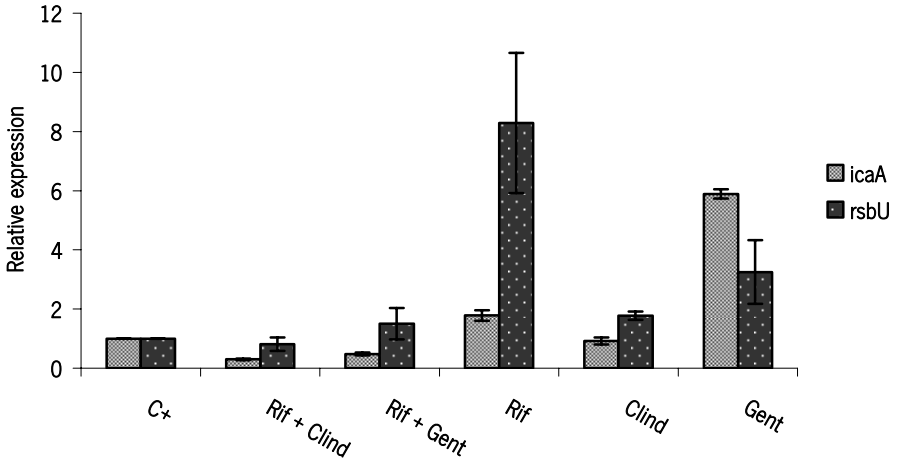
A


Relative expression
35
30
25
20
15
10
5
0
C+
Rif + Clind
Rif + Gent
Rif
Clind
Gent
icaA
rsbU

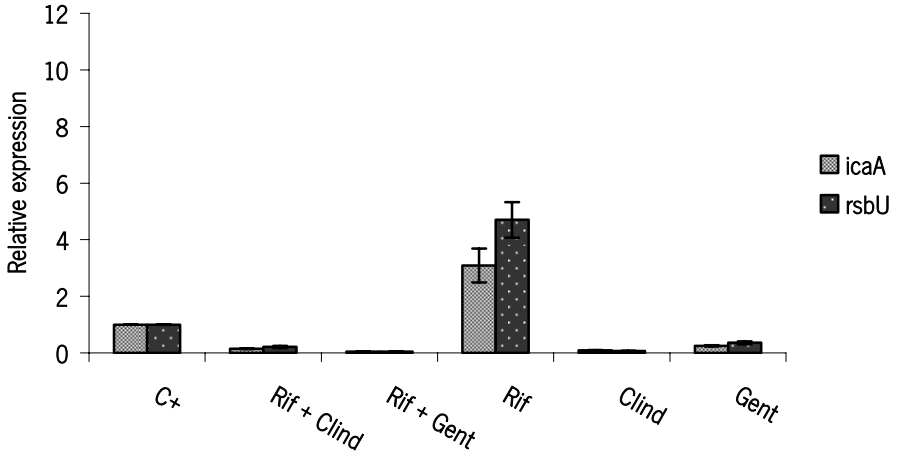
B


Relative expression
35
30
25
20
15
10
5
0
C+
Rif + Clind
Rif + Gent
Rif
Clind
Gent
icaA
rsbU

C


12
10
8
6
4
2
0
Relative expression
icaA
rsbU
C+
Rif + Clind
Rif + Gent
Rif
Clind
Gent

D


12
10
8
6
4
2
0
Relative expression
icaA
rsbU
C+
Rif + Clind
Rif + Gent
Rif
Clind
Gent

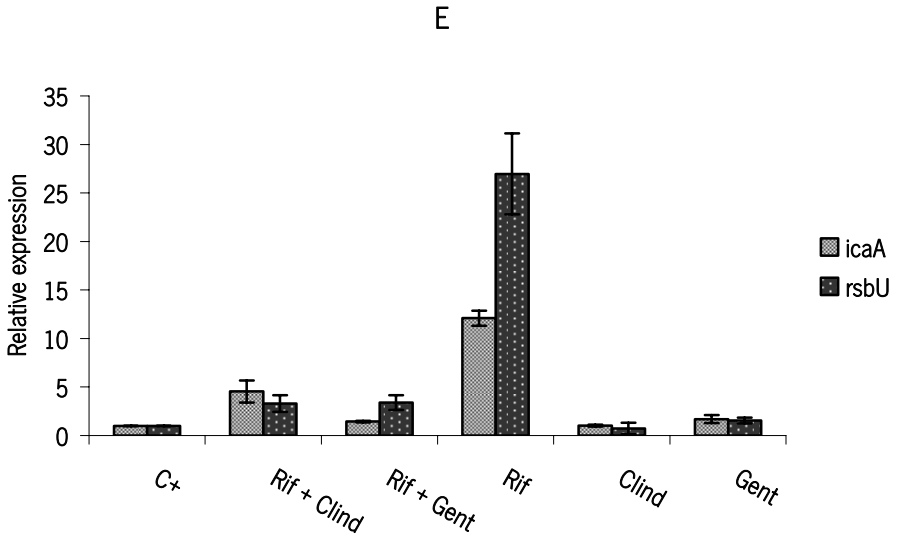


Figure. VII.1. Expression of *icaA* and *rsbU* in *S. epidermidis* strains (A) 117977, (B) 132034, (C) 150271, (D) 1457 and (E) 9142, in response to rifampicin (Rif), Clindamycin (Clind), gentamicin (Gent), Rifampicin combined with clindamycin (Rif + Clind) and rifampicin with gentamicin (Rif + Gent). The relative expression of *icaA* and *rsbU* in biofilms exposed to antibiotics was plotted against unexposed control biofilms (C+), using *16S* as the reference gene. The $2^{-\Delta\Delta_{CT}}$ was calculated from the average CT values of two reactions. Error bars represent standard deviation.

Relative to the unexposed control, *icaA* and *rsbU* were overexpressed in all strains (although at different extents) when exposed to rifampicin at break point concentration ($p < 0.05$), except for strain 132034 (Figure VII.1.B). The same was observed for gentamicin, which caused the overexpression of both genes in four strains ($p < 0.05$) with the exception of 1457 *S. epidermidis.* Generally, no overexpression of *icaA* and *rsbU* genes was observed in biofilm cells exposed to clindamycin. Relatively to combinations of antibiotics, the response of *S. epidermidis* was strain dependent. Three strains demonstrated an overexpression of both genes after exposure to rifampicin + clindamycin and rifampicin + gentamicin when compared with untreated biofilms ($p < 0.05$). On the contrary, these two combinations induced a subexpression of *icaA* and *rsbU* on 1457 *S. epidermidis*. On the other hand, in strain 150271, both combinations provoked a subexpression of *icaA* and the expression of *rsbU* was unaffected comparatively to positive control ($p < 0.05$). Normally, the expression of *icaA* is accompanied by the expression of *rsbU*. Pintens *et al.* (2008) demonstrated that the increase in *icaA* expression clearly preceded

the increase in *rsbU* expression *in vitro* as well as *in vivo*. This is consistent with factors other than *sigB* inducing *icaADBC* operon expression (Conlon *et al.*, 2002; Mack *et al.*, 2000; Mack *et al.*, 2007).

Comparing the effect of antibiotics alone and in combination it was observed that the combinations always expressed less *icaA* and *rsbU* genes than antibiotics individually. Since clindamycin and gentamicin are not active antimicrobial agents against *S. epidermidis*, as previously showed, and rifampicin was the only antibiotic presenting similar effect to the most active combinations tested, it seems important to compare the genetic expression of both genes assessed after biofilm exposure to these antimicrobial treatments. Therefore, although the two combinations of antibiotics induced an increase of *icaA* and *rsbU* expression, this increase was not as marked as in cells exposed to rifampicin. In fact, comparing the *icaA* and *rsbU* gene expression after treatment with rifampicin and rifampicin combined with clindamycin and gentamicin, we can conclude that with the exception of 132034, there is always a general decrease of the expression of these two genes after biofilm exposure to both antibiotics combinations ($p < 0.05$) (Figure VII.1.).

RsbU is a positive regulator of the alternative sigma factor *sigB*, known to control PNAG/PIA synthesis at the level of transcription. Moreover, the global stress response regulator σ^B and *rsbU* control biofilm development in *S. epidermidis* (Knobloch *et al.*, 2001). The present results clearly indicated that the expression of *icaA* and *rsbU* is generally inferior in the presence of break point concentrations of rifampicin combined with clindamycin or gentamicin in comparison to rifampicin alone. Although all treatments studied induced the expression of the two genes assayed, after overnight treatment, rifampicin is a stronger inducer of *icaA* and *rsbU* genes expression, possibly inducing in *S. epidermidis* persister cells a high ability for biofilm formation. In fact, biofilm formation has been reported to be induced by conditions that are potentially toxic for bacterial cells, such as high levels of osmolarity, detergents, urea, ethanol, oxidative stress, and the presence of sub-MICs of some antibiotics (Cramton *et al.*, 2001; Knobloch *et al.*, 2002, Rachid *et al.*, 2000). In *S. epidermidis*, the induction of PNAG/PIA production and biofilm formation can improve the ability of biofilm immune evasion. PNAG/PIA is crucial for connection of cells in a biofilm, avoiding the detachment of biofilm cells, mechanism that can make the cells more susceptible to antimicrobial agents. Furthermore, this exopolymer has also a protective function. Consequently, the induction of PNAG/PIA formation can be a possible defence and resistance mechanism of cells.

Therefore, in addition to the already known advantages of antibiotics combinatorial therapy, namely as a strategy to reduce resistance development, our results show a decreased expression of *icaA* and *rbsU*, biofilm-related genes when combining rifampicin (the most efficient antibiotic against *S. epidermidis* used in clinical practice) with clindamycin or gentamicin, ensuring a more efficient control of *S. epidermidis* biofilms associated infections.

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VIII. CONCLUSIONS AND FINAL REMARKS

In this last chapter, a summary of the most important conclusions of the present thesis is presented. Additionally, some work perspectives are suggested for further research in this field.

| FINAL CONCLUSIONS

The main aim of the present thesis was to study/develop new therapeutic strategies against *S. epidermidis* biofilms. In order to achieve this objective several approaches were tested *in vitro* namely: the use of a sesquiterpenoid with antimicrobial properties, farnesol; the use of this compound in combination with antibiotics presenting different mechanisms of action, and in combination with another antimicrobial compound, *N*-acetylcysteine. Moreover, the use of double combinations of traditional antibiotics as a possible therapeutic strategy against *S. epidermidis* biofilms was also studied as well as the assessment of the genetic expression of some genes (*icaA*-virulence gene and *rsbU*-stress regulator) after treatment with two of the most effective antibiotic combinations tested.

The main conclusions of this thesis are the following:

Small concentrations (100 μM) of farnesol were sufficient to exhibit antibacterial effect on planktonic cells. In biofilm cells, farnesol was not so effective and its effect seems to be strongly dependent on the cells metabolic activity and amount of polymeric matrix. Farnesol induced a reduction of biofilm biomass, caused a notable change in the composition of the biofilm matrix, and in the spatial structure of the biofilm, supporting the hypothesis that farnesol causes disruption of the cytoplasmic membrane and consequently release of cellular content. Therefore, in addition to cell death, farnesol seems to destroy the biofilm structure. It should be noted that above 100 μM farnesol effect is almost concentration and time independent.

Overall, the results indicate a potential antibacterial effect of farnesol against *S. epidermidis*, and therefore the possible action of this molecule on the prevention of *S. epidermidis* related infections. Additionally, the loss of cellular viability and consequently the loss of biofilm formation ability (main virulence factor) induced by farnesol suggest a potential use of this molecule in the prevention of *S. epidermidis* infection. This general promotion of biofilm

weakness may be a potential help to the human immune system to eradicate focus of *Staphylococcus epidermidis* infection.

Farnesol showed a combined effect with some antibiotics against planktonic cells although this was not so evident against biofilm cells. Despite the reduced efficacy against biofilm cells, farnesol seems to be a potential adjuvant therapeutic agent to antibiotics for the treatment of *S. epidermidis* systemic related infections. Moreover, its long PAE makes farnesol a promising candidate in the prevention of biofilm formation and to be used as an alternative to antibiotics since alone it also showed to be very effective against planktonic cells.

Although, NAC was not so effective against biofilm cells as on planktonic cells, this compound exhibited a high antibactericidal effect against the two *S. epidermidis* clinical isolates tested on both lifestyle. In the concentrations assayed NAC was more active against *S. epidermidis* cells than farnesol. Some synergy was evidencied by NAC at MIC concentration combined with farnesol in planktonic cells. However, when tested in biofilm cells generally no synergy was observed between NAC and farnesol. In summary, NAC at 40 mg mL^{-1} was the only of the tested treatments that was bactericidal against *S. epidermidis* cells both in planktonic or in biofilm form. Moreover, although NAC and farnesol have different modes of action, the combination of both has no significant synergistic effect.

Albeit none of the antibiotics tested have demonstrated an antimicrobial effect (log reduction ≥ 3) against all *S. epidermidis* strains biofilms, rifampicin alone was the most effective of the antibiotics tested. Rifampicin combined with clindamycin or with gentamicin showed to have the broadest range of action, considering the strains tested. Levofloxacin in combination with rifampicin displayed an antimicrobial effect against three strains but failed to attain a bactericidal action against the other two. Rifampicin is one of the constituents of almost all combinations active and presenting a high range of action against *S. epidermidis* strains tested. So, rifampicin should be part of any antibiotic therapy directed against *S. epidermidis* biofilms.
Comparatively to rifampicin alone, the cells after exposure to rifampicin+clindamycin and rifampicin+gentamicin exhibited a lower genetic expression of the two biofilm-genes studied, responsible for PNAG/PIA production, which lowers the virulence of persister cells, ensuring a more efficient control of *S. epidermidis* biofilms associated infections. This reinforces the potential use of combinatorial therapy as alternative to monotherapy.

In conclusion, there are some combinations of more traditional antibiotics that can be strongly considered as therapeutic strategies for an efficient control of *S. epidermidis* biofilms

associated infections. Moreover as an alternative to monotherapy, these combinations can be advantageous avoiding the likehood of resistance development. A point to have in mind is that *S. epidermidis* biofilm control is strongly strain dependent.

| WORK PERSPECTIVES

In this work, some new therapeutic strategies were studied *in vitro* and several points about have been clarified. Nevertheless, much work can still be done in this field.

| To complement the work already done

- To study the genetic expression of some genes of virulence of cells after being exposed to farnesol and NAC.

- To study the effect of farnesol produced by *Candida albicans* when growing with *S. epidermidis*. *S. epidermidis* can co-inhabit with *Candida albicans* forming mixed biofilms and causing polymicrobial infections involving catheters and orthopaedic prostheses. The study of the interspecies interactions and the possible competition between these two microorganisms and the effect of farnesol and other quorum-sensing molecules produced by *Candida albicans* on *S. epidermidis* cells would be interesting.

- *In vivo* assays testing the antimicrobial effect of farnesol and NAC. Although the antimicrobial activity of farnesol and NAC has been shown, additional studies involving animal models need to be performed to assess the potential effects of farnesol and NAC *in vivo*.

| OTHER THERAPEUTIC STRATEGIES

- Use of dispersin B in combination with antibiotics, farnesol and NAC.

Use of enzymes able to destroy the biofilm matrix, might also be assessed in combination with antibiotics or antimicrobial agents such as farnesol or NAC, thus aiming to contribute for new and improved therapies. One example is dispersin B, a glycoside hydrolase enzyme produced by the periodontal pathogen *Aggregatibacter actinomycetemcomitans*. Dispersin B catalyzes the hydrolysis of poly-*N*-acetylglucosamine, a sticky extracellular polysaccharide produced by various Gram-positive bacteria including *Staphylococcus epidermidis*.

- Test new antibiotics (antibiotics of novel generation) such as linezolid, tigecycline, daptomycin, etc. alone and in combination with farnesol and NAC.

- Test naturally occurring agents such as tobramycin (aminoglycoside); *melaleuca alternifolia* (tea tree) oil; two olive *(Olea europaea)* secoiridoides, oleuropein (the bitter principle of olives) and hydroxytyrosol (derived from oleuropein by enzymatic hydrolysis and responsible for the high stability of olive oil), etc. on *S. epidermidis* biofilms.

- Test the use of phage in the combat of *S. epidermidis*-related infections, by isolating anti-staphylococcal phages and testing the effectiveness of this possible antimicrobial therapy (phage therapy).

There is still a lot of work to be done.