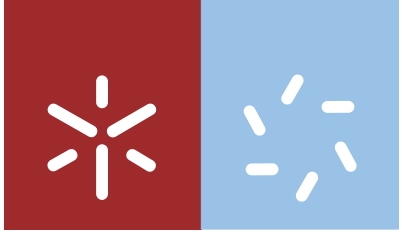
Universidade do Minho
Escola de Ciências

Nathalie de Sá Lopes

Quantification of biofilm-associated genes in *S. epidermidis* biofilms: its impact in biofilm formation and 3D structure

June 2014

Nathalie de Sá Lopes **Quantification of biofilm-associated genes in *S. epidermidis* biofilms: its impact in biofilm formation and 3D structure** UMinho|2014



Universidade do Minho
Escola de Ciências

Nathalie de Sá Lopes

Quantification of biofilm-associated genes in *S. epidermidis* biofilms: its impact in biofilm formation and 3D structure

Master Thesis
Master Degree in Molecular Genetics

Work conducted under the supervision of
Doutor Nuno Miguel Dias Cerca
and co-supervision of
Doutor Björn Fredrik Johansson

June 2014

Nome: Nathalie de Sá Lopes

Endereço electrónico: nat_lopes90@hotmail.com

Número do Bilhete de Identidade: 13708123

Título dissertação:

Quantification of biofilm-associated genes in *Staphylococcus epidermidis* biofilms: its impact in biofilm formation and 3D structure

Orientadores:

Doutor Nuno Miguel Dias Cerca

Doutor Björn Fredrik Johansson

Ano de conclusão: 2014

Designação do Mestrado:

Mestrado em Genética Molecular

Universidade do Minho, ____/____/_____

Assinatura: _____

ACKNOWLEDGMENTS

É com muita satisfação que expresso neste pequeno espaço o mais profundo agradecimento a todas as pessoas que contribuíram direta ou indiretamente na realização da minha dissertação de Mestrado.

Em primeiro lugar começo por agradecer ao meu orientador, Doutor Nuno Cerca, pela oportunidade que me deu para participar neste projeto, pelos conhecimentos transmitidos, acompanhamento e disponibilidade ao longo deste percurso. Quero também agradecer pela exigência continua, de forma a preparar me o melhor possível para qualquer obstáculo que possa surgir no meu percurso cientifico, tornando-me mais dinâmica, confiante, autónoma e autocritica.

Aos elementos pertencentes ao grupo do Nuno Cerca pela forma como me integraram, pela disponibilidade e ajuda prestada, pelo bom ambiente e convívio. Agradeço de uma forma especial ao Fernando Oliveira, pela disponibilidade em ajudar-me a iniciar o meu projeto, pelos conhecimentos transmitidos e pela confiança que depositou em mim, por toda a dedicação e troca de ideias ao longo do meu trabalho, e obviamente pelo companheirismo, paciência e momentos de descontração. À Ana Isabel pela partilha de conhecimentos, pelo auxílio e acompanhamento neste projeto, pela boa disposição e encorajamento, e principalmente pela oportunidade que me ofereceu para colaborar em outro projeto, contribuindo para a minha evolução cientifica. À Cármen Sousa que me deu a conhecer este projeto e o meu orientador, pelo apoio e ajuda prestada durante este percurso, pelos momentos de descontração e convivência. Aos restantes membros deste grupo, que fui conhecendo ao longo deste pequeno percurso, pela boa disposição, espirito de equipa e convívio.

A todos os colegas de laboratório pelo companheirismo, disponibilidade prestada, ótimo ambiente de trabalho e convívio.

Agradeço a todos os meus amigos, que apesar de estarem distantes, me acompanharam e apoiaram durante todo o meu Mestrado, e pelos momentos especiais que me proporcionaram. À Joana Guedes, a minha colega de quarto e amiga, pela paciência e apoio, pela integração no seu grupo de amigas e pelos momentos fantásticos que todas passamos ao longo destes dois anos de Mestrado.

Por fim, agradeço de forma muito especial aos meus Pais e Irmão por todo o apoio, incentivo, dedicação, carinho e confiança depositada em mim, dando me a oportunidade de seguir e alcançar os meus objetivos.

This thesis was financed by the FCT Strategic Project PEst-OE/EQB/LA0023/2013 and the Project "BioHealth - Biotechnology and Bioengineering approaches to improve health quality'', Ref. NORTE-07-0124-FEDER-000027, co-funded by the Programa Operacional Regional do Norte (ON.2 – O Novo Norte), QREN, FEDER. I also acknowledge the project "Consolidating Research Expertise and Resources on Cellular and Molecular Biotechnology at CEB/IBB", Ref. FCOMP-01-0124-FEDER-027462 and the FCT grant SFRH/BD/66166/2009.

Quantification of biofilm-associated genes in *Staphylococcus epidermidis* biofilms: its impact in biofilm formation and 3D structure

ABSTRACT

Staphylococcus epidermidis is a common commensal coloniser of the human skin and is currently the most frequent cause of biomaterial associated infections. Several studies have attempted to identify the determinants that distinguish invasive from commensals *S. epidermidis* strains. Its pathogenesis is directly related to its ability to establish multi-layered and highly structured biofilms, resistant to antimicrobial agents. This bacteria expresses several protein factors that are responsible for the development of the biofilm, including the contribution of specific factors (*icaA*, *aap* and *bhp* genes) in the accumulation phase. In the last years, several research groups have been trying to understand the contribution of biofilm-associated genes involved in biofilm formation. Therefore, the aim of this thesis was to analyse the gene expression of *icaA*, *aap* and *bhp* and compare with the formation of the biofilm structure. Two *S. epidermidis* strains, one isolated from a hospital environment and another from the skin of a healthy person were characterized at the level of biofilm formation, at different times of incubation. According to our results, both strains demonstrated an increase of biomass production over time, revealing the importance to use screening assays with more than 24 h of incubation. A biofilm structure analysis was also performed to detect the presence of poly-N-acetylglucosamine (PNAG), the major component of *S. epidermidis* biofilm matrix. The results demonstrated a higher production of PNAG only after 48 h for SECOMO034.A1. Due to the low sensitivity of the method or low quantity of proteins produced, it was not possible to determine the concentration of proteins in the biofilm matrix. Finally, the gene expression at two different biofilm formation times were determined, confirming the importance of the *icaA* gene in the accumulation stage, explaining the high production of biomass and PNAG. On the other hand, the *aap* and *bhp* expression levels raised some questions about their role in the biofilm process.

Quantificação dos genes associados à formação do biofilme de *Staphylococcus epidermidis*: o seu impacto na formação do biofilme e na estrutura 3D

RESUMO

Staphylococcus epidermidis é um colonizador comensal comum da pele humana e é atualmente a causa mais frequente de infeções associadas a biomateriais. Vários estudos têm tentado identificar os fatores determinantes que diferenciam as estirpes invasivas de *S. epidermidis* das comensais. A patogenicidade desta bactéria está diretamente relacionada com a sua capacidade de formar biofilmes altamente estruturados e resistentes a agentes antimicrobianos. Esta bactéria expressa diversos fatores que são responsáveis pelo desenvolvimento do biofilme, incluindo fatores específicos (genes *icaA*, *aap* e *bhp*) na fase de acumulação. Nos últimos anos, vários grupos de investigação têm tentado compreender a contribuição dos genes que estão envolvidos na formação do biofilme. O objetivo desta dissertação consistiu na análise da expressão dos genes *icaA*, *aap* e *bhp* e sua comparação com a formação e a estrutura do biofilme. Duas estirpes de *S. epidermidis*, uma isolada de um ambiente hospitalar e outra a partir de uma pessoa saudável, foram caracterizadas ao nível da formação de biofilme, a diferentes tempos de incubação. De acordo com os nossos resultados, ambas as estirpes demonstraram um aumento de produção de biomassa ao longo do tempo, revelando a importância de utilizar ensaios de rastreio com mais de 24 h de incubação. Uma análise da estrutura do biofilme também foi realizada para detetar a presença de poly-N-acetilglucosamina (PNAG), o componente principal da matriz de biofilmes de *S. epidermidis*. Os resultados demonstraram uma elevada produção de PNAG somente após 48 h, na estirpe SECOMO034.A1. Em conjunto, também se tentou visualizar as proteínas extra-celulares na matriz do biofilme. Contudo, não foi possível esta análise, provavelmente devido à baixa sensibilidade do método. Por fim, foi determinada a expressão dos genes a dois tempos de formação de biofilme diferentes, confirmando a importância do gene *icaA* na fase de acumulação e explicando a elevada produção de biomassa e PNAG. Por outro lado, os níveis de expressão dos genes *aap* e *bhp* não foram claramente associados à crescente acumulação de biofilme ao longo do tempo.

TABLE OF CONTENTS

LIST OF ABREVIATIONS

Aae	Adhesion protein
Aap	Accumulation-associated protein
Agr	Accessory gene regulator
AIP	Autoinducing peptide
AMP	Antimicrobial protein
Atle	Autolysin protein
BAIs	Biomaterial-associated infections
Bap	Biofilm-associated protein
Bhp	Bap homologue protein
CoNS	Coagulase-negative Staphylococci
CVCs	Central intravenous catheters
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
eDNA	Extracellular DNA
EDTA	Ethylenediaminetetraacetic acid
FAME	Fatty acid modifying enzyme
GlcNAc	N-acetylglucosamine
HGSA	Hospital Geral de Santo António
MSCRAMM	Microbial surface components recognizing adhesive matrix molecules
NaCl	Sodium chloride
OD	Optical density
PCR	Polymerase chain reaction
PGA	Poly-γ-glutamic acid

PIA	Polysaccharide intercellular adhesion
PNAG	Poly-N-acetylglucosamine
PSM	Phenol Modulins
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
SCCmec	Staphylococcal cassete chromosome mec
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSBg	Tryptic soy broth with 0.4% extra glucose
WGA	Wheat germ agglutinin

LIST OF FIGURES

LIST OF TABLES

CHAPTER 1

INTRODUCTION

1.1 Staphylococci

Staphylococci represent a group of bacteria found in human normal microflora and other animals and are divided into the coagulase-positive (e.g. *Staphylococcus aureus*) and coagulase-negative species (Roth and James, 1988), according to the presence or absence of the coagulase enzyme, respectively (Wang *et al.,* 2003). Kloos and Musselwhite (1975) defined through quantitative studies, that the staphylococci group comprises 50% of bacterial isolates from the head, nares and axillae and 10 to 70% isolates from legs and arms (Kloos and Musselwhite, 1975). Coagulase-negative staphylococci (CoNS) are a group of bacteria that commensally inhabit the human skin and mucous membrane (Roth and James, 1988). They belong to the genus *Staphylococcus* with approximately 39 species and 21 subspecies (Euzeby, 2013; Tille, 2013). The microorganisms that belong to this genus are Gram-positive cocci (0.5 to 1.5 μm of diameter), non-motile, non-spore forming and generally unencapsulated (Götz *et al.*, 2006; Tille, 2013). Most species are also facultative anaerobes, catalase positive and oxidase negative (Götz *et al.*, 2006). CoNS were divided into two groups according to their resistance or susceptibility to novobiocin. The novobiocin susceptible species include *S. epidermidis*, *S. haemolyticus*, *S. hominis*, *S. lugdunensis* and *S. schleiferi* while the novobiobin resistant species are *S. saprophyticus* and *S. xylosus* (von Eiff *et al.,* 2002).

1.2. CoNS role in skin colonization and nosocomial infections

The human skin microflora is colonized by a large amount of microorganisms that live harmlessly as commensals on the skin surface. CoNS species make part of this group of organism (Roth and James, 1988) and skin colonization by CoNS may have a crucial role in normal human skin microflora maintenance, inhibiting the colonization and enhancing the killing of other pathogenic microorganism (Otto, 2009).

Initially CoNS were considered non-pathogenic being dismissed as culture contaminants (Eng *et al.*, 1982). However, several years later CoNS were identified as the causative of various nosocomial infections (Dandalides *et al.,* 1986; von Eiff *et al.,* 2001). Subsequently the distinction among contaminating (commensal) and clinical significant CoNS isolates (invasive) became a major challenge (O'Gara and Humphreys, 2001). Therefore the development of effective methods to properly differentiate commensal from invasive isolates is critical (Gu *et al.,* 2005). CoNS infections (including *S. epidermidis*) arise usually in immunecompromised host including human immunodeficiency virus

positive (HIV-positive) patients, premature newborns, intravenous drugs abusers and patients with malignant diseases or under immune-suppressive therapy (Longauerova, 2006). In situations of trauma, inoculation or implantation of medical devices, *S. epidermidis* can also influence healthy patients after penetrating the skin barrier or the mucous membranes (Otto, 2008). Clinical manifestations of CoNS infections generally are subtle and non-specific and can be considered subacute or even chronic. Different infection symptoms can arise depending on the device implanted, place of insertion and on the patients conditions (von Eiff *et al.,* 2002).

Therefore, the high incidence of these infections is generally associated with the developments in medicine namely the implantation of biomedical devices. Consequently, the risks of developing serious diseases has raised, affecting the quality of life and increasing the mortality rate (Mack *et al.,* 2013). CoNS, especially *S. epidermidis*, have been considered the most frequently microorganisms linked to nosocomial infections (Table 1.1) (Vuong and Otto, 2002). Among the nosocomial infections causes, biomaterial-associated infections (BAIs) can reach one million cases per year (Darouiche, 2004). *S. epidermidis* is responsible for approximately 90% of the infections related with artificial joints (Ehrlich *et al.,* 2004).

Table 1.1 Prevalence of CoNS and *S. epidermidis* in BAIs.

INFECTIONS (PERCENTAGE)	REFERENCE
CoNS	
Endocartis	(Lalani *et al.*, 2006; Wang *et al.,* 2007; Murdoch *et al.,* 2009)
- Prosthetic valve infections (17%; 15%-40%) - Intracardiac devices (26%)	(Murdoch *et al.*, 2009)
Catheter related infections (50-70%)	(O'Gara and Humphreys, 2001)
Joint replacement infections (20-50%)	(O'Gara and Humphreys, 2001)
Cardiac pacemaker infections (25%)	(Rogers *et al.,* 2009)
S. epidermidis	
Bloodstream infections	(Simon *et al.,* 2005; Gordon *et al.,* 2006)
- Cardiac assist devices (38%) - Intravascular devices (87%)	(Dufour *et al.,* 2012)
Catheter-related infections (50-70%)	(von Eiff *et al.,* 2002)
Prosthetic valve infection (82%)	(Chu *et al.,* 2009)
Urinary tract infections (95%)	(Dufour *et al.,* 2012)

The medical devices related to the BAIs include intravascular, cardiovascular, neurosurgical, urological, orthopaedic and dental devices (von Eiff *et al.,* 2005). There are different types of devices: intravascular devices, that interact with coagulation factors and circulating blood cells; and extravascular devices, which interact with the adjacent tissue, interstitial fluid and attracted phagocytes (Zimmerli and Trampuz, 2013). These devices may contain abiotic material, such as metals or polymers and some of them may also contain biological materials, e.g. blood vessels, allogenic or xenogeneic sources (Anderson *et al.,* 2008; Anderson and McNally, 2011). The majority of the medical devices must be removed to treat the local infection, following a frequent surveillance after the new device implantation (Brooks *et al.,* 2013).

1.3. *S. epidermidis* infections, pathogenesis and virulence factors

S. epidermidis is the most common organisms responsible for nosocomial infections, as mentioned above. The infections caused by this bacterium normally occur when the integrity of the skin barrier is disturbed by the insertion of medical devices. Its pathogenesis is mainly linked with the biofilm formation in the surface of medical devices (Vuong and Otto, 2002) such as central intravenous catheters (CVCs), prosthetic joints, cardiac pacemakers, heart valves and vascular grafts (Table 1.2) (Rogers *et al.,* 2009). Besides biofilms, *S. epidermidis* pathogenesis is also related to the capacity of antibiotic resistance (Rogers *et al.,* 2009) and to the ability to avoid the immune system response (Kong *et al.,* 2006).

Table 1.2 Examples of nosocomial infections associated with *S. epidermidis* biofilms (adapted from Costerton *et al.,* 1999) .

NOSOCOMIAL INFECTIONS	BACTERIAL SPECIES
Arteriovenous shunts	*S. epidermidis* and *S. aureus*
Central venous catheters	*S. epidermidis* and others
Hickman catheters	*S. epidermidis* and *C. albicans*
Mechanical heart valves	*S. aureus* and *S. epidermidis*
Orthopedic devices	*S. aureus* and *S. epidermidis*
Penile prostheses	*S. aureus* and *S. epidermidis*

The most studied *S. epidermidis* virulence factors are mainly related to the components involved in the biofilm formation process, namely factors contributing in the adhesion, intercellular aggregation and

dissembling (Otto, 2004). Although, certain factors are found on both clinical and commensal isolates thus demonstrating also a role in the commensal lifestyle and evidencing that CoNS are indeed "accidental pathogens" (Rohde *et al.,* 2004; Kocianova *et al.,* 2005). The majority of *S. epidermidis* virulent factors have a role during its commensal lifestyle such as poly-N-acetylglucosamine (PNAG), poly-γ-glutamic acid (PGA) and metalloprotease of *S. epidermidis* (SepA) protease, which are responsible for the protection of the bacteria from antimicrobial proteins (AMPs), where AMPs are a major determinant of host immune system (Vuong *et al.,* 2004a; Kocianova *et al.,* 2005; Lai *et al.,* 2007). The presence of these factors on both lifestyles of *S. epidermidis* further complicates the distinction between commensal and invasive isolates. The production of lipases, proteases, toxins and other exoenzymes contribute to the persistence of *S. epidermidis* in the organism and possibly also in degradation of host tissue, thus being associated to bacterial virulence (Otto, 2004). In Table 1.3 are represented some of the virulence factors of *S. epidermidis.*

Table 1.3 *Staphylococcus* virulence factors and functions (adapted from Longauerova *et al.,* 2006 and Otto, 2012).

VIRULENCE FACTOR	GENE	FUNCTION
Protective exopolymers		
PNAG	*icaA, icaD, icaB* and *icaC*	
PGA	*capA, capB, capC* and *capD*	
Resistant to AMPs		
SepA protease	*sepA*	
Aps system	*apsR, apsS* and *apsX*	
Exoenzymes		
Lipases	*gehC* and *gehD*	Persistence in fatty acid secretions
Cystein proteases	*sspB*	Possibly tissue damage
Serin protease	*sspA*	Degradation of fibrinogen
FAME	unknown	Detoxification of bactericidal fatty acid
Metaloproteinase	*sepA*	Involved in lipase maturation, AMP resistance and, potentially, tissue damage
Other factors		
Staphyloferrins A and B	*sfna locus*	Siderophores involved in iron acquisition
SitA, SitB and SitC	*sitA, sitB* and *sitC*	Involved in iron uptake
Peptidoglycan/lipoteichoic acid	*tagF, femA* and others	

AMP, antimicrobial protein; FAME, fatty acid modifying enzyme; PGA, poly-γ-glutamic acid; PNAG, poly-N-acetylglucosamine

1.4. Biofilm Formation

Bacterial species, until a few years ago, were characterized as an independent unicellular organism, without the capacity to communicate or interact with equal species in the vicinity (Shapiro, 1988). Further observations, revealed that bacterial species eventually lived within a dynamic structure, so-called biofilm, appearing to have a similar behaviour as multicellular organism (Costerton *et al.,* 1999). Subsequently, the organization of bacterial species in biofilms was defined by Costerton *et al.* (1999) as protective mode of growth by forming a complex agglomerate of adherent multiple microbial species enclosed in a polymeric matrix.

The ability of bacteria to adhere to medical devices and form a complex biofilm are the main causes of the BAIs. However studies revealed that most of bacterial species (approximately 99%) live naturally organized in biofilms, indicating that biofilms are not always definitely linked to infections (Costerton *et al.,* 1987; Dalton and March, 1998). The development of the biofilm matrix increases the antibiotic resistance when compared with the planktonic bacteria (free-floating bacteria) (Gilbert *et al.,* 1990; Smith, 2005), as well as the capacity to escape the host immune response (Cerca *et al.,* 2006). The poor efficiency of antimicrobial antibiotics can be explain due a notable adaptation to the biofilm growth by downregulation of basic cell processes and upregulation of numerous factors that are involved in resistance and defensive mechanisms (Yao *et al.,* 2005). In addition, the planktonic bacteria have also a higher growth rate, as compared with the microorganism encased in the biofilm (Cerca *et al.,* 2005). Since microorganism usually live in environments with low nutrients concentrations, therefore it is more advantageous to the bacteria to grow in biofilms (Cerca and Jefferson, 2012).

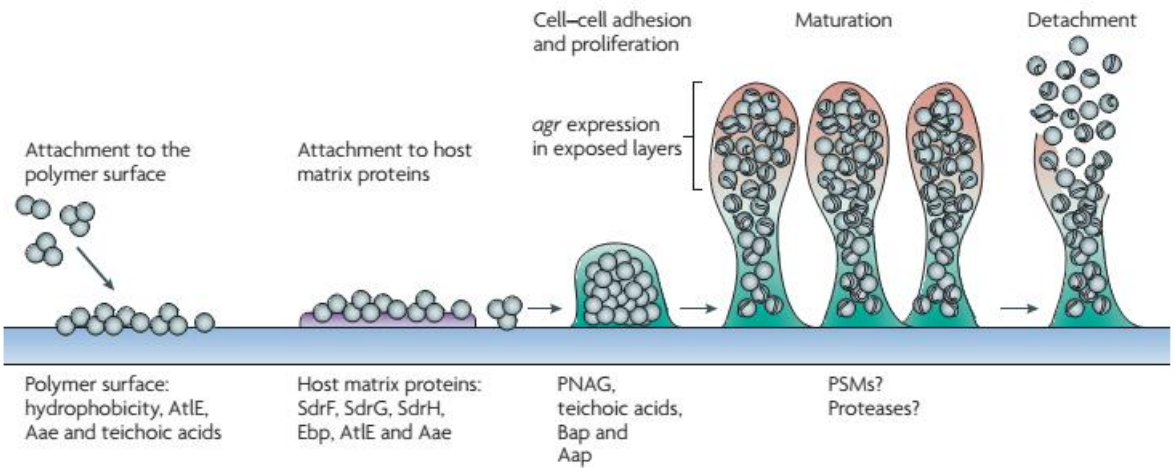


Figure 1.1 *Staphylococcus epidermidis* biofilm formation stages (adapted from Otto, 2009).

S. epidermidis biofilm formation follows four distinct main phases (Fig. 1.1). Initially the planktonic cells attach to the medical device surface and then the bacteria begin to accumulate, forming a biofilm with multiple layers, following the growth and maturation of the biofilm. The final stage of the biofilm cycle is the detachment of single cells or cells clusters which are responsible for the development of new biofilms (Rohde *et al.,* 2006).

1.4.1. Initial attachment

Initial attachment of bacterial planktonic cells into the devices surface is the most crucial stage to induce the BAIs. The surface condition of the medical devices influences the biofilm formation, existing two types of adhesion. The adhesion can occur right after the implantation (early stage – adhesion to abiotic surfaces) or later, reaching even months after the implantation (late stage – adhesion to biotic surfaces) (Dunne, 2002). In later stages, the device surface has already suffered several alterations forming the conditioning film, composed with proteinaceous macromolecules components of body fluids (e.g. blood, urine, saliva or mucous). Each macromolecular component has a specific role that differs according to the organism involved and to the type of tissue cell (Gristina, 1987; Choong and Whitfield, 2000). The attachment of bacteria to abiotic surfaces occurs directly to native polymers surface and requires also the intervention of several physiochemical variables, such as hydrophobic and electrostatic interactions, Van der Waals forces, surface tension, stearic hindrance and temperature, to enhance the planktonic cells attachment (Dunne, 2002; Longauerova, 2006).

Among the two mechanisms that initiate the biofilm formation, the adhesion to biotic surfaces is more important. In order to reach an effective adhesion through this mechanism, a vast group of surface proteins, so called microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Table 1.4), are associated and expressed by *S. epidermidis* (Otto, 2009).

The adhesion of *S. epidermidis* to abiotic surfaces is more specific and is normally mediated by hydrophobic interactions (Vacheethasanee *et al.,* 1998). In case of abiotic surfaces, various bacterial surface structures and proteins are involved in the adhesion, including autolysin protein (atlE) (Heilmann *et al.,* 1997), adhesion protein (aae) (Heilmann *et al.,* 2003) and teichoic acids (Schoenfelder *et al.,* 2010). Moreover the adhesion to biotic surfaces is mediated by cell wall-associated proteins, from *S. epidermidis,* that will interact with host extracellular matrix proteins such as collagen, fibrinogen, fibronectin and vitronectin (Rohde *et al.,* 2010). The cell wall-associated proteins involved in this process are fibrinogen-binding protein (Fbe/SdrG) (Davis *et al.,* 2001), SdrF

(Arrecubieta *et al.,* 2007), fibronectin-binding protein (Embp) (Williams *et al.,* 2002), GehD (Bowden *et al.,* 2002) and other surface proteins of *S. epidermidis*.

Table 1.4 *S. epidermidis* factors involved in biofilm formation (adapted from McCann *et al.*, 2008 and Otto, 2009).

	FACTOR	FUNCTION
Initial attachment		
Adhesion to abiotic surfaces	AtlE/Aae*	Autolysin and adhesion: binds to fibrinogen, fibronectin and vitronectin; promotes binding to polystyrene surfaces
	Teichoic acids*	Interacts with immobilized fibronectin
Adhesion to biotic surfaces	Fbe (SdrG)*	Binds to fibrinogen
(MSCRAMMs)	SdrF*	Binds to collagen
	Embp*	Binds to fibronectin
	GehD*	Binds to collagen
Accumulation	PIA*	Forms a polysaccharide extracellular biofilm matrix
	Aap*	Mediates PIA-independent biofilms
	Bap/Bhp*	Mediates PIA-independent biofilms
	SarA	Regulate PIA and *ica* gene
	SarZ	
	σ^B	
	LuxS	
Detachment	Agr	Quorum sensing system; controls production of enzymes and cell-cell communication
	PSMs*	α-type, β-type, δ-toxin and PSMδ; proinflamatory properties

* - also considered as virulent factors

1.4.2. Biofilm accumulation

The accumulation stage is characterized by a significant increase of adhered bacterial cells into the devices surface, following proliferation and accumulation of bacterial species. An extensive network of multi-layered cellular clusters is developed, representing the extracellular matrix. The biofilm biomass is generally composed with 80 to 90% of matrix material (extracellular polymeric substance) and 10 to 20% of microbial cells (Kokare *et al.,* 2009). The extracellular matrix usually comprises proteins, polysaccharides, extracellular DNA (eDNA) and apparently host factors (Izano *et al.,* 2008; Boles and Horswill, 2011), providing a complete protection from any mechanism capable of interfering with its

development, like response of the immune system or the action of the antimicrobial agents (Mah and O'Toole, 2001; Qin *et al.,* 2007). The conformation of the extracellular matrix can differ, depending on the *staphylococcus* strains and on the microenvironment conditions (Boles and Horswill, 2011). Normally strains that form more robust biofilms are composed mainly with polysaccharides while strains forming more weak biofilms are mostly composed with proteins and eDNA (Rohde *et al.,* 2007; Izano *et al.,* 2008; Boles and Horswill, 2011).

In *S. epidermidis* biofilms, the accumulation phase is accomplished by the production of factors that mediates the intercellular adhesion. The main factor regulating this process is the polysaccharide intercellular adhesion (PIA) (Mack *et al.,* 1996) also recognized as poly-N-acetylglucosamine (PNAG) (Maira-Litran *et al.,* 2002). PIA is a linear ß-1,6-linked glucosaminoglycan, possessing two charges: negative charges from O-succinylation and positive charges due to partial de-N-acetylation (Rohde *et al.,* 2010). It is synthesized through enzymes encoded by the ica operon (Mack *et al.,* 1996).

In the *ica* locus is represented the structural genes required for the PIA synthesis. This operon comprises four reading frames (*icaA*, *icaB*, *icaC* and *icaD*) and a fifth gene (*icaR*) located upstream of *icaA* (Fig. 1.2a and b), responsible for the regulation of *icaADBC* expression and also of *S. epidermidis* biofilm formation (Conlon *et al.,* 2002). Each reading frame has a particular function on the PIA synthesis. IcaA and IcaD are membrane proteins whose function is to produce a chain of N-acetylglucosamine (GlcNAc) monomers from UDP-GlcNAc, where IcaA represents the catalytic enzyme and requires IcaD for full activity. IcaC is also a membrane protein and is responsible for the elongation and carriage of the chain through the cytoplasmatic membrane (Gerke *et al.,* 1998). Once exported IcaB located in the cell surface, deacetylates the GlcNAc residues giving cationic charges that are essential for the surface binding of PIA supporting also biofilm formation, surface colonization and avoiding host immune response (Vuong *et al.,* 2004a).

Several factors are involved on the regulation of *ica* gene expression, thus in PIA synthesis, highlighting SarA, sigmaB (σβ) (Handke *et al.,* 2007), SarZ (Wang *et al.,* 2008) and LuxS (Fig. 1.2b) (Xu *et al.,* 2006). The quorum sensing system luxS is known to repress *ica* transcription and consequently decrease biofilm formation (Xu *et al.,* 2006), while SarA and sigmaB regulatory proteins up-regulate *ica* transcription (Tormo *et al.,* 2005a; Handke *et al.,* 2007).

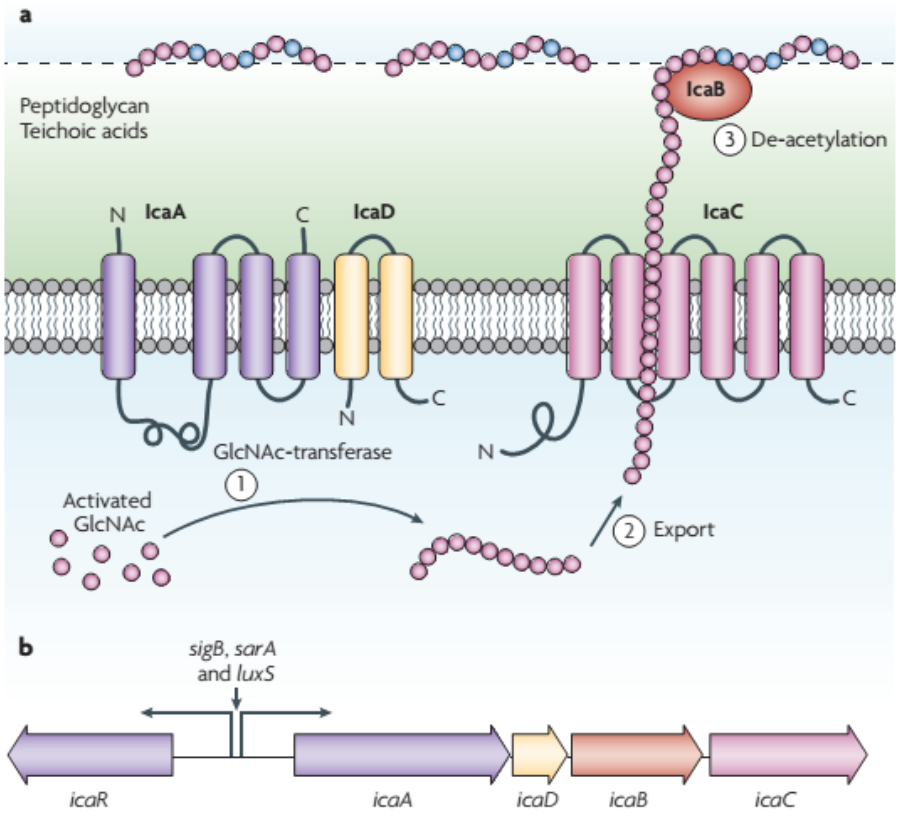


Figure 1.2 Polysaccharide intercellular adhesin synthesis: **(a)** *icaADBC gene* structure and role in PIA synthesis; **(b)** genetic organization of *icaADBC gene* and PIA regulator factors of *Staphylococcus epidermidis* (adapted from Otto, 2009).

Furthermore, the presence of environmental growth conditions such as sub-inhibitory concentrations of antibiotics, anaerobic growth conditions, high temperatures or osmolarity may enhance the PIA function in the biofilm formation (Boles and Horswill, 2011).

Besides *ica* gene, additional genes were considered to be involved in *S. epidermidis* biofilm formation. Studies demonstrated that some *S. epidermidis* strains are *icaADBC*-negative thus without the ability to produce PIA, but are able to developing PIA-independent biofilms. PIA-independent biofilms use alternative mechanism to mediate the accumulation process by the presence of additional intercellular adhesins such as accumulation associated protein (Aap) (Rohde *et al.,* 2005) and biofilm associated protein (Bap/Bhp) (Tormo *et al.*, 2005b).

Aap is a 220-kDa fibrillar protein anchored via a LPXTG motif, processed by both bacterial and host proteases and is implicated in *S. epidermidis* biofilms as a putative cell wall receptor for PIA (Hussain *et al.,* 1997; Mack, 1999). A smaller length of Aap protein (≈140-kDa) is required to induce biofilm formation (Hussain *et al.,* 1997). In addition Aap may also be exogenously activated by adding

granulocyte proteases (Rohde *et al.,* 2005). The mechanism responsible for regulating Aap expression is not completely understood. Aap contain two domains: a) an N-terminal A domain, which may also have a lectin-like domain to mediate adherence to skin and b) a B domain, with a variable number of 128bp amino acid repeats (Bowden *et al.,* 2005). Domain B repeats integrate G5 domains, which are zinc (Zn^{2+})-dependent, with the affinity to incorporate 2-3 Zn^{2+} ions (Conrady *et al.,* 2008). The deletion of Aap domain A results in domain B exposure, providing intercellular adhesion properties and, consequently, biofilm accumulation. This study enhances the role of domain B in intercellular adhesion and biofilm process demonstrating evidences that domain A is not specifically required to promote *S. epidermidis* biofilms formation (Rohde *et al.,* 2005). The domain A seems to have role in skin colonization by mediating the adhesion to corneocytes (Macintosh *et al.,* 2009), revealing that Aap is a bifunctional molecule important for both commensal and pathogenic *S. epidermidis* lifestyles. Since Aap has the capacity to mediate the intercellular adhesion, in a PIA-negative background, but also function as a putative cell wall receptor for PIA, leading to biofilm accumulation, it was attributed a bimodal role to this protein in *S. epidermidis* biofilm accumulation (Rohde *et al.,* 2005).

Studies of Aap expression during different *S. epidermidis* biofilms stages have been performed. They revealed the role of this gene in early stages of biofilm formation, since in later stages a downregulation of the expression was observed (Vandecasteele *et al.,* 2003), emphasising the idea that Aap is mainly essential in the accumulation phase rather than maintaining the biofilm development.

Another surface protein, Bap, is also thought to be involved in PIA-independent biofilms. It was initially found in bovine mastitis *S. aureus* isolates (Cucarella *et al.*, 2001) and lately detected in the genome of *S. epidermidis* (Tormo *et al.,* 2005b). The *bap* gene present in *S. epidermidis* has 8226bp, encoding a protein with 2742 amino acids residues and approximately 284.4 kDa of molecular mass. The protein contains an N-terminal signal sequence with two domains (A and B), for extracellular secretion and a C-domain, composed by 16 tandem repeat units with 86 amino acids each. The C-terminal sequence integrates a LPXTG motif (Cucarella *et al.,* 2001; Tormo *et al.,* 2005b). In *S. epidermidis* isolates from humans, a similar protein was discovered and named as Bap homologue protein (Bhp). Bhp composition is basically identical to Bap protein (Fig. 1.3) having also an N-terminal signal sequence and a putative carboxy-terminal segment with an LPXTG motif. In addition, it also contains a hydrophobic membrane-spanning domain and sequences of positively charged residues (Tormo *et al.,* 2005b). However, further studies are necessary to gain access the role of Bhp protein in *S. epidermidis* biofilm formation, the mechanism that regulates its expression and also its pathogenicity towards BAIs.

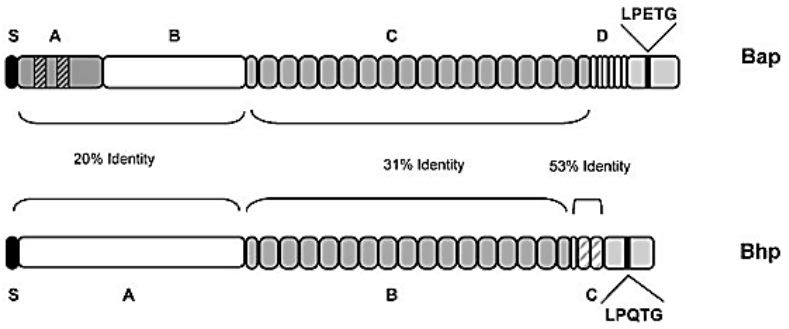


Figure 1.3 Structure similarities between Bap and Bhp proteins in *S. epidermidis* strains (adapted from Tormo *et al.,* 2005).

Among these two main proteins, linked with biofilm accumulation in PIA-independent isolates, Aap is encoded in about 90% of *S. epidermidis* isolates while Bhp is found in only 15 to 45% isolates, however these estimations may not be precise depending on the study performed (Rohde *et al.,* 2007; Fey and Olson, 2010).

1.4.3. Maturation

Once bacteria attach and accumulate on the devices surface, the biofilm begins to form a more dynamic and robust structure. The maturation consists on the generation of a slime glycocalyx to encase bacterial species linked in the surface, establishing a tree-dimensional structure with representative mushroom-like cells appearance nearby the fluid-filled channels (Dunne, 2002). The glycocalyx appears to increase the stability of the biofilm structure, thus influencing the BAIs treatment with antimicrobial agents and even blocking the host immune response (Vuong *et al.,* 2004b; Patel *et al.,* 2007). The channels represent an optimum hydrodynamic flow, intended to deliver all the conditions necessary to improve the growth potential, including nutrients, oxygen and also enable the removal of metabolic waste. Moreover, internal pH, carbon source and osmolarity likewise regulate the progression of biofilm maturation (Dunne, 2002). A mature biofilm is characterized by a set of layers: the main bulk, a linking film, a conditioning film and the surface where the bacterial species initially attached (Fig. 1.4) (Habash and Reid, 1999).

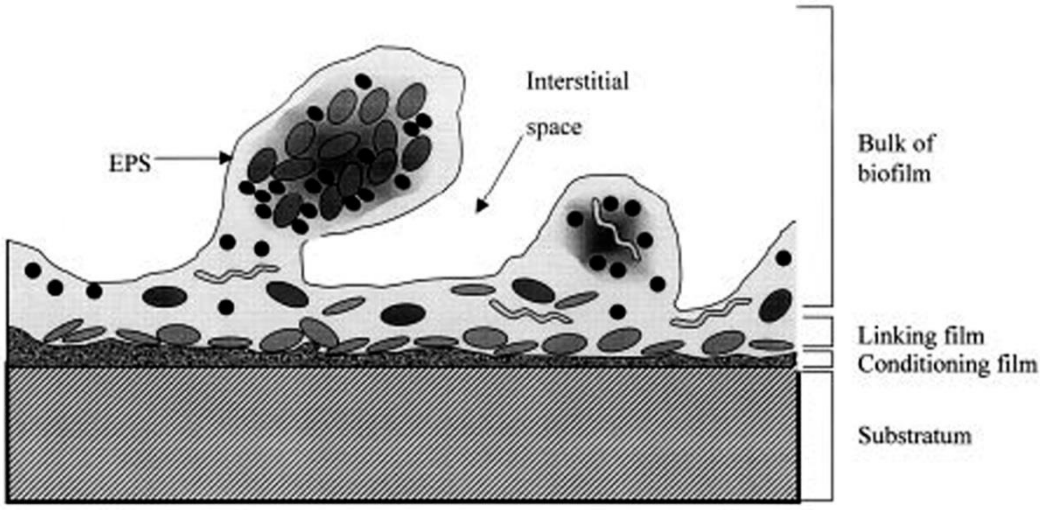


Figure 1.4 Mature biofilm structure representing the different layers (adapted from Habash and Reid, 1999).

Yao *et al.* (2005), and other authors, believed that the characteristics and behaviours of both planktonic cells and bacterial cells incorporated within the biofilm matrix were significantly distinct. Several factors evidenced this hypothesis: a) adjust their physiology to anaerobic metabolism, b) downregulate protein, and cell wall and DNA synthesis (Yao *et al.,* 2005) and c) have spatial and temporal response according to their specific environment (Stewart and Franklin, 2008). In particular *S. epidermidis* cells living inside the biofilm bulk can be at four different physiological states (aerobic, anaerobic, dormant cells and dead cells) and these metabolic conditions may contribute for antibiotic resistant (Rani *et al.,* 2007).

1.4.4. Disassembly

In the detachment stage, a dynamic equilibrium is achieved and individual cells or cells aggregates dissipate from the superior biofilm layer (bulk of biofilm) colonizing and inducing the establishment of a new biofilm in other sites or organs (Yao *et al.,* 2005). Detachment of biofilm cells may implicate the degradation of biofilm extracellular matrix and some physiological modifications, to promote bacterial cells adaptation to the external conditions. In order to break through the biofilm matrix, *S. epidermidis* produce a group of extracellular enzymes, or surfactants, such as proteases and DNases (Boles and Horswill, 2011).

A regulatory system is necessary to control all the processes during the biofilm formation phases, including the production of enzymes, cell-cell communication and other mechanisms. The quorum sensing system is responsible for that regulation and is encoded by an accessory gene regulator (agr) system, which is activated during the transition from exponential to stationary phase, i.e., when the mature biofilm has achieved a state of equilibrium (Arciola *et al.,* 2012). *Agr* gene contains two transcription units (RNAII and RNAIII) that are regulated by their specific promoters (P2 and P3) (Novick *et al.,* 1993). The transcription unit RNAII comprises four genes: AgrA and AgrC, assembled forming a transmembrane transduction complex (Lina *et al.,* 1998), AgrD, a pro-signalling peptide and AgrB, a membrane component, whose function consist on the exportation of post-translationally modified signalling peptide (Kong *et al.,* 2006). RNAIII is also an effector molecule of agr system, controlling the transcription of target genes, such as virulence factors (Mayville *et al.,* 1999; Thoendel *et al.,* 2011). The activation of the quorum sensing agr system is obtained by a signalling molecule named autoinducing peptide (AIP), produced by AgrD, that is released from the biofilm to signalize the system (Kong *et al.,* 2006). After reaching a critical threshold concentration, AIP activates a two-component signal transduction cascade (AgrC and then AgrA) promoting the production of virulent factors (Fig. 1.5) (Boles and Horswill, 2011).

The agr system in *S. epidermidis* enhances the biofilm detachment, spread and also contributes their virulence capacity by producing multiple-proteases and small forming toxins named as phenol modulins (PSMs) (Otto *et al.,* 2004). PSMs are compounds of amphiphilic peptides, with inflammatory properties and are subdivided in different types: α-type peptides (≈20 amino acids; PSMα), β-type peptides (40-45 amino acids; PSMβ1, 2 and 3), δ-toxin (25 amino acids; PSMγ) and recently PSMδ (23 amino acids) (Otto *et al.,* 2004; Otto, 2008). Curiously, under biofilm conditions, PSMβ expression is dominant in comparison with the other classes (Otto, 2008). PSMβ stimulates dissemination of cells by forming holes in biofilms. This will result in the modulation of the typical structure with cell towers and fluid-filled channels (Otto, 2008). Wang *et al.* (2011) also showed the role of PSMβ in *S. epidermidis* biofilm dissembling, in vitro, as well as the dissemination from colonized catheters, in vivo. Additionally, they tested the efficiency of antibodies against PSMβ to block bacterial dissemination from catheters, establishing a powerful tool to manipulate biofilms cells spread and subsequently reduce the incidence of BAIs (Wang *et al.*, 2011). Relatively to δ-toxin, this PSM functions as a detergent that disrupts the biofilm polysaccharide matrix and has been hypothesized to have a role in necrotizing enterocolitis in neonates (Otto, 2009). δ-toxin also prevent hydrophobic interactions, among bacterial cells surfaces,

reducing the surface tension in biofilm interface, improving the cells detachment (McCann *et al.,* 2008).

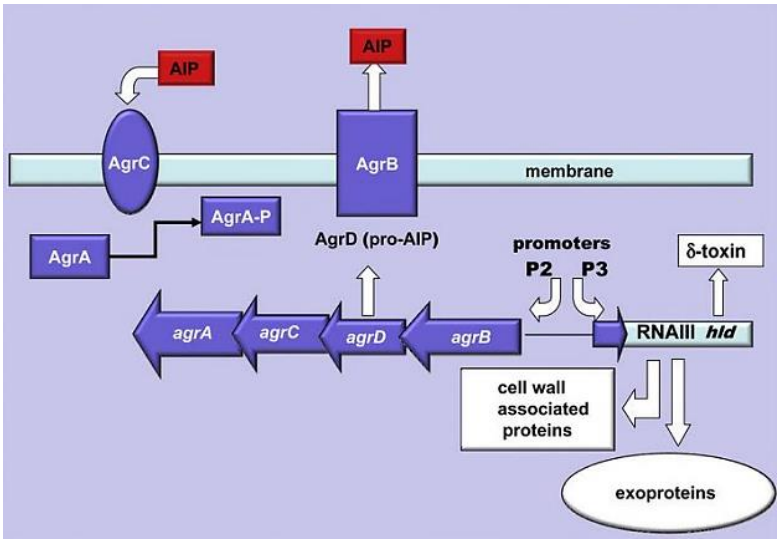


Figure 1.5 Quorum sensing agr system (adapted from Arciola *et al.,* 2012).

1.5. Antimicrobial resistance

The resistance to antibiotics is the main challenge in infections associated with biofilm formation. The antibiotic resistance, acquired by bacteria, may be associated with some factors: a) the abuse of antibiotics, incorrect diagnosis or treatment and disobedience of the antibiotic therapy by patients (Otto, 2004); b) increase of immune-compromised patients, use of invasive procedures or devices, inappropriate disinfectants and default of practices to control diseases/infections in hospital environment (McCann *et al.,* 2008). In the particular case of *S. epidermidis,* resistance to antimicrobial agents may be linked to several characteristics such as highly adaptive nature, inherit genetic variability, great recombination potential and the capacity to shift genetic material (Ziebuhr *et al.,* 2006). These characteristics demonstrate its capacity to adapt new environments and to escape the antibiotic action, leading to advanced risks, inefficiency therapy and high mortality rates (Ziebuhr *et al.,* 2006).

An important feature of biofilms is that the antimicrobial resistance is higher for bacterial cells within the biofilm, as compared with the planktonic population (Cerca *et al.,* 2005). Antibiotics may eradicate the planktonic cells near surface or released from the biofilm, however no effect is obtained for biofilm

population regardless of the size of the antibiotic molecule (Cerca *et al.,* 2005), allowing them to re-establish the biofilm and cause other infections. Subsequent treatments with antibiotics only reduce a minority of bacterial cells, enhancing these populations to be more resistant to antimicrobial agents (Ehrlich *et al.,* 2004). Besides bacterial population and their diversity, additional factors can also contribute to high tolerance including: the diffusion barrier imparted by the exopolysaccharide matrix preventing some antibiotic penetration (Dufour *et al.,* 2012), physiological conditions of biofilm-growing cells since growth conditions are different in the biofilm layers (Francolini and Donelli, 2010), induction of particular resistance mechanisms and/or the development of dormant persister cells (Dufour *et al.,* 2012). Despite these tolerant factors, the antibiotic entrance may be facilitated through the disruption of one particular region of biofilm layers improving their efficiency against biofilm-mediated infections (Rani *et al.,* 2007).

Many antibiotics are used to reduce the biofilms biomass as well as control *S. epidermidis* infections such as methicillin, rifamycin, quilonone, gentamycin, tetracycline, erythromycin, sulfonamides and glycopeptide antibiotics (Rogers *et al.,* 2009). However, the most studied antimicrobial agent in nosocomial infections caused by *S. epidermidis* and other CoNS is methicillin. It is mediated by the *mecA* gene, located on a particular molecular vector, named as staphylococcal cassete chromosome mec (*SCCmec*), which encodes a penicillin-binding protein (Ziebuhr *et al.,* 2006). Among most frequently isolated nosocomial pathogens 59.5% of *S. epidermidis* isolates are resistant to methicillin (Otto, 2008). Michelim *et al.* (2005) selected 98 *S. epidermidis* clinical isolates obtained from blood, catheters and other materials and evaluated their resistant to several antibiotics. The results revealed that 82.6% were resistant to gentamycin, 79.6% to erythromycin and 71.4% to ciprofloxaxin, excluding vancomycin that was vulnerable for all 98 isolates (Michelim *et al.,* 2005). Oliveira and Cerca (2013) also assessed some antibiotic resistance in *S. epidermidis* (n=31) and other CoNS isolates (n=30). In particularly *S. epidermidis* demonstrated a higher rate of resistance for penicillin with 52%, following 48% to erythromycin, 42% gentamicin and 6% to ciprofloxacin. All isolates were also susceptible for vancomycin (Oliveira and Cerca, 2013).

1.6. Aims

S. epidermidis biofilms are involved on the majority of infections linked to implantation on medical devices. Among the different biofilm stages, biofilm attachment and accumulation are the most critical, allowing the production of a more stable and robust biofilm. While the role of the *ica* operon has been

extensively investigated, other biofilm forming factors are not fully explored. Therefore, the main objective of this work was to explore the role of three genes that have been considered the most important in *S. epidermidis* biofilm accumulation (*icaA, aap* and *bhp*). To determine the relative contribution of each gene in biofilm accumulation, biomass quantification and the 3D structure of the biofilms were related with the expression of those genes, overtime. Furthermore, to understand if the observed phenomenon was strictly present in clinical isolates, a commensal strain was used, in order to compare with the clinical isolate.

CHAPTER 2

MATERIAL AND METHODS

2.1. *S. epidermidis* isolates

The *S. epidermidis* strains used in this study were either isolated from blood of patients with *S. epidermidis* infections (Laboratory of Microbiology of Santo Antonio General Hospital (HGSA), Oporto, Portugal) or from healthy volunteers (Oliveira, 2013).

2.2. Planktonic growth

Individual cells from each isolate were inoculated in a 10 mL tube with 1 mL of Tryptic Soy Broth (TSB, Liofilchem, Teramo, Italy) at 37 °C with agitation at 120 rpm (ES-20 Shaker-Incubator, BioSan, Riga, Latvia) in order to obtain a starter culture. After reaching the exponential phase the pre-inoculums were diluted until the measured optical density (OD; 640 nm) was between 0,25 and 0,30 (approximately 2 x 10^8 CFU/ml) (Cerca *et al.,* 2004). The starter culture was diluted 1:100 in TSB supplemented with 0.4% (w/v) glucose (TSBG) in a 25 mL Erlenmeyer flask and incubated at 37° C with agitation at 120 rpm.

2.3. Biofilm formation

Bacterial biofilms were grown as previously described (Cerca *et al.,* 2004). A starter culture was prepared and adjusted to the desired OD as described above. Subsequently, 10 µL of the started culture was inoculated in a 24-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium) plus 990 µL of TSB, supplement with 0.4% of glucose (TSBG) to induce the biofilm formation. The cultures were grown for 12, 24, 36, 48, 60 and 72 h at 37 °C on an orbital shaker at 120 rpm. At each 24 h, the growth medium was removed and exchanged by fresh TSBG. Biofilm formation assay was repeated no less than three times.

2.4. Biofilm Characterization

2.4.1. Biofilm quantification

Biofilm quantification for each time point was determined by OD measurement, as described before (Freitas *et al.,* 2013). First the growth medium was removed and the biofilms were washed with 0.9% NaCl in order to remove all the detached cells. The biofilms were scraped and resuspended into 1 mL

of the same saline solution, following sonication at different time and amplitude: 10 seconds at 30%, 20 seconds at 30% and 40 seconds at 40%. After sonication, the biomass quantification of each biofilm was determined by measuring the OD at 640 nm. The cell suspensions were diluted with 0.9% of NaCl until the OD measurement was below 0.8 and the OD determination was calculated by multiplying the dilution factor by the OD measurement obtained.

2.5. Detection of biofilm-associated genes

The DNA extraction was performed by suspending five colonies of an overnight culture on Tryptic Soy Agar (TSA) plates, in 200 µL of ultrapure water. The suspension was heated at 100 °C for 15 minutes in a thermal block, kept on ice for 5 minutes, in order to disrupt the cells, and then centrifuged at maximum speed (16100 g) for 10 minutes at 4 °C. The supernatant was transferred to a new tube and stored at -20 °C, until further use.

Table 2.1 Information of the primers.

TARGET GENE	OLIGONUCLEOTIDE PRIMERS SEQUENCE (5' TO 3')	AMPLICON SIZE (BP)
icaA	Fw TGC ACT CAA TGA GGG AAT CA Rv TCA GGC ACT AAC ATC CAG CA	417
	Fw TGC ACT CAA TGA GGG AAT CA Rv TAA CTG CGC CTA ATT TTG GAT T	134
	Fw CAA GCG AAG TCA ATC TCT TGC Rv GCG GCA TTG ATA ACC CAG TA	582
aap	Fw GCT CTC ATA ACG CCA CTT GC Rv GGA CAG CCA CCT GGT ACA AC	617
	Fw GCA CCA GCT GTT GTT GTA CC Rv GCA TGC CTG CTG ATA GTT CA	190
bhp	Fw TGG ACT CGT AGC TTC GTC CT Rv TCT GCA GAT ACC CAG ACA ACC	213
	Fw CGT TCC CTT GAT TGA GGT GT Rv GTT ACG TGA ACG GGT CGA TT	404
mecA	Fw CCG AAA CAA TGT GGA ATT GG Rv TCA CCT GTT TGA GGG TGG AT	600
	Fw GGC CAA TAC AGG AAC AGC AT Rv CGT CAA CGA TTG TGA CAC G	425
rpoB	Fw CAA TTC ATG GAC CAA GC RV CCG TCC CAT GTC ATG AAA C	899

bp – base pairs, Fw – forward, Rv – reverse

PCR reactions were performed with a final volume of 10 µL according to the following conditions: 5 µL of DreamTaq Master Mix (x2) (Thermo Scientific, USA), 2 µL of ultrapure water, 1 µL of primer mixture (forward and reverse primers) and up to 1 µL of DNA template. PCR amplifications were obtained by using the MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA) with the following conditions: 94 °C for 5 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 45 s, and 72 °C for 10 min. The information of the primers is described in Table 2.1. A positive control (S. *epidermidis* RP62A), a negative control (water) and an internal control (*rpoB* gene) were also included in each PCR run (Henriques *et al.,* 2012).

Amplified products were analysed in 2% agarose gel stained with Midori Green DNA stain (Nippon Genetics Europe GmbH, Germany), for 45 minutes at 70 volts. Bromophenol blue (x6) (Fisher Scientific) was used as loading dye and NZYDNA Ladder V (Nzytech, Lisboa, Portugal) as molecular weight marker.

2.6. Quantification of gene expression

The protocols used for RNA extraction, DNase treatment, cDNA synthesis and real-time PCR assays were previously optimized for *S. epidermidis* biofilms (França *et al.,* 2012).

2.6.1. RNA extraction

The RNA extraction protocol combines a mechanical (glass beads) and a chemical (phenol) lysis to improve the RNA extraction from the biofilms (França *et al.,* 2011) along with a silica-membrane RNA isolation which minimizes the time required to extract the RNA (E.Z.N.A. Total RNA Kit, Omega Bio-Tek, GA, USA).

The bacterial biofilms were washed to remove all the detached cells, resuspended in 2 mL of 0.9% NaCl (pull of 10 biofilms) and directly stored on ice. The cell suspension was centrifuged at maximum speed (16100 g) for 10 minutes at 4 °C. Once centrifuged the supernatant was discarded and the bacterial pellet was resuspended in 500 µL of TRK lysis buffer supplemented with β-mercaptoethanol plus 500 µL of phenol (Applichem, Darmstadt, Germany). The suspension was transferred into 2 mL safeLock tubes with 0.5 g of acid-washed glass beads (150 – 212 µm) (Sigma, USA) and placed into the FastPrep Cell disruptor (MP Biomedicals, BIOPORTUGAL, Portugal) for 35 seconds at 6.5 m/s.

Subsequently the samples were immediately placed on ice for 5 minutes. The cell disruption and the cooling step were repeated two more times.

Afterwards the samples were centrifuged at maximum speed (12300 g) for 1.5 minutes at 4 °C. The samples was transferred for a 2 mL DNase/RNase free tube (avoiding the aspiration of glass beads) adding later an equal volume of 70% ethanol. The RNA solution was transferred into a RNA isolation column (including any precipitate), centrifuged at 12300 g for 30 seconds at room temperature (RT) and the flow-through was discarded. Columns were then washed with 500 µL of wash buffer I, centrifuged at 10000 g for 30 seconds at RT. The flow-through was discarded and the columns placed in the same collection tube. A second wash with 500 µL of wash buffer II, centrifuged at 10000 g for 30 seconds at RT. The flow-through was discarded and the columns placed in the same collection tube. The columns were washed once again with wash buffer II, centrifuged at 10000 g for 30 seconds at RT. The flow-through and the collection tubes were discarded and the columns placed into a new collection tube. In order to remove any trace of the wash buffer II which contains ethanol, the columns were centrifuged at 12300 g for 2 minutes at RT and then transferred into a 1.5 mL DNase/RNase free tube. Finally, to elute the RNA from the columns 50 µL of DEPC-treated were added into the centre of the column and then centrifuged at 16000 g for 2 minutes. The RNA tube was immediately placed on ice.

2.6.2. DNase treatment

Degradation of genomic DNA was achieved by adding 5 µL of DNase buffer and 2 µL of DNase I (Fermentas, Ontario, Canada) to each RNA sample. Samples were then incubated at 37 °C for 30 minutes. DNase I activity was deactivated with 5 µL of 50 mM EDTA and incubation at 65 °C for 10 minutes.

2.6.3. RNA quantification

The total RNA concentration and purity was determined by a NanoDrop 1000 (Thermo Scientific, USA). For each RNA sample, duplicate measurements were performed and averaged. The absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} were considered to confirm potential protein and chemical contamination (polysaccharide, phenol), respectively.

2.6.4. cDNA synthesis

cDNA synthesis was performed following the protocol of the RevertAid First Strand cDNA Synthesis kit (Fermentas, Ontario, Canada). The same concentration of total RNA (250 ng) from each sample was reverse transcribed in a 10 µL reaction volume. A control reaction was performed under the same conditions but lacking the reverse transcriptase enzyme (no-RT control) to determine the possibility of genomic DNA residues.

2.6.5. Real-Time PCR

The quantification of biofilm gene expression was determined by quantitative real-time PCR (qPCR). qPCR analysis was performed using iQ™ SYBR® Green Supermix (Bio-Rad, Hercules, CA, USA) in a 10 µL reaction tube. Each PCR reaction contained 2 µL diluted cDNA or no-RT control (1:200 in DEPC-treated water), 5 µL of master mix, 1 µL of primer mixture (10 µM of each forward and reverse primers), and 2 µL of nuclease-free water. The primer efficiency was determined by the dilution method. Information of the primers used is described in Table 2.2.

Table 2.2 Primers used in qPCR assays.

TARGET GENE	OLIGONUCLEOTIDE PRIMERS SEQUENCE (5' TO 3')	AMPLICON SIZE (BP)
16S rRNA	FW GGG CTA CAC ACG TGC TAC AA Rv GTA CAA GAC CCG GGA ACG TA	176
icaA	Fw TGC ACT CAA TGA GGG AAT CA Rv TAA CTG CGC CTA ATT TTG GAT T	134
aap	Fw GCA CCA GCT GTT GTT GTA CC Rv GCA TGC CTG CTG ATA GTT CA	190
bhp	Fw TGG ACT CGT AGC TTC GTC CT Rv TCT GCA GAT ACC CAG ACA ACC	213

bp – base pairs, Fw – forward, Rv – reverse

qPCR run was performed on a CFX96 (BioRad, Hercules, CA, USA) with the following cycle parameter: 94 °C for 10 min, 39 cycles of 94 °C for 15 s, 58 °C for 20 s and 72 °C for 25 s. qPCR products were analysed by melting curves to confirm the amplification of the desired product and detect possible unspecific products or primer dimer formation. Each experiment was performed in triplicate and a no template control (nuclease-free water) for each primer mixture was included to assess reagent

contamination. The expression of *icaA*, *aap* and *bhp* was normalised in relation to the housekeeping gene expression *16S rRNA* through the $n^{\Delta Ct}$ method (Livak method), where n stands for the reaction efficiency (n = 1.89 for *icaA* gene, n = 1.94 for *aap* gene and n = 1.96 for *bhp* gene) and $\Delta Ct = Ct_{16S\ rRNA} - Ct_{target\ gene}$. The data analysis was based at least on 3 independent experiments.

2.7. Confocal laser scanning microscopy (CLSM)

Biofilms were prepared as described in the biofilm formation section although in a 6-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium) with 20x20 mm cover glass (Labbox). Cultures were diluted 1:100 in fresh TSBG and incubated at 37 °C with shaking at 120 rpm for 12 and 48 h. At 48 h, the growth medium was replaced with an equal volume of fresh TSBG, every 24 h. After incubation time, the medium was removed and the biofilms stained for confocal microscopy analysis.

Biofilms staining was performed in the dark using three stains (Invitrogen, Table 2.3) to analyse the biofilm structure and composition. The DAPI stain was used to mark the cells by binds to the DNA; while SYPRO and WGA were used to detect the presence of proteins and polysaccharides, respectively. Two combinations were used: DAPI and WGA, SYPRO and WGA. Two independent biofilms of each isolate and time-point were analysed for DAPI+WGA while for SYPRO+WGA was only one biofilm at each time-point. Once the incubation time was completed the stain was removed and then washed with sterile water. The biofilm images were acquired in an Olympus™ FluoView FV1000 (Olympus, Lisboa, Portugal) confocal scanning laser microscope. Biofilms were observed using a 60x water-immersion objective (60x/1.2 W).

Table 2.3 Information of the stains used in CLSM.

STAIN	EXCITATION/EMISSION WAVELENGTHS	INCUBATION (MINUTES)
FilmTracer SYPRO Rubi Biofilm Matrix Stain	Excitation: 450 nm Emission: 610 nm	20–30
Wheat Germ Agglutinin (WGA)	Excitation: 488 nm Emission: 524 nm	10
DAPI Nucleic Acid Stain	Excitation: 358 nm Emission: 461 nm	5

2.8. Statistical Analysis

Statistical significance of all results was determined by using Graphpad Prism version 6.02. Biomass quantification results of biofilm formation were analysed by two-way analysis of variance (ANOVA). The mean normalised gene expression was compared among isolates at different time-points by applying the student's t test. All tests were performed with a confidence level of 95%.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Isolates characterization

Clinical isolates were provided from the Laboratory of Microbiology of the Santo António General Hospital (Oporto, Portugal) from patients with *Staphylococcus epidermidis* infections. The isolates were streaked in plates with TSA growth medium and incubated at 37 °C, until colonies were completely formed. After incubation, the colony morphology of all isolates was analysed and registered, such as colour, shape, elevation and pigment characteristics. Further characterization methods were performed to characterize the clinical isolates: a) quantification of the biofilm formation at 24 h of incubation, by OD measurement, b) presence of biofilm-associated genes, by PCR and c) presence of the methicillin gene (*mecA*), which is responsible for methicillin resistance, by PCR (Table 3.1).

Table 3.1 Characterization of *S. epidermidis* clinical strains.

STRAINS	STRAIN ORIGIN	BIOFILM FORMATION AT 24 H (a)	PRESENCE OF BIOFILM-ASSOCIATED GENES (b)	DETECTION *mecA* GENE
PT13031	Bloodstream infections	1,81***	*icaA, aap*	No
PT11007	Bloodstream infections	0,70***	*icaA, aap*	Yes
PT13018	Bloodstream infections	0,44**	*icaA, aap*	Yes
PT12006	Bloodstream infections	0.42**	*icaA, aap, bhp*	Yes
PT12037	Bloodstream infections	0,35*	*icaA, aap, bhp*	Yes
PT13016	Bloodstream infections	0,31*	*aap, bhp*	Yes
PT13022	Central line-associated bloodstream infections	0,30*	*aap, bhp*	Yes
PT13007	Bloodstream infections	0,29*	*aap, bhp*	Yes
PT12032	Central line-associated bloodstream infections	0,16*	*icaA, aap, bhp*	Yes
PT12054	Bloodstream infections	0,15*	*icaA, aap, bhp*	Yes
PT13017	Bloodstream infections	0,14*	*icaA, aap, bhp*	Yes
PT12053	Bloodstream infections	0,14*	*icaA, aap, bhp*	Yes
PT13014	Bloodstream infections	0,13*	*icaA, aap, bhp*	Yes
PT12063	Respiratory tract infections	0,13*	*aap*	No

(a) Biofilm formation was quantified by OD measurement. (b) The detection of the genes was determined by PCR using two independent primer sets. *** strong biofilm producer, ** moderate biofilm producer, * weak and no-biofilm producers.

According to our results, 71% of the clinical strains showed a weak biofilm formation, 14% were moderate biofilm producers and only 14% were strong biofilm producers. The *aap* and *icaA* genes were detected in all the clinical strains analysed. Regarding the *bhp* gene, it was detected in 71% of the clinical strains; however *bhp* gene was absent in the strong biofilm producers. Okee *et al.* (2012) analysed the presence of *icaA*, *aap* and *bhp* genes in 30 *S. epidermidis* clinical isolates, from which 70% of the isolates were *icaA* positives, 17% possessed the *aap* gene and only 10% had the *bhp* gene (Okee *et al.,* 2012). Gad *et al.* (2009), characterized 35 *S. epidermidis* isolates from urinary tract catheterized patients regarding the capacity of biofilm formation and also the *icaA* gene presence. Their results demonstrated that 31 isolates were biofilms producers, from which 51,4% were strong biofilm producers and 37,1% were moderate biofilm producers. Additionally, all biofilms producers were positive for *icaA* gene, therefore enhancing that this gene is important in biofilm formation (Gad *et al.,* 2009). Both authors had verified the absence of *aap* and *bhp* genes in most *S. epidermidis isolates*, although in our study, these genes were present in the majority of the strains. However, the role of these genes in biofilm formation of clinical strains is not yet fully understood.

Also important, the majority of the characterized strains are resistant to methicillin since approximately 86% revealed the presence of the *mecA* gene. Other authors verified the same high percentage of methicillin resistance in *S. epidermidis* clinical isolates, thus suggesting that its presence may be also related to the bacteria pathogenesis (Kitao *et al.,* 2010; Iorio *et al.,* 2011).

After the clinical strains characterization, one *S. epidermidis* strain was selected for further studies, taking into account the formation of a moderate biofilm and the presence of the three genes of interest, which are regarded as important in the biofilm accumulation stage.

Several studies have been performed to determine factors that may discriminate between invasive and commensal strains of *S. epidermidis* (Frebourg *et al.,* 2000; Galdbart *et al.,* 2000). The elucidation of the major differences between these two types of strains may help to better understand the *S. epidermidis* pathogenesis. Therefore, a *S. epidermidis* commensal strain was also selected from a previously characterized collection of CoNS (Oliveira, 2013) (appendix A), following the same considerations. Thus, *S. epidermidis* clinical strain PT12006 and the commensal strain SECOMO034.A1 were used in additional studies, in order to accomplish the objectives of this thesis.

3.2. Biofilm formation

The biofilm formation, for both strains, was quantified by the measurement of the OD at 640 nm, for six timepoints: 12, 24, 36, 48, 60 and 72 h (Fig. 3.1). The TSB medium was supplemented with 0,4% of glucose and changed every 24 h. A sonication stage was applied, to reduce and eliminate the cell clusters present in the biofilms, improving the biofilm quantification by OD measurement, as previously described (Freitas *et al.,* 2013).

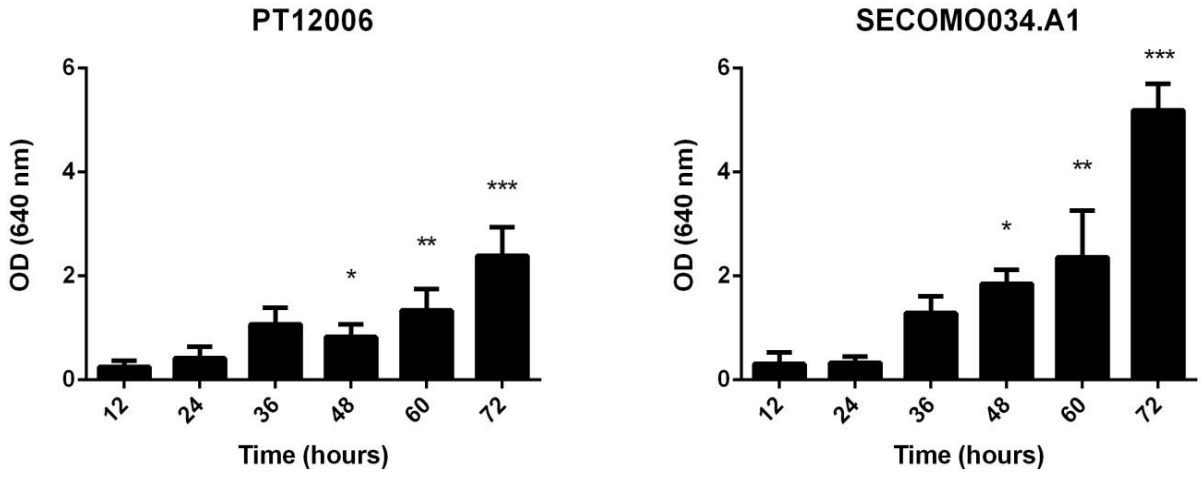


Figure 3.1 Biofilm formation quantification determined by OD measurement of *S. epidermidis* PT12006 and SECOMO034.A1; * represents statistical significant differences between the strains at the different times of incubation ($p < 0,05$).

At an early stage (12-24 h) biofilm formation was not statistically different on both strains, although there was a notable statistical difference after 48 h. Biofilm formation of PT12006 seems to have a slight increase overtime, except at 72 h of incubation, which has almost double of the biomass. However, strain SECOMO034.A1 had significant differences only at a later stage of biofilm formation, demonstrating an evident twofold increase of biomass at 72 h in comparison with the 60 h incubation time.

In general, the commensal strain seems to have more biomass production, when compared with the clinical strain. However, this finding was only observed with the prolongation of the incubation time up to 72 h. According to our results, it is possible to conclude that screening assay for biofilm formation up to 24 h of incubation is not suitable to determine the real ability of a bacterial strain to form biofilm, not being able to distinguish between a strong or a moderate producer. On the other hand, increasing the incubation time allows a better discrimination of the biofilm formation ability.

3.3. Analysis of the biofilm structure by CLSM

Recently, confocal laser scanning microscopy (CLSM) has been used for biofilm analysis, mainly to study the biofilm structure and also their changes due to antibiotics exposure or susceptibility to phagocytosis (Schommer *et al.*, 2011; Cerca *et al.*, 2012). It is a powerful tool to study bacterial biofilms by enabling in-depth analysis of biological structures, without killing or damaging the biological structure (Lawrence and Neu, 1999; Cerca *et al.,* 2012). For this study, the CLSM was used to analyse the biofilm structure in two different stages of biofilm formation (12 and 48 h). Two different analyses were performed: staining with DAPI+WGA or staining with SYPRO+WGA.

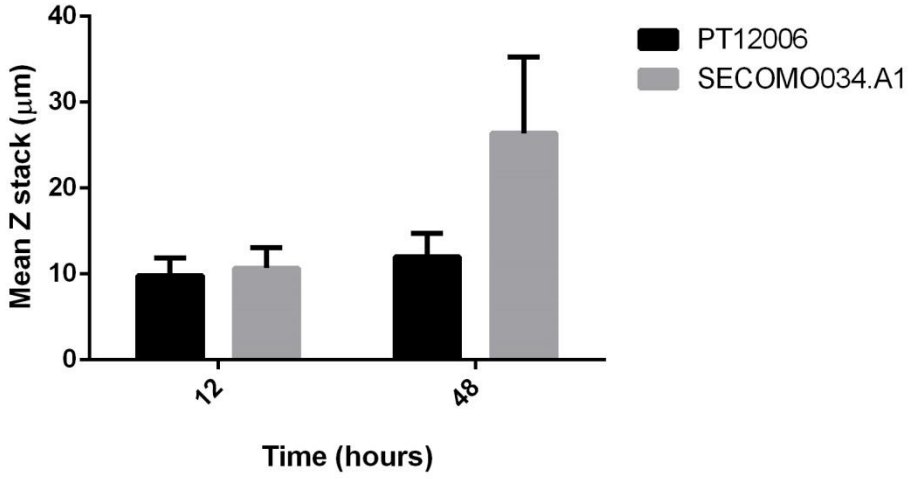


Figure 3.2 Mean of z stack obtained at different focus depths, for each strain at two biofilm formation stages.

We first analysed the maximum deepness of each biofilm by quantifying the z stacks from two independent biofilms in each strain, for both biofilm formation stages. The figure 3.2 demonstrates that the maximum biofilm depthness of the strain PT12006 is almost similar at 12 and 48 h. Conversely, the biofilm structure of the strain SECOMO034.A1 demonstrated a remarkable increase, approximately two-fold increase, between the two different biofilm formation stages. These results are in agreement with the results obtained in the quantification of the biofilm formation (Fig. 3.1), since the biomass quantification was also equal for both strains at 12 h and distinct between 12 and 48 h for SECOMO034.A1. Examples of these biofilms can be visually confirmed through the figures obtained by CLSM (Fig. 3.3 and 3.4).

CLSM also allows the discrimination of specific molecules within the biofilm, depending on the stains used. Since the biofilm matrix in *S. epidermidis* comprises several factors such as *ica* locus encoding the PIA (PNAG) and others proteins like Aap, Bhp, EmbP and also eDNA (Frank and Patel, 2007; Izano *et al.*, 2008; Boles and Horswill, 2011), we performed two independent stains. For the analysis of the matrix composition, the biofilms were first stained with DAPI (5 minutes) to mark cells and with WGA (10 minutes), which binds to *N*-acetyl-d-glucosamine and *N*-acetylneuraminic acid residues, indicating the presence of PNAG in the biofilm matrix. The DAPI+WGA staining results of the PT12006 biofilms, demonstrated basically the same quantification of DAPI and WGA staining either between the z stacks and the two biofilm formations stages (12 and 48 h). The strain SECOMO34.A1 showed an increase in both number of DAPI-labeled cells and presence of PNAG stained by WGA at either biofilm formation stages. Additionally, between the z stacks, a higher presence of PNAG was detected, since cells within the biofilm produce more factors for the biofilm development (Fig. 3.3 and 3.4). After 48 h of biofilm incubation, the SECOMO034.A1 cells seem to be organized in agglomerates with the PNAG surrounding them, showing a more complex and mature structure.

There are a limited number of studies available, using the CLSM method to evaluate the biofilm structure of *S. epidermidis* strains. Schommer *et al.* (2011) characterized the structure of three *S. epidermidis* strains, by detecting the presence of PIA. Their results demonstrated significant structural differences between strains, due to differential distribution and localization of intercellular adhesins within the multilayered biofilm architecture. The cells were surrounded with tether-like PIA fibers, permeating the biofilm in a string-like manner (Schommer *et al.,* 2011). According to our results, PNAG also seems to be localized around the biofilm cells.

However, PNAG is not the only component of the biofilm structure; there is also a wide range of proteins involved in the formation of the extracellular matrix. Therefore, the extracellular proteins among biofilm cells were visualized through the incubation with SYPRO Ruby (30 minutes), at 12 and 48 h, in the biofilms of both clinical and commensal strains. Surprisingly, the SYPRO+WGA staining was inefficient, since there was no detection of the SYPRO fluorescence on both *S. epidermidis* strains, even after 48 h of growth. A positive control with known proteinaceous content was included and a moderate to week signal was detected (data not shown). This suggests that our CLSM might not have the sensibility or detection limit sufficient to detect smaller amounts of proteins in the matrix.

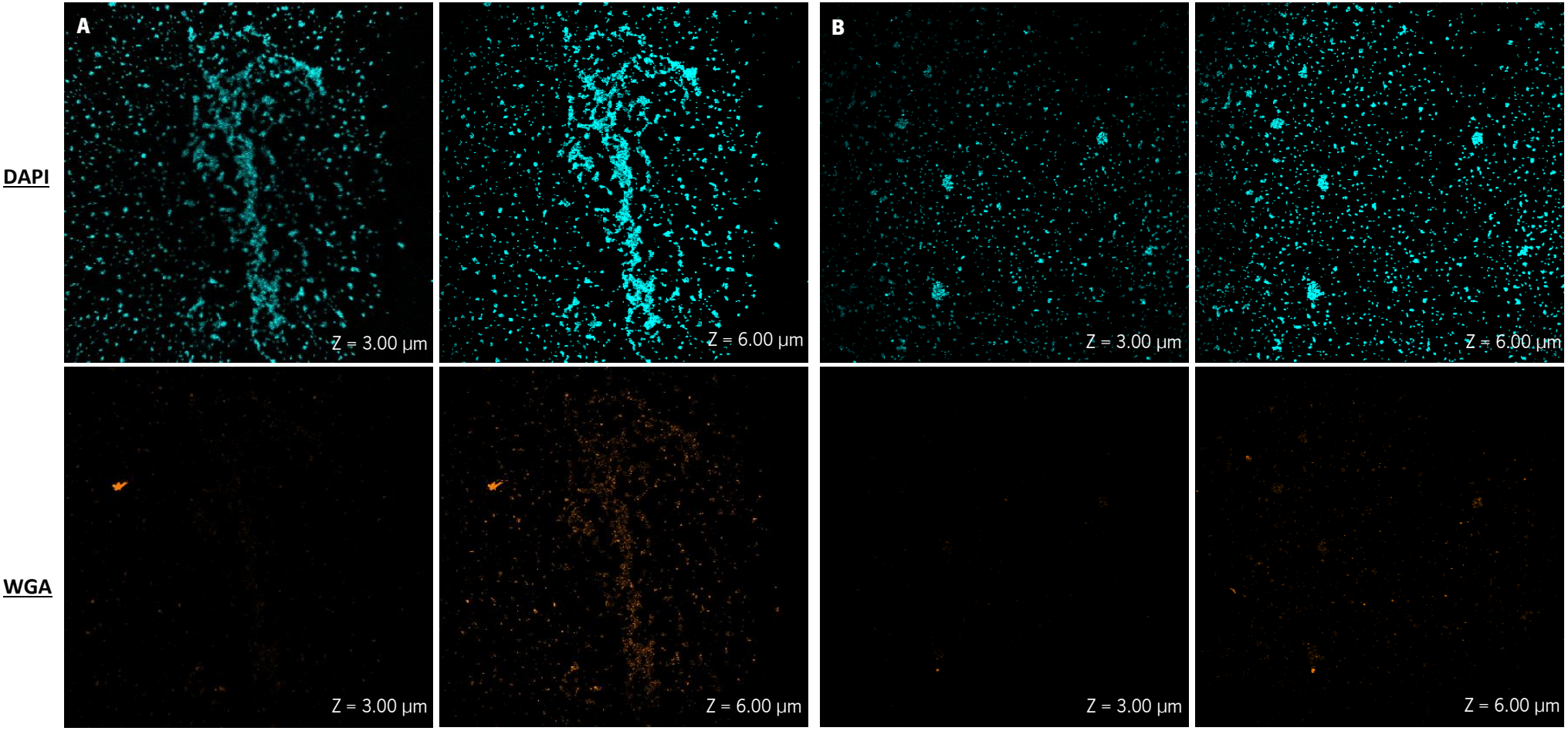


Figure 3.3 Analysis of DAPI+WGA staining of biofilm formation at 12 h for both strains (A) PT12006 and (B) SECOMO034.A1.

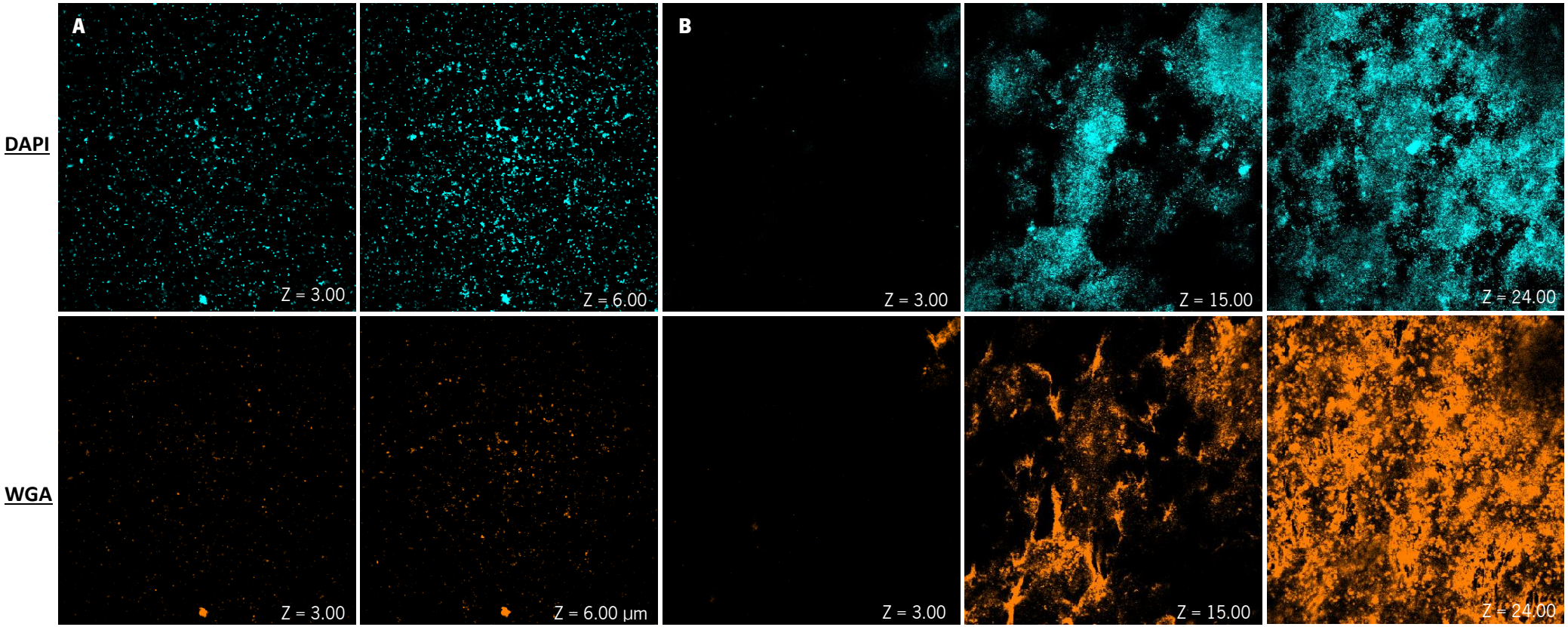


Figure 3.4 Analysis of DAPI+WGA staining of biofilm formation at 48 h for both strains (A) PT12006 and (B) SECOM0034.A1.

The identification of extracellular proteins in the biofilm matrix was also evaluated in a few studies. In a group of CoNS isolates (including *S. epidermidis*), Tremblay *et al.* (2013), studied the biofilm structure and the biofilm morphology, using the CLSM method. The bacterial biofilms grew for 24 h and were stained with WGA with SYPRO Ruby. According to their results, the *S. epidermidis* isolate had different biofilm morphology and a low quantity of cells when compared with the others CoNS. The biofilm structure appeared to be composed with some aggregates or microcolonies (Tremblay *et al.,* 2013), which is in accordance with our results. Additionally, they also detected in the biofilm of *S. epidermidis* isolate, largest amounts of WGA and SYPRO Ruby, suggesting that the biofilm matrix contains PNAG and proteins (Tremblay *et al.,* 2013). In another published article, the authors also characterized the biofilm morphology and structure of a strong *S. epidermidis* biofilm producer, *S. epidermidis* RP62A, after 24 h of incubation. The biofilms were stained also with WGA and SYPRO Ruby. Their results demonstrated that RP62A developed a thick, multilayered biofilm combined with large and abundant structures of PNAG, and also visualized the presence of extracellular proteins in the biofilm matrix (Frank and Patel, 2007).

3.4. Gene expression

After analysing biofilm quantification, by two different methods, we quantified gene expression of the three best studied genes involved in biofilm formation in *S. epidermidis.* Biofilm formation is achieved through three distinct stages: initial attachment, accumulation and maturation and finally detachment (Otto, 2009). The accumulation and maturation stage is the most critical and several genes are involved in this stage, mainly *icaA*, *aap* and *bhp* genes (Rohde *et al.,* 2006). Their expression was analysed at an early (12 h) and late (54 h) stage of biofilm formation.

We first determined the relative expression of biofilms, by comparing the expression of the biofilm with the planktonic growth, in order to determine how these genes would be under- or overexpressed during the biofilm development. Not surprisingly, our results demonstrated that the expression of the tested genes is higher in the biofilm as comparison with the expression of the planktonic growth (figure 3.5). This increase in expression was expected, since the main role of these genes is the regulation of the biofilm accumulation phase.

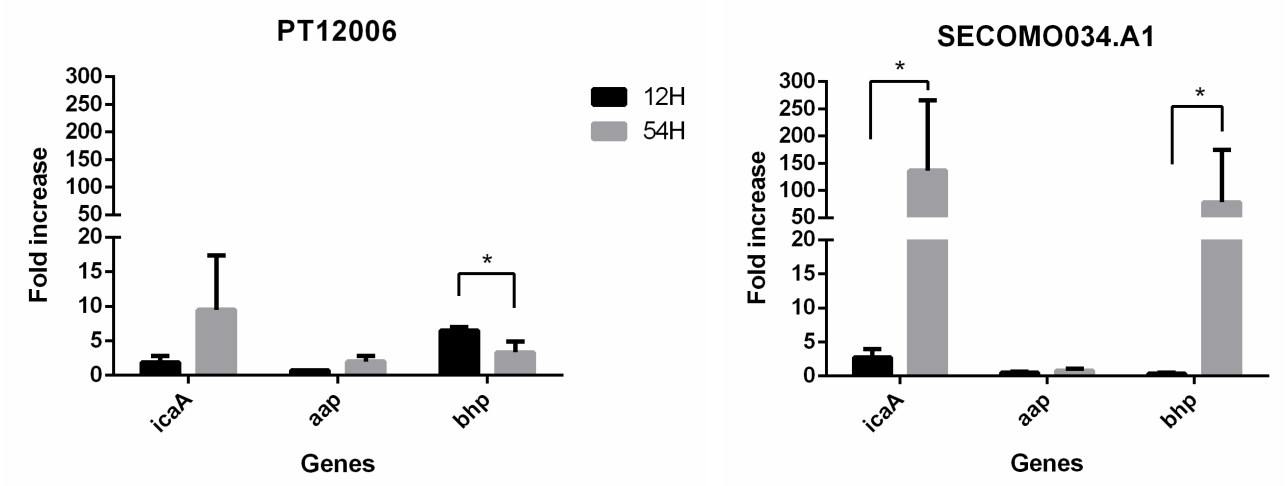


Figure 3.5 Relative expression of PT12006 and SECOMO034.A1 for *icaA*, *aap* and *bhp* genes, in two different development stages of biofilm formation; * represents statistical significant differences between the two times of incubation for each gene and strain ($p < 0,05$). The fold increase is related to the expression in planktonic cultures.

However, our goal was to determine the relative importance of each gene during biofilm formation. For that, we also performed the normalization of gene expression regarding the *16S* expression (housekeeping gene) through the Livak method (Fig. 3.6).

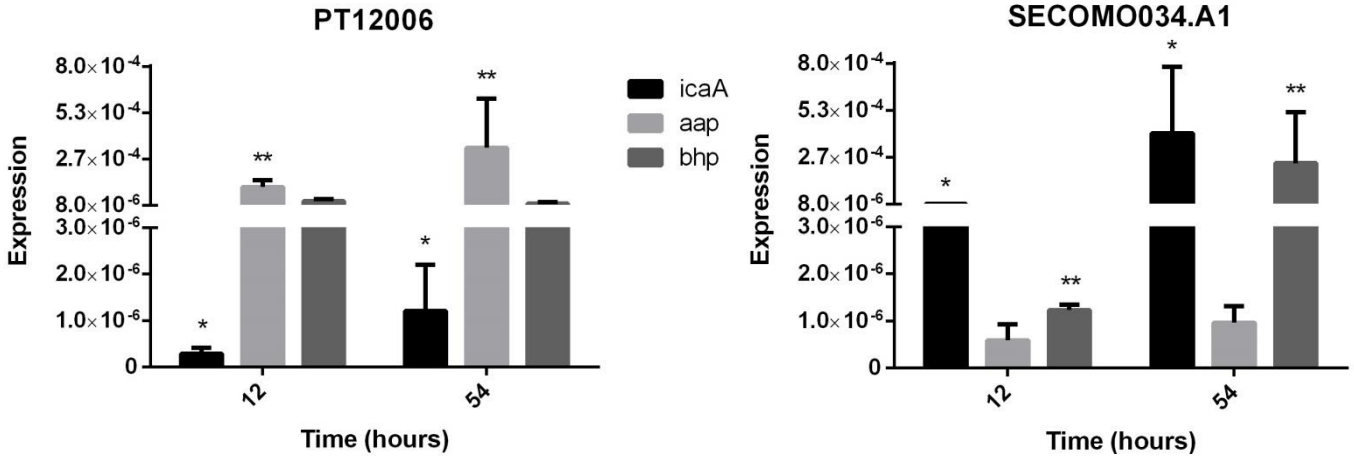


Figure 3.6 Normalized expression of the genes of interest at 12 and 54 h of incubation by qPCR, on both PT12006 and SECOMO034.A1 strains; *, ** represents statistical significant differences between the two times of incubation for each gene and strain ($p < 0,05$).

The strain PT12006 demonstrated a higher expression of *aap* and *bhp* genes than *icaA* gene, independent of the biofilm formation stage. Interestingly, after 54 h of incubation *icaA* and *aap* genes increased, however *aap* gene revealed almost a 100-fold increased while *icaA* gene revealed only a two-fold increase. In the contrary, at an early stage, SEMCOMO034.A1 demonstrated a higher *icaA* gene

expression than *aap* and *bhp* genes. At 54 h, SECOMO034.A1 gene expression increased for *icaA* and *bhp* genes, but *aap* gene expression was not statistically different, showing a slight increase.

The *ica* gene, which encodes PIA, is considered the most important gene involved in biofilm formation (Mack *et al.,* 1996). Interestingly, the *icaA* gene expression of PT12006 between 12 and 54 h seems to be in agreement with the increase obtained in the biofilm formation. Also, the SECOMO034.A1 demonstrated almost a 100-fold increase between both early and later stages and that can explain the remarked increase of the biofilm formation and the DAPI concentration, on those two incubation times. Furthermore, the number of *icaA* mRNA molecules that were present in SECOMO034.A1 was significantly higher than in strain PT12006, further suggesting that enhancing *icaA* gene expression is associated with an higher biofilm accumulation.

Despite the different levels of *icaA* expression of both strains at 12 h, these expression levels are not displayed in the WGA staining, which could be explained, since the biofilm cells were still at an early stage of attachment, producing more proteins to support adhesion than for the accumulation process. The higher presence of PNAG at 48 h obtained by SECOMO034A.1 (Fig. 3.4) seems to be strictly associated to the high levels of expression of the *icaA* gene, after reaching 54 h of biofilm formation, since it is responsible to encode the proteins that produce PNAG.

Besides *ica* gene, several studies demonstrated the role of additional intercellular adhesins like *aap* and *bhp* in *ica*-negative strains, with the capacity to mediate the accumulation process (Rohde *et al.,* 2005; Tormo *et al.,* 2005b). However, despite the detection of the expression of both *aap* and *bhp*, we were not able to visualize those proteins on the biofilm matrix. Therefore, we can deduce that the SYPRO stain had some problems related with the sensibility and detection limit by the CSLM method. Interestingly, despite the role of *bhp* in biofilm formation is still not well understood, we detected an notable increase in bhp expression after 54 h of incubation, but just in the strain SECOMO034.A,1. Therefore, the *bhp* gene, in this strain, may have some contribution in a later stage of biofilm formation.

According to the selected clinical and commensal strains, when comparing the biofilm formation and gene expression, the most evident relationship was related to the *icaA* gene, apparently being essential in the biofilm accumulation step. *In vitro* studies have demonstrated that *S. epidermidis* mutants lacking PIA are not able to accumulate into multilayered biofilms (Mack *et al.,* 1994). Additional studies relating the biofilm formation and gene expression are required, to fully understand the main function of these genes during the biofilm development process.

Patel *et al.* (2012) also evaluated *icaA* and *aap* gene expression of *S. epidermidis* strain RP62A at different biofilm formation stages. They demonstrated approximately a 100-fold increase in the expression of *icaA* gene between 12 to 48 h but the higher increase occurred from 12 to 24 h. Importantly, their results of the *aap* gene expression revealed a 10-fold increase between 12 and 48 h, however demonstrating a 100-fold increase of *aap* gene expression in comparison with the *icaA* gene (Patel *et al.,* 2012). The results obtained by Patel *et al.* (2012) are consistent with our results of the strain PT12006, since the expression of the *aap* gene was also higher than the *icaA* gene. However, an important question remains to be answered: are the high levels of *aap* expression responsible for the biofilm increase or it is overexpressed as a consequence of biofilm increase? Although, a more specific research need to be performed with a higher number of clinical and commensal *S. epidermidis* strains, in order to understand whether or not commensal strains form more biomass than clinical strains, overtime. Unfortunately, the detection of proteins in the biofilm structure was not possible, and consequently we could not determine the complexity of the biofilm matrix or relate the expression of Aap and Bhp proteins. To overcome this issue, further analysis should be performed with the use of other efficient stains for the detection of these proteins.

CHAPTER 4

MAIN CONCLUSIONS AND FUTURE PERSPECTIVES

S. epidermidis have been associated in the development of several infections. Over the years, researchers had focused on the main factors responsible for the *S. epidermidis* ability to form strong and resistant biofilms. The progress of the biofilm formation is mainly associated with a range of genes that are expressed by the bacteria, such as *icaA*, *aap* and *bhp*. Recognizing the importance of these genes, the main objective of this thesis was to evaluate their function during the biofilm formation, and also in development of the biofilm matrix.

The majority of the studies that analyse the biofilm formation are performed only at 24 h and may not be appropriate to determine the real ability of a bacterial strain to form biofilm. This was shown through our results of biofilm quantification overtime. Both strains selected have the three genes of interest, however exhibited different phenotypes and biomass production, after extending the incubation time.

In general, our results of the biofilm formation and structure were in accordance with the expression levels obtained. The gene expression results enhanced the extreme importance of the *icaA* gene, which demonstrated to be responsible for the increase of the biomass production during the accumulation stage and also for the PNAG presence in the biofilm matrix in both strains. Moreover, our results raised some questions about the real importance of *aap* and *bhp* genes in the biofilm process, appearing to have some minor function in a later stage.

A major limitation of our study was the inclusion of only two isolates, due to economic constraints. Therefore, in the future, more experiments on biofilm formation should be performed with a larger number of isolates to ensure more significant conclusions and also with both clinical and commensal isolates, in order to identify the factors of the *S. epidermidis* pathogenesis. Moreover, further studies of gene expression and biofilm quantification at different stages of biofilm development are required to elucidate the specific contribution of *aap* and *bhp* genes during the biofilm process. Also, we could better understand the role of these genes by analysing the biofilm formation with *S. epidermidis* mutants lacking *aap* or *bhp*.

Additionally, it would be interesting to evaluate the presence of other proteins such as *aap* and *bhp* in the biofilm matrix, using more specific markers, such as antibodies, in order to determined their precisely concentration and localization. Furthermore, this method would promote a better comparison with the gene expression assay.

REFERENCE LIST

Anderson, J.M., McNally, A.K., 2011. Biocompatibility of implants: lymphocyte/macrophage interactions. Seminars in immunopathology 33, 221-233.

Anderson, J.M., Rodriguez, A., Chang, D.T., 2008. Foreign body reaction to biomaterials. Seminars in immunology 20, 86-100.

Arciola, C.R., Campoccia, D., Speziale, P., Montanaro, L., Costerton, J.W., 2012. Biofilm formation in *Staphylococcus* implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. Biomaterials 33, 5967-5982.

Arrecubieta, C., Lee, M.H., Macey, A., Foster, T.J., Lowy, F.D., 2007. SdrF, a *Staphylococcus epidermidis* surface protein, binds type I collagen. The Journal of biological chemistry 282, 18767-18776.

Boles, B.R., Horswill, A.R., 2011. Staphylococcal biofilm disassembly. Trends in microbiology 19, 449-455.

Bowden, M.G., Chen, W., Singvall, J., Xu, Y., Peacock, S.J., Valtulina, V., Speziale, P., Hook, M., 2005. Identification and preliminary characterization of cell-wall-anchored proteins of *Staphylococcus epidermidis*. Microbiology 151, 1453-1464.

Bowden, M.G., Visai, L., Longshaw, C.M., Holland, K.T., Speziale, P., Hook, M., 2002. Is the GehD lipase from *Staphylococcus epidermidis* a collagen binding adhesin? The Journal of biological chemistry 277, 43017-43023.

Brooks, B., Brooks, A., Grainger, D., 2013. Antimicrobial Medical Devices in Preclinical Development and Clinical Use, in: Moriarty, T.F., Zaat, S.A.J., Busscher, H.J. (Eds.), Biomaterials Associated Infection, Springer New York, pp. 307-354.

Cerca, N., Gomes, F., Pereira, S., Teixeira, P., Oliveira, R., 2012. Confocal laser scanning microscopy analysis of *S. epidermidis* biofilms exposed to farnesol, vancomycin and rifampicin. BMC research notes 5, 244.

Cerca, N., Jefferson, K.K., 2012. Que vantagens têm os microrganismos em formar um biofilme?, in: Cerca, N., Azevedo, F. (Eds.), Biofilmes - Na Saúde, no Ambiente, na Indústria, Publindústria - Edições Técnicas.

Cerca, N., Jefferson, K.K., Oliveira, R., Pier, G.B., Azeredo, J., 2006. Comparative antibody-mediated phagocytosis of *Staphylococcus epidermidis* cells grown in a biofilm or in the planktonic state. Infection and immunity 74, 4849-4855.

Cerca, N., Martins, S., Cerca, F., Jefferson, K.K., Pier, G.B., Oliveira, R., Azeredo, J., 2005. Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry. The Journal of antimicrobial chemotherapy 56, 331-336.

Cerca, N., Pier, G.B., Vilanova, M., Oliveira, R., Azeredo, J., 2004. Influence of batch or fed-batch growth on *Staphylococcus epidermidis* biofilm formation. Letters in applied microbiology 39, 420-424.

Choong, S., Whitfield, H., 2000. Biofilms and their role in infections in urology. BJU International 86, 935-941.

Chu, V.H., Miro, J.M., Hoen, B., Cabell, C.H., Pappas, P.A., Jones, P., Stryjewski, M.E., Anguera, I., Braun, S., Munoz, P., Commerford, P., Tornos, P., Francis, J., Oyonarte, M., Selton-Suty, C., Morris, A.J., Habib, G., Almirante, B., Sexton, D.J., Corey, G.R., Fowler, V.G., Jr., 2009. Coagulase-negative staphylococcal prosthetic valve endocarditis--a contemporary update based on the International Collaboration on Endocarditis: prospective cohort study. Heart 95, 570-576.

Conlon, K.M., Humphreys, H., O'Gara, J.P., 2002. icaR encodes a transcriptional repressor involved in environmental regulation of ica operon expression and biofilm formation in *Staphylococcus epidermidis*. Journal of bacteriology 184, 4400-4408.

Conrady, D.G., Brescia, C.C., Horii, K., Weiss, A.A., Hassett, D.J., Herr, A.B., 2008. A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. Proceedings of the National Academy of Sciences of the United States of America 105, 19456-19461.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., Marrie, T.J., 1987. Bacterial biofilms in nature and disease. Annual review of microbiology 41, 435-464.

Costerton, J.W., Stewart, P.S., Greenberg, E.P., 1999. Bacterial biofilms: a common cause of persistent infections. Science 284, 1318-1322.

Cucarella, C., Solano, C., Valle, J., Amorena, B., Lasa, I., Penades, J.R., 2001. Bap, a *Staphylococcus aureus* surface protein involved in biofilm formation. Journal of bacteriology 183, 2888-2896.

Dalton, H.M., March, P.E., 1998. Molecular genetics of bacterial attachment and biofouling. Current opinion in biotechnology 9, 252-255.

Dandalides, P.C., Rutala, W.A., Thomann, C.A., Sarubbi, F.A., 1986. Serious postoperative infections caused by coagulase-negative staphylococci: an epidemiological and clinical study. The Journal of hospital infection 8, 233-241.

Darouiche, R.O., 2004. Treatment of infections associated with surgical implants. The New England journal of medicine 350, 1422-1429.

Davis, S.L., Gurusiddappa, S., McCrea, K.W., Perkins, S., Hook, M., 2001. SdrG, a fibrinogen-binding bacterial adhesin of the microbial surface components recognizing adhesive matrix molecules subfamily from *Staphylococcus epidermidis*, targets the thrombin cleavage site in the Bbeta chain. The Journal of biological chemistry 276, 27799-27805.

Dufour, D., Leung, V., Lévesque, C.M., 2012. Bacterial biofilm: structure, function, and antimicrobial resistance. Endodontic Topics 22, 2-16.

Dunne, W.M., Jr., 2002. Bacterial adhesion: seen any good biofilms lately? Clinical microbiology reviews 15, 155-166.

Ehrlich, G.D., Hu, F.Z., Lin, Q., Costerton, J.W., Post, J.C., 2004. Intelligent Implants to Battle Biofilms. Self-diagnosing, self-treating, self-monitoring artificial joints could combat postimplant infections attributable to biofilms. American Society for Microbiology 70, 7.

Eng, R.H., Wang, C., Person, A., Kiehn, T.E., Armstrong, D., 1982. Species identification of coagulase-negative staphylococcal isolates from blood cultures. Journal of clinical microbiology 15, 439-442.

Euzeby, J., 2013. List of Prokaryotic names with Standing in Nomenclature - Genus Staphylococcus. Available at http://www.bacterio.cict.fr/s/staphylococcus.html [Acessed 18 December 2013].

Fey, P.D., Olson, M.E., 2010. Current concepts in biofilm formation of *Staphylococcus epidermidis*. Future microbiology 5, 917-933.

França, A., Freitas, A.I., Henriques, A.F., Cerca, N., 2012. Optimizing a qPCR gene expression quantification assay for *S. epidermidis* biofilms: a comparison between commercial kits and a customized protocol. PloS one 7, e37480.

França, A., Melo, L.D., Cerca, N., 2011. Comparison of RNA extraction methods from biofilm samples of *Staphylococcus epidermidis*. BMC research notes 4, 572.

Francolini, I., Donelli, G., 2010. Prevention and control of biofilm-based medical-device-related infections. FEMS immunology and medical microbiology 59, 227-238.

Frank, K.L., Patel, R., 2007. Poly-N-acetylglucosamine is not a major component of the extracellular matrix in biofilms formed by icaADBC-positive *Staphylococcus lugdunensis* isolates. Infection and immunity 75, 4728-4742.

Frebourg, N.B., Lefebvre, S., Baert, S., Lemeland, J.F., 2000. PCR-Based assay for discrimination between invasive and contaminating *Staphylococcus epidermidis* strains. Journal of clinical microbiology 38, 877-880.

Freitas, A.I., Vasconcelos, C., Vilanova, M., Cerca, N., 2013. Optimization of an automatic counting system for the quantification of *Staphylococcus epidermidis* cells in biofilms. Journal of Basic Microbiology, n/a-n/a.

Gad, G.F., El-Feky, M.A., El-Rehewy, M.S., Hassan, M.A., Abolella, H., El-Baky, R.M., 2009. Detection of icaA, icaD genes and biofilm production by *Staphylococcus aureus* and *Staphylococcus epidermidis* isolated from urinary tract catheterized patients. Journal of infection in developing countries 3, 342-351.

Galdbart, J.O., Allignet, J., Tung, H.S., Ryden, C., El Solh, N., 2000. Screening for *Staphylococcus epidermidis* markers discriminating between skin-flora strains and those responsible for infections of joint prostheses. The Journal of infectious diseases 182, 351-355.

Gerke, C., Kraft, A., Sussmuth, R., Schweitzer, O., Gotz, F., 1998. Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the *Staphylococcus epidermidis* polysaccharide intercellular adhesin. The Journal of biological chemistry 273, 18586-18593.

Gilbert, P., Collier, P.J., Brown, M.R., 1990. Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. Antimicrobial agents and chemotherapy 34, 1865-1868.

Gordon, R.J., Quagliarello, B., Lowy, F.D., 2006. Ventricular assist device-related infections. The Lancet infectious diseases 6, 426-437.

Götz, F., Bannerman, T., Schleifer, K.-H., 2006. The Genera Staphylococcus and Macrococcus, in: Dworkin, M., Falkow, S., Rosenberg, E., Schleifer, K.-H., Stackebrandt, E. (Eds.), The Prokaryotes, Springer US, pp. 5-75.

Gristina, A.G., 1987. Biomaterial-centered infection: microbial adhesion versus tissue integration. Science 237, 1588-1595.

Gu, J., Li, H., Li, M., Vuong, C., Otto, M., Wen, Y., Gao, Q., 2005. Bacterial insertion sequence IS256 as a potential molecular marker to discriminate invasive strains from commensal strains of *Staphylococcus epidermidis*. The Journal of hospital infection 61, 342-348.

Habash, M., Reid, G., 1999. Microbial biofilms: their development and significance for medical device-related infections. Journal of clinical pharmacology 39, 887-898.

Handke, L.D., Slater, S.R., Conlon, K.M., O'Donnell, S.T., Olson, M.E., Bryant, K.A., Rupp, M.E., O'Gara, J.P., Fey, P.D., 2007. SigmaB and SarA independently regulate polysaccharide intercellular adhesin production in *Staphylococcus epidermidis*. Canadian journal of microbiology 53, 82-91.

Heilmann, C., Hussain, M., Peters, G., Gotz, F., 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. Molecular microbiology 24, 1013-1024.

Heilmann, C., Thumm, G., Chhatwal, G.S., Hartleib, J., Uekotter, A., Peters, G., 2003. Identification and characterization of a novel autolysin (Aae) with adhesive properties from *Staphylococcus epidermidis*. Microbiology 149, 2769-2778.

Henriques, A., Cereija, T., Machado, A., Cerca, N., 2012. In silico vs in vitro analysis of primer specificity for the detection of *Gardnerella vaginalis*, *Atopobium vaginae* and *Lactobacillus spp.* BMC research notes 5, 637.

Hussain, M., Herrmann, M., von Eiff, C., Perdreau-Remington, F., Peters, G., 1997. A 140-kilodalton extracellular protein is essential for the accumulation of *Staphylococcus epidermidis* strains on surfaces. Infection and immunity 65, 519-524.

Iorio, N.L., Azevedo, M.B., Frazao, V.H., Barcellos, A.G., Barros, E.M., Pereira, E.M., de Mattos, C.S., dos Santos, K.R., 2011. Methicillin-resistant *Staphylococcus epidermidis* carrying biofilm formation genes: detection of clinical isolates by multiplex PCR. International microbiology : the official journal of the Spanish Society for Microbiology 14, 13-17.

Izano, E.A., Amarante, M.A., Kher, W.B., Kaplan, J.B., 2008. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Applied and environmental microbiology 74, 470-476.

Kitao, T., Ishimaru, M., Nishihara, S., 2010. Detection of biofilm-producing and methicillin resistance genes in *Staphylococcus epidermidis* isolated from healthy humans and in blood culture tests. Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy 16, 170-173.

Kloos, W.E., Musselwhite, M.S., 1975. Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. Applied microbiology 30, 381-385.

Kocianova, S., Vuong, C., Yao, Y., Voyich, J.M., Fischer, E.R., DeLeo, F.R., Otto, M., 2005. Key role of poly-gamma-DL-glutamic acid in immune evasion and virulence of *Staphylococcus epidermidis*. The Journal of clinical investigation 115, 688-694.

Kokare, C.R., Chakraborty, A.N., Khopade, A.N., Mahadik, K.R., 2009. Biofilms: Importance and applications. Indian Journal Biotechnology 8, 10.

Kong, K.F., Vuong, C., Otto, M., 2006. *Staphylococcus* quorum sensing in biofilm formation and infection. International journal of medical microbiology : IJMM 296, 133-139.

Lai, Y., Villaruz, A.E., Li, M., Cha, D.J., Sturdevant, D.E., Otto, M., 2007. The human anionic antimicrobial peptide dermcidin induces proteolytic defence mechanisms in staphylococci. Molecular microbiology 63, 497-506.

Lalani, T., Kanafani, Z.A., Chu, V.H., Moore, L., Corey, G.R., Pappas, P., Woods, C.W., Cabell, C.H., Hoen, B., Selton-Suty, C., Doco-Lecompte, T., Chirouze, C., Raoult, D., Miro, J.M., Mestres, C.A., Olaison, L., Eykyn, S., Abrutyn, E., Fowler, V.G., Jr., 2006. Prosthetic valve endocarditis due to coagulase-negative staphylococci: findings from the International Collaboration on Endocarditis Merged Database. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology 25, 365-368.

Lawrence, J.R., Neu, T.R., 1999. Confocal laser scanning microscopy for analysis of microbial biofilms. Methods in enzymology 310, 131-144.

Lina, G., Jarraud, S., Ji, G., Greenland, T., Pedraza, A., Etienne, J., Novick, R.P., Vandenesch, F., 1998. Transmembrane topology and histidine protein kinase activity of AgrC, the agr signal receptor in *Staphylococcus aureus*. Molecular microbiology 28, 655-662.

Longauerova, A., 2006. Coagulase negative staphylococci and their participation in pathogenesis of human infections. Bratislavske lekarske listy 107, 448-452.

Macintosh, R.L., Brittan, J.L., Bhattacharya, R., Jenkinson, H.F., Derrick, J., Upton, M., Handley, P.S., 2009. The terminal A domain of the fibrillar accumulation-associated protein (Aap) of *Staphylococcus epidermidis* mediates adhesion to human corneocytes. Journal of bacteriology 191, 7007-7016.

Mack, D., 1999. Molecular mechanisms of *Staphylococcus epidermidis* biofilm formation. The Journal of hospital infection 43 Suppl, S113-125.

Mack, D., Davies, A.P., Harris, L.G., Jeeves, R., Pascoe, B., Knobloch, J.K.-M., Rohde, H., Wilkinson, T.S., 2013. *Staphylococcus epidermidis* in Biomaterial-Associated Infections, in: Moriarty, T.F., Zaat, S.A.J., Busscher, H.J. (Eds.), Biomaterials Associated Infection, p. 30.

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H., Laufs, A., 1996. The Intercellular Adhesin Involved in Biofilm Accumulation of *Staphylococcus epidermidis* Is a Linearb-1,6-Linked Glucosaminoglycan: Purification and Structural Analysis. Journal of bacteriology 178, 9.

Mack, D., Nedelmann, M., Krokotsch, A., Schwarzkopf, A., Heesemann, J., Laufs, R., 1994. Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. Infection and immunity 62, 3244-3253.

Mah, T.F., O'Toole, G.A., 2001. Mechanisms of biofilm resistance to antimicrobial agents. Trends in microbiology 9, 34-39.

Maira-Litran, T., Kropec, A., Abeygunawardana, C., Joyce, J., Mark, G., 3rd, Goldmann, D.A., Pier, G.B., 2002. Immunochemical properties of the staphylococcal poly-N-acetylglucosamine surface polysaccharide. Infection and immunity 70, 4433-4440.

Mayville, P., Ji, G., Beavis, R., Yang, H., Goger, M., Novick, R.P., Muir, T.W., 1999. Structure-activity analysis of synthetic autoinducing thiolactone peptides from *Staphylococcus aureus* responsible for virulence. Proceedings of the National Academy of Sciences of the United States of America 96, 1218-1223.

McCann, M.T., Gilmore, B.F., Gorman, S.P., 2008. *Staphylococcus epidermidis* device-related infections: pathogenesis and clinical management. The Journal of pharmacy and pharmacology 60, 1551-1571.

Michelim, L., Lahude, M., Araújo, P.R., Giovanaz, D.S.H., Müller, G., Delamare, A.P.L., Costa, S.O.P., Echeverrigaray, S., 2005. Pathogenicity Factors and antimicrobial resistance of *Staphylococcus epidermidis* associated with nosocomial infections occurring in intensive care units. Brazilian Journal of Microbiology 36, 7.

Murdoch, D.R., Corey, G.R., Hoen, B., Miro, J.M., Fowler, V.G., Jr., Bayer, A.S., Karchmer, A.W., Olaison, L., Pappas, P.A., Moreillon, P., Chambers, S.T., Chu, V.H., Falco, V., Holland, D.J., Jones, P., Klein, J.L., Raymond, N.J., Read, K.M., Tripodi, M.F., Utili, R., Wang, A., Woods, C.W., Cabell, C.H., 2009. Clinical presentation, etiology, and outcome of infective endocarditis in the 21st century: the International Collaboration on Endocarditis-Prospective Cohort Study. Archives of internal medicine 169, 463-473.

Novick, R.P., Ross, H.F., Projan, S.J., Kornblum, J., Kreiswirth, B., Moghazeh, S., 1993. Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. The EMBO journal 12, 3967-3975.

O'Gara, J.P., Humphreys, H., 2001. *Staphylococcus epidermidis* biofilms: importance and implications. Journal of medical microbiology 50, 582-587.

Okee, M.S., Joloba, M.L., Okello, M., Najjuka, F.C., Katabazi, F.A., Bwanga, F., Nanteza, A., Kateete, D.P., 2012. Prevalence of virulence determinants in *Staphylococcus epidermidis* from ICU patients in Kampala, Uganda. Journal of infection in developing countries 6, 242-250.

Oliveira, F., 2013. Diversity and characterisation of coagulase negative staphylococci isolated from healthy individuals in Portugal, Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto, p. 111.

Oliveira, F., Cerca, N., 2013. Antibiotic resistance and biofilm formation ability among coagulase-negative staphylococci in healthy individuals from Portugal. The Journal of antibiotics 66, 739-741.

Otto, M., 2004. Virulence factors of the coagulase-negative staphylococci. Frontiers in bioscience : a journal and virtual library 9, 841-863.

Otto, M., 2008. Staphylococcal biofilms. Current topics in microbiology and immunology 322, 207-228.

Otto, M., 2009. *Staphylococcus epidermidis*--the 'accidental' pathogen. Nature reviews. Microbiology 7, 555-567.

Otto, M., 2012. Molecular basis of *Staphylococcus epidermidis* infections. Seminars in immunopathology 34, 201-214.

Otto, M., O'Mahoney, D.S., Guina, T., Klebanoff, S.J., 2004. Activity of *Staphylococcus epidermidis* phenol-soluble modulin peptides expressed in Staphylococcus carnosus. The Journal of infectious diseases 190, 748-755.

Patel, J.D., Colton, E., Ebert, M., Anderson, J.M., 2012. Gene expression during *S. epidermidis* biofilm formation on biomaterials. Journal of biomedical materials research. Part A 100, 2863-2869.

Patel, J.D., Ebert, M., Ward, R., Anderson, J.M., 2007. *S. epidermidis* biofilm formation: effects of biomaterial surface chemistry and serum proteins. Journal of biomedical materials research. Part A 80, 742-751.

Qin, Z., Ou, Y., Yang, L., Zhu, Y., Tolker-Nielsen, T., Molin, S., Qu, D., 2007. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology 153, 2083-2092.

Rani, S.A., Pitts, B., Beyenal, H., Veluchamy, R.A., Lewandowski, Z., Davison, W.M., Buckingham-Meyer, K., Stewart, P.S., 2007. Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. Journal of bacteriology 189, 4223-4233.

Rogers, K.L., Fey, P.D., Rupp, M.E., 2009. Coagulase-negative staphylococcal infections. Infectious disease clinics of North America 23, 73-98.

Rohde, H., Burandt, E.C., Siemssen, N., Frommelt, L., Burdelski, C., Wurster, S., Scherpe, S., Davies, A.P., Harris, L.G., Horstkotte, M.A., Knobloch, J.K., Ragunath, C., Kaplan, J.B., Mack, D., 2007. Polysaccharide intercellular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. Biomaterials 28, 1711-1720.

Rohde, H., Burdelski, C., Bartscht, K., Hussain, M., Buck, F., Horstkotte, M.A., Knobloch, J.K., Heilmann, C., Herrmann, M., Mack, D., 2005. Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. Molecular microbiology 55, 1883-1895.

Rohde, H., Frankenberger, S., Zahringer, U., Mack, D., 2010. Structure, function and contribution of polysaccharide intercellular adhesin (PIA) to *Staphylococcus epidermidis* biofilm formation and pathogenesis of biomaterial-associated infections. European journal of cell biology 89, 103-111.

Rohde, H., Kalitzky, M., Kroger, N., Scherpe, S., Horstkotte, M.A., Knobloch, J.K., Zander, A.R., Mack, D., 2004. Detection of virulence-associated genes not useful for discriminating between invasive and commensal *Staphylococcus epidermidis* strains from a bone marrow transplant unit. Journal of clinical microbiology 42, 5614-5619.

Rohde, H., Mack, D., Christner, M., 2006. Pathogenesis of staphylococcal device-related infections: from basic science to new diagnostic, therapeutic and prophylactic approaches. Medical Microbiology 17, 10.

Roth, R.R., James, W.D., 1988. Microbial ecology of the skin. Annual review of microbiology 42, 441-464.

Schoenfelder, S.M., Lange, C., Eckart, M., Hennig, S., Kozytska, S., Ziebuhr, W., 2010. Success through diversity - how *Staphylococcus epidermidis* establishes as a nosocomial pathogen. International journal of medical microbiology : IJMM 300, 380-386.

Schommer, N.N., Christner, M., Hentschke, M., Ruckdeschel, K., Aepfelbacher, M., Rohde, H., 2011. *Staphylococcus epidermidis* uses distinct mechanisms of biofilm formation to interfere with phagocytosis and activation of mouse macrophage-like cells 774A.1. Infection and immunity 79, 2267-2276.

Shapiro, J.A., 1988. Bacteria as Multicellular Organisms, Scientific American, p. 8.

Simon, D., Fischer, S., Grossman, A., Downer, C., Hota, B., Heroux, A., Trenholme, G., 2005. Left ventricular assist device-related infection: treatment and outcome. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America 40, 1108-1115.

Smith, A.W., 2005. Biofilms and antibiotic therapy: is there a role for combating bacterial resistance by the use of novel drug delivery systems? Advanced drug delivery reviews 57, 1539-1550.

Stewart, P.S., Franklin, M.J., 2008. Physiological heterogeneity in biofilms. Nature reviews. Microbiology 6, 199-210.

Thoendel, M., Kavanaugh, J.S., Flack, C.E., Horswill, A.R., 2011. Peptide signaling in the staphylococci. Chemical reviews 111, 117-151.

Tille, P., 2013. Bailey and Scott's Diagnostic Microbiology. Elsevier Science Health Science Division.

Tormo, M.A., Knecht, E., Gotz, F., Lasa, I., Penades, J.R., 2005b. Bap-dependent biofilm formation by pathogenic species of *Staphylococcus*: evidence of horizontal gene transfer? Microbiology 151, 2465-2475.

Tormo, M.A., Martí, M., Valle, J., Manna, A.C., Cheung, A.L., Lasa, I., Penadés, J.R., 2005a. SarA is an essential positive regulator of *Staphylococcus epidermidis* biofilm development. Journal of bacteriology 187, 9.

Tremblay, Y.D., Lamarche, D., Chever, P., Haine, D., Messier, S., Jacques, M., 2013. Characterization of the ability of coagulase-negative staphylococci isolated from the milk of Canadian farms to form biofilms. Journal of dairy science 96, 234-246.

Vacheethasanee, K., Temenoff, J.S., Higashi, J.M., Gary, A., Anderson, J.M., Bayston, R., Marchant, R.E., 1998. Bacterial surface properties of clinically isolated *Staphylococcus epidermidis* strains determine adhesion on polyethylene. Journal of biomedical materials research 42, 425-432.

Vandecasteele, S.J., Peetermans, W.E., Merckx, R., Van Eldere, J., 2003. Expression of biofilm-associated genes in *Staphylococcus epidermidis* during in vitro and in vivo foreign body infections. The Journal of infectious diseases 188, 730-737.

von Eiff, C., Jansen, B., Kohnen, W., Becker, K., 2005. Infections associated with medical devices: pathogenesis, management and prophylaxis. Drugs 65, 179-214.

von Eiff, C., Peters, G., Heilmann, C., 2002. Pathogenesis of infections due to coagulase-negative staphylococci. The Lancet infectious diseases 2, 677-685.

von Eiff, C., Proctor, R.A., Peters, G., 2001. Coagulase-negative staphylococci. Pathogens have major role in nosocomial infections. Postgraduate medicine 110, 63-64, 69-70, 73-66.

Vuong, C., Kocianova, S., Voyich, J.M., Yao, Y., Fischer, E.R., DeLeo, F.R., Otto, M., 2004a. A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. The Journal of biological chemistry 279, 54881-54886.

Vuong, C., Otto, M., 2002. *Staphylococcus epidermidis* infections. Microbes and infection / Institut Pasteur 4, 481-489.

Vuong, C., Voyich, J.M., Fischer, E.R., Braughton, K.R., Whitney, A.R., DeLeo, F.R., Otto, M., 2004b. Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. Cellular microbiology 6, 269-275.

Wang, A., Athan, E., Pappas, P.A., Fowler, V.G., Jr., Olaison, L., Pare, C., Almirante, B., Munoz, P., Rizzi, M., Naber, C., Logar, M., Tattevin, P., Iarussi, D.L., Selton-Suty, C., Jones, S.B., Casabe, J., Morris, A., Corey, G.R., Cabell, C.H., 2007. Contemporary clinical profile and outcome of prosthetic valve endocarditis. JAMA : the journal of the American Medical Association 297, 1354-1361.

Wang, L., Li, M., Dong, D., Bach, T.H., Sturdevant, D.E., Vuong, C., Otto, M., Gao, Q., 2008. SarZ is a key regulator of biofilm formation and virulence in *Staphylococcus epidermidis*. The Journal of infectious diseases 197, 1254-1262.

Wang, R., Khan, B.A., Cheung, G.Y., Bach, T.H., Jameson-Lee, M., Kong, K.F., Queck, S.Y., Otto, M., 2011. *Staphylococcus epidermidis* surfactant peptides promote biofilm maturation and dissemination of biofilm-associated infection in mice. The Journal of clinical investigation 121, 238-248.

Wang, X.M., Noble, L., Kreiswirth, B.N., Eisner, W., McClements, W., Jansen, K.U., Anderson, A.S., 2003. Evaluation of a multilocus sequence typing system for *Staphylococcus epidermidis*. Journal of medical microbiology 52, 989-998.

Williams, R.J., Henderson, B., Sharp, L.J., Nair, S.P., 2002. Identification of a fibronectin-binding protein from *Staphylococcus epidermidis*. Infection and immunity 70, 6805-6810.

Xu, L., Li, H., Vuong, C., Vadyvaloo, V., Wang, J., Yao, Y., Otto, M., Gao, Q., 2006. Role of the luxS quorum-sensing system in biofilm formation and virulence of *Staphylococcus epidermidis*. Infection and immunity 74, 488-496.

Yao, Y., Sturdevant, D.E., Otto, M., 2005. Genomewide analysis of gene expression in *Staphylococcus epidermidis* biofilms: insights into the pathophysiology of *S. epidermidis* biofilms and the role of phenol-soluble modulins in formation of biofilms. The Journal of infectious diseases 191, 289-298.

Ziebuhr, W., Hennig, S., Eckart, M., Kranzler, H., Batzilla, C., Kozitskaya, S., 2006. Nosocomial infections by *Staphylococcus epidermidis*: how a commensal bacterium turns into a pathogen. International journal of antimicrobial agents 28 Suppl 1, S14-20.

Zimmerli, W., Trampuz, A., 2013. Biomaterial-Associated Infection: A Perspective from the Clinic, in: Moriarty, T.F., Zaat, S.A.J., Busscher, H.J. (Eds.), Biomaterials Associated Infection, Springer New York, pp. 3-24.

SUPPLEMENTARY INFORMATION

Appendix A Characterization of *S. epidermidis* commensal isolates (adapted by Oliveira, 2013).

STRAIN	ORIGIN	BIOFILM FORMATION	GENES
SECOM005.A	Healthy 23 years old female	Strong	*icaA, aap*
SECOM020.A1	Healthy 15 years old male	Strong	*icaA, bhp*
SECOM003.A	Healthy 35 years old male	Moderate	*aap*
SECOM004.A	Healthy 54 years old male	Moderate	*icaA, aap*
SECOM034.A1	Healthy 19 years old male	Moderate	*icaA, aap, bhp*
SECOM066.A	Healthy 19 years old female	Moderate	*aap*
SECOM001.B	Healthy 56 years old female	Weak	*None*
SECOM023.A	Healthy 22 years old male	Weak	*aap*
SECOM053.A	Healthy 27 years old male	Weak	*icaA, aap*