Universidade do Minho
Escola de Ciências

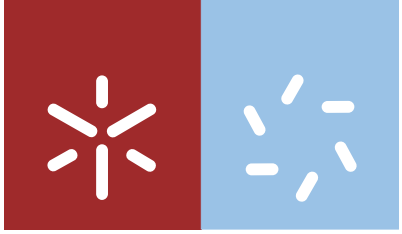
Cármen Sofia Vieira de Sousa

Analysis of gene expression variability in *Staphylococcus epidermidis* biofilms

June 2014

Cármen Sofia Vieira de Sousa **Analysis of gene expression variability in *Staphylococcus epidermidis* biofilms**

UMinho | 2014



Universidade do Minho
Escola de Ciências

Cármen Sofia Vieira de Sousa

Analysis of gene expression variability in *Staphylococcus epidermidis* biofilms

Master Thesis
Master in Molecular Genetics

Work performed by supervisor of
Doctor Nuno Cerca

Work performed by co-supervisor of
Doctor Björn Johansson

June 2014

DECLARAÇÃO

NOME: Cármen Sofia Vieira de Sousa

ENDEREÇO ELECTRÓNICO: carmen.sousa@live.com.pt

BILHETE DE IDENTIDADE: 13367698

TÍTULO DA TESE DE MESTRADO:

Analysis of gene expression variability in *Staphylococcus epidermidis* biofilms

Análise da variabilidade da expressão genética em biofilmes de *S. epidermidis*

ORIENTADOR: Doutor Nuno Cerca

CO-ORIENTADOR: Doutor Björn Johansson

ANO DE CONCLUSÃO: 2014

MESTRADO EM:

Mestrado Genética Molecular

Universidade do Minho, ___/___/_____

Assinatura:_____

ACKNOWLEDGMENTS

The realization of this master thesis was possible due to the contribution of many people, which I´m very grateful.

It´s an honour for me that I acknowledge, with deep gratitude, my supervisor Professor Nuno Cerca, for receiving me at his laboratory and group, for the support and knowledge that made me overcome any difficulty along the way. His guidance was a good beggining to my scientific career and inspired me to working in research. I can´t forget some opportunities that he gave me along my degree.

To all Nuno Cerca group members, for all support and for providing and excellent and friendly atmosphere, specially to Ângela França by the support of procedures, methodologies and her knowledge in this research area, which were very important to me, for my improvement and growth.

To all colleagues of the laboratory and Master course.

Finally, the most important thanks goes to my parents because, what I am today I owe to them and without them none of this would be possible. So, the least I can do is dedicate this thesis to them.

This thesis was funded by European Union funds (FEDER/COMPETE) and by national funds (FCT) under the projects with reference FCOMP-01-0124-FEDER-014309 (PTDC/BIA-MIC/113450/2009).

Análise da variabilidade da expressão genética em biofilmes de *S. epidermidis*

RESUMO

Staphylococcus epidermidis é um agente bacteriano comensal que coloniza, maioritariamente, o epitélio humano mas, mediante determinadas condições, pode tornar-se num agente patogénico oportunista. Atualmente, esta espécie é causadora da maior parte de infeções adquiridas em ambiente hospitalar, especificamente em dispositivos médicos. Os mecanismos de colonização e adaptação desta bactéria não estão totalmente estudados, no entanto é conhecido que esta bactéria torna-se patogénica quando o hospedeiro está imunodeprimido e o processo infecioso ocorre devido à inserção do dispositivo médico e, consequentemente, à formação de agregados tridimensionais denominados de biofilme. Os biofilmes fornecem inúmeras vantagens às bactérias nomeadamente, adaptação a novos ambientes, proteção física, resistência a antibióticos e mecanismos de defesa ao sistema imunitário do hospedeiro. Todas estas interações resultam de diferentes perfis de expressão genética e, desta forma, é extremamente importante o estudo das alterações na expressão dos genes, quando os biofilmes estão presentes. Para tal, são fundamentais três etapas experimentais: extração de RNA, síntese de cDNA e PCR quantitativo em tempo real. No entanto, sabe-se que há variabilidade inerente a cada uma das etapas, bem como relativo ao crescimento das estruturas heterogéneas dos próprios biofilmes. O principal objetivo deste trabalho foi estudar a influência das várias etapas experimentais na variabilidade na expressão genética no crescimento bacteriano. Ao longo deste trabalho, estudou-se a capacidade de formação de biofilme e a presença dos genes de interesse, entre diferentes estirpes de *S. epidermidis*. De seguida, selecionou-se as estirpes mais indicadas para a quantificação da expressão genética. Os nossos resultados demonstraram que a variabilidade biológica foi o principal causador da variabilidade na quantificação da expressão dos genes. Por conseguinte, propusemos a otimização de um simples protocolo experimental, de modo a minimizar esta variabilidade. Esta otimização baseou-se, na utilização de uma mistura de 20 biofilmes independentes, utilizados num único passo de extração de RNA. Foi observado, com sucesso, a redução da variabilidade biológica, utilizando duas estirpes independentes de *S. epidermidis*.

Analysis of gene expression variability in *Staphylococcus epidermidis* biofilms

ABSTRACT

S. epidermidis is the most frequently bacteria isolated from human epithelia, and for a long time, it has been regarded as an innocuous commensal bacterium. However, currently, this bacteria persist as a major cause of hospital and community-acquired infections. It is primarily associated with infections of indwelling medical devices, by the formation of a structure called a biofilm. The biofilms can quickly adapt to new conditions and consequently, can result in the appearance of infections, that are resistance to many antibiotics and mechanisms of the host immune defense. So, it is crucial to study gene expression and their influence in biofilm formation. In gene expression studies, there are three fundamental experimental steps which have some variability: RNA extraction, reverse-transcriptase reaction and quantitative polymerase chain reaction (qPCR). However, since biofilms are very heterogeneous communities, often gene expression studies reveal a high variability. The objective of this work was to demonstrate and understand the influence in the variability of gene expression quantification, by the individual experimental steps required for gene expression quantification, namely bacterial growth, RNA extraction, reverse transcriptase and real-time quantitative PCR. We tested the biofilm formation and the presence of key genes in several strains of *S. epidermidis* in order to select the strains to be used in the gene expression studies. Our results demonstrated that biologic variability was the step which more influence gene expression quantification. Additionally, we proposed an optimized protocol to enhance gene expression reproducibility in *S. epidermidis* biofilms and our results were favorable since we reduced the biologic variability with a pool of 20 biofilms, as determined by the quantification of gene expression in two independent strains.

CONTENTS

NOMENCLATURE

Abbreviations

A	absorbance
Aap	accumulation associated protein
Aap	accumulation associated gene
ADI	arginine deiminase operon
Agr	accessory gene regulator
AHIs	acylated homoserine lactones
AI	autoinducers
AIP	auto-inducing peptide
AMPs	antimicrobial peptides
ATCC	american type culture collection
AtlE	autolysin E
ATP	adenosine triphosphate
Aur	gene that encodes metalloprotease
Bap	biofilm associated protein
Bp	base pairs
Cap	complete capsule
cDNA	complementary DNA
ClpP	caseinolytic Clp protease
CoNS	coagulase-negative staphylococci
CT	cycle threshold
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid

dNTPs	deoxynucleotide triphosphates
eDNA	environmental DNA
EDTA	ethylenediamine tetraacetic acid
Embp	binding protein to the extracellular matrix of the host
EPS	Extracellular polymeric substances
Fmtc	multiple peptide resistance factor gene
GC	guanidine-citosine
G5	short tandem repeat
GlcNAc	*N*-acetilglucosamine monomers
Ica	intercellular adhesion gene
IL	interleukins
LrgB	antiholin-like proteins gene
*Lux*S	*quorum-sensing* system in Staphylococci
LuxS	protein
MASCRAMM	microbial surface components recognizing adhesive matrix molecules
µL	microliter
mL	milliliter
mM	milimol
MGEs	mobile genetic elements
MIQE	minimum information for publication
mRNA	messenger RNA
n	number
NaCl	sodium chloride
nm	nanometers

NRT	reverse transcriptase minus negative control
NTC	no-template control
O.D.	optical density
P	promoter
PBP2a	penicillin-binding protein
PCR	polymerase chain reaction
PGA	poly-*γ*-glutamic acid
Pgi	glycolytic / gluconeogenic gene
PIA	polysaccharide intercellular adhesion
PNAG	poly-*N*-acetylglucosamine
PSM	phenol soluble modulins
qPCR	quantitative PCR
QS	quorum-sensing
RNA	ribonucleic acid
RPM	rotation per minute
rRNA	ribossomic RNA
RT	room temperature
RT-PCR	reverse transcriptase PCR
S.	*staphylococcus*
*Sar*A	*staphylococcus aureus* protein A
SarZ	*staphylococcus aureus* protein Z
SCC*mec*	*staphylococcal* cassette chromosome *mec*
SdrF	colagen-binding protein
SdrG	fibrinogen-binding protein

SdrH	elastin-binding protein
SigB	sigma factor ∂^{B}
Spl	serine protease-like proteins
TCA	tricarboxylic acid
TLR2	toll-like receptor 2
TSA	tryptic soy agar
TSB	tryptic soy broth
TSBG	tryptic soy broth supplemented with glucose
Zn^{2+}	zinc ions

LIST OF PUBLICATIONS

Abstracts and Posters

Sousa, C.; França, A. and Cerca, N. (2013). Analysis of gene expression variability in *Staphylococcus epidermidis* biofilms. *MICROBIOTEC´13*, 6-8 December, Aveiro, Portugal.

LIST OF FIGURES

LIST OF TABLES

“Remember that people can take everything from you,

unless your knowledge”

Albert Einstein

CHAPTER 1

Introduction

1.1 *Staphylococcus epidermidis*

Staphylococci is a group of bacteria that are responsible for causing a variety of diseases, ranging from minor skin infections to life-threatening bacteremia and may cause a significant morbidity and/or mortality (Gill, 2005; CDC, 2012). Currently, they persist as a major cause of hospital and community-acquired infections worldwide which represents an increasing problem in modern medicine (O´Gara, 2001). Nevertheless, some species of Staphylococci have a good symbiotic relationship with their host (Azevedo, 2012; Li, 2000; Baldassarri, 1997).

A major nosocomial and opportunistic pathogen of this genus is *Staphylococcus epidermidis* that belongs to the genus *Staphylococcus,* family Staphylococcaceae. This genus has leastwise 30 species whereupon *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Staphylococcus saprophyticus* which are commonly associated with human infections (Azevedo, 2012; Euzeby, 2014).

S. epidermidis is the most frequently isolated from human epithelia, and colonizes the axillae, head and nares (Kloos and Musselwhite, 1975) and for a long time, it has been regarded as an innocuous commensal bacterium (Vuong and Otto, 2002). Epidemiological studies have demonstrated that healthy people carry between 10 and 24 different strains of *S. epidermidis* at any one time (Kloos and Musselwhite, 1975). This specie is a gram-positive (like all *Staphylococcus*) which means that the cell hall is constituted by a thick layer of peptidoglycan and is known as coagulase-negative staphylococci (CoNS), since it does not produce the coagulase enzyme (Wang, 2003; Wisplinghoff, 2003). In clinical microbiology, CoNS are used to distinguish between *S. aureus* (produces coagulase enzyme) and other staphylococci. However, based in some studies that did a species identification, it his proposed that most of non-specified CoNS infections are due to *S. epidermidis* (CDC, 2004; Rogers *et al.*, 2009).

The cells of *S. epidermidis* are spherical, encapsulated and frequently, in the form of irregular curls. The colonies are smooth, high, shiny and opaque, varying it´s color from white to golden yellow (Götz *et al.*, 2006). From a biochemical point of view, *S. epidermidis* is facultative anaerobic, which means that they can grow in the absence of oxygen or having aerobic respiration; catalase positive responsible for catalyze the decomposition of hydrogen peroxide to water and oxygen and oxidase negative which means that not produce cytochrome c oxidase (Götz *et al.*, 2006). This specie colonizes the skin of human population as previously mentioned (symbiotic relationship). Although the mechanisms of adaption and colonization are not fully understood,

analysis of *S. epidermidis* genome revealed the carriage of genes with predicted function in the protection (Gill *et al.*, 2005; Zhang *et al.*, 2003). This bacterium is primarily associated with infections of indwelling medical devices (pathogen relationship), such as prosthetic heart valves, peripheral or central intravenous catheters and joint prostheses (Dai *et al.*, 2012). These infections occur during device insertion and form multilayered agglomerations called biofilms that will be further discussed below (Chu *et al.*, 2009).

1.2 Microbial biofilms

The majority of bacteria (around 99%) are normally found in biofilm (O´Gara and Humphreys, 2001). It has been suggested that biofilms can provide many advantages to bacteria: adaptation at new environments, such as mutations in genome (Percival *et al.*, 2000); physic protection by tridimensional biofilm and their matrix (Donlan and Costerton, 2002); metabolic potential (heterogeneity and stratification) that indicates different genetic expressions of microorganisms and source of genetic information that can be an advantage to evolution of species (Costerton *et al.*, 1999). For the same reason, several reports demonstrated that pathogenic bacteria form biofilms preferentially, which is recognized as the major of virulence factor (O´Gara and Humphreys, 2001). These biofilms are extremely difficult to threat and represents a serious burden to the public health system (Chu *et al.*, 2009).

1.2.1 General model of biofilm formation

A biofilm is normally defined as a structured and three-dimensional community of bacteria attached to an abiotic or living surface and surrounded by a matrix of extracellular polymeric substances (EPS) (Percival *et al.*, 2012) such as proteins, lipids, polysaccharides, nucleic acids (Donlan and Costerton, 2002).

Biofilm formation occurs in several stages: (1) contact of planktonic bacteria with a surface, mediated by non-specific interactions such as hydrophobic and electrostatic interactions; (2) growth in clusters with the presence of specific molecules, such as adhesins; (3) the matrix becomes to be formed; (4) biofilm maturation (more thicker) and the matrix more proeminent; (5) bacteria will then detach from the biofilm and (6) will colonize different surfaces or regions of a specific surface as can see in Figure 1.1 (Fey and Olson, 2010; Stoodley *et al.*, 2002).

The first contact of planktonic bacteria may occur by a direct adhesion to the polymer surface or adhesion to host matrix proteins that cover the polymer surface, often associated with medical device-related infections. After this stage, structures, named microcolonies, are formed by the multiplication of bacteria and the production of extracellular matrix. These two stages constitute the initial adhesion. The biofilm maturation consists in the formation of channels of water, ion and nutrient exchange (three-dimensional appearance). Finally, the dispersal of single bacteria cells, or large cell clusters, may occur and consequently, initiate a new cycle of biofilm formation anywhere else in the host (Rohde *et al.*, 2006; Schoenfelder *et al.*, 2010).

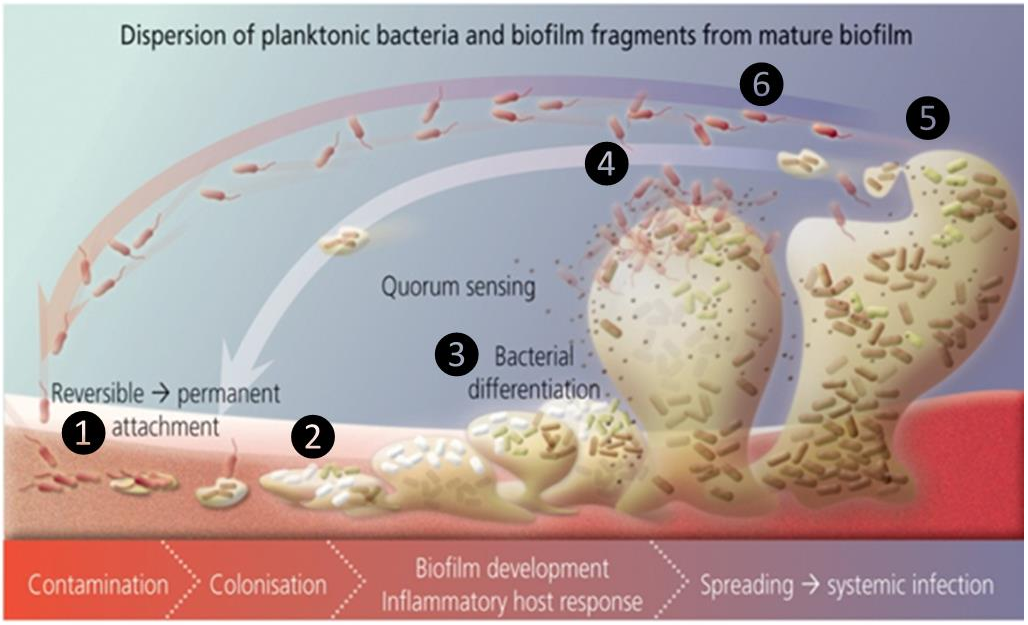


Figure 1.1 – Different stages of biofilm formation (Adapted from *http://woundsinternational.wordpress.com*).

1.2.2 Biofilm matrix

The biofilm matrix, sometimes also referred as the biofilm EPS, is very important for the survival of bacteria inside the biofilm, against adverse conditions (Flemming *et al.*, 2000). These conditions can be low nutrient availability (Cerca and Jefferson, 2012), high osmotic stress (Hamilton and Characklis, 1989) or susceptibility of antibiotics (Howden *et al.*, 2010).

EPS varies in physical (such as diffusivity, thermal conductivity and rheological properties) and chemical properties and can account for 50 %-90 % of the total organic carbon of biofilms (Flemming *et al.*, 2000; Sutherland *et al.*, 2001). These substances are highly hydrated and may be hydrophilic and hydrophobic with different degrees of solubility (Sutherland *et al.*, 2001). In

Gram-positive bacteria, such as *S. epidermidis*, the EPS produced are primarily cationic which produces exopolymers such as poly-*γ*-glutamic acid (PGA) and poly-*N*-acetylglucosamine (PNAG) (PNAG will be further discussed in the biofilm development of *S. epidermidis*) (Percival *et al.*, 2000).

The main function of the matrix is to ensure cohesion of the biofilm structure since this structure is not rigid and the exposure to the mechanical stresses can lead a part of biofilm will break and release from the surface (Rohde *et al.*, 2006; Schoenfelder *et al.*, 2010). There are more functions, not least important, such as initial adhesion (polysaccharides, proteins, DNA, amphiphilic molecules) (Rhode *et al.*, 2007); intercellular adhesion (polysaccharides, proteins and DNA); water retention (hydrophilic polysaccharides) (Heilmann *et al.*, 2006); protective barrier (polysaccharides and proteins) (Gross *et al.*, 2001); adsorption of compounds (polysaccharides with charge and proteins) (Cockayne *et al.*, 1998); enzymatic activity (proteins); nutrients source (any components of the matrix) (Cerca and Jefferson, 2012); exchange of genetic information (DNA) (Molin *et al.*, 2003) and storage energetic (polysaccharides) (Sadovskaya *et al.*, 2005). Some reports have been shown to protect bacteria against antibiotics therapy and host immune attack that will be further discussed (Arciola *et al.*, 2005; Beenken *et al.*, 2004; Cerca *et al.*, 2006; Nagarajan *et al.*, 2009; Yao *et al.*, 2005).

1.3 How S. epidermidis forms biofilms?

Many bacteria use signaling systems to adapt gene expression to environmental changes (Chen *et al.*, 2002). Staphylococci group have two known *quorum sensing* (QS) systems (*agr* and *lux*S systems) which can be defined as bacterial intercommunication systems that control the expression of multiple genes, in response to cell density (Fuqua *et al.*, 1994). These systems use autoinducers (AI), which are small signal molecules and when accumulated to a threshold concentration activates the system, directly or indirectly, controlling the transcription of specific genes (Yarwood and Schlievert, 2003). Gram-positive bacteria, such as *S. epidermidis*, use oligopeptide AIs that moves athwart two-component phosphorely cascades while gram-negative bacteria use acylated homoserine lactones (AHLs) (Carmody *et al.*, 2004).

The importance of *agr* system in biofilm formation was first shown by the group of Otto (Otto *et al.*, 2001). The QS is encoded by the accessory gene regulator (*agr*) locus and consists of

four genes (*agr*A, *agr*B, *agr*C and *agr*D) that are co-transcribed. The system is organized in a two-component of transmembrane transduction complex (*agr*A and *agr*C), a pro-signalling peptide (*agr*D) and a membrane component (*agr*B), responsible for the externalization of the post-translationally modified signaling peptide which consists of an auto-inducing peptide (AIP) (Vuong *et al.*, 2000; Otto *et al.*, 2001). This system are regulated directly by DNA-binding protein *agr*A or through the regulatory RNAIII (Novick *et al.*, 1993). The *agr*A activates the P2 promoter which controls expression of *agr*B, *agr*C, *agr*D, *agr*A and it also activates P3 promoter which drives expression of RNAIII and the embedded phenol-soluble modulin ∂–toxin (Queck *et al.*, 2008). The QS *agr* system is activated during the transition from exponential to a growth stationary phase. Despite, the role of *agr* during infection is controversial (Qin *et al.* 2007), some studies reported that controls the expression of a serie of toxins (Vuong *et al.* 2004), virulence factors (Janzon *et al.*, 1989), represses biofilm formation (Vuong *et al.*, 2000; Otto *et al.*, 2001) and the interaction with innate immune system (Otto *et al.*, 2001) that will be further discussed below.

The QS system more recently described was *lux*S and this role was not well studied. However, it is known that *lux*S uses autoinducer 2 (AI-2), limits biofilm formation, virulence, regulate the *ica* gene (represses the *ica*ADBC transcription) and production of PIA (synthesis) (Lin *et al.*, 2006; Xu *et al.*, 2006). This system is not always accompanied by endogenous AI-2 activity suggesting that AI-2 QS system may not be operative in some bacteria. The same study showed that *lux*S in *S. epidermidis* is functional and *lux*S-dependent gene regulation represses biofilm formation *in vitro* and pathogen success during biofilm-associated infection. Thus, by regulating different biofilm factors, the two QS systems of *Staphylococcus*, have very similar effects on the biofilm mode of growth (Lin *et al.*, 2006).

1.3.1 Initial Adhesion

Initial adhesion is characterized by the bacterial adherence to foreign body or biomaterials (Vacheethasanee *et al.*, 1998). In *S. epidermidis* hydrophobic interactions (biofilm associated protein – Bap) and adhesins/autolysins (Autolysin E – AtlE)) are involved adhesion to uncoated surfaces (Vacheethasanee *et al.*, 1998). The human body quickly reacts to the presence of medical devices by coating them with glycoproteins and proteins, such as fibronectin, vitronectin, fibrinogen, albumin and immunoglobulins (Begun *et al.*, 2007). The specific binding with fibronectin is mediated by a surface proteins group characterized by the presence of various serine-

aspartate repetitions. The interaction with fibronectin seems be get by techoic acids and also by a binding protein to the extracellular matrix of the host (Embp) (Gross *et al.*, 2011). AtlE also mediate the interaction and this protein is connected to the Bap. Both proteins are responsible for contributing to the hydrophobic character of the cell surface, that will have an impact on the initial adhesion to surfaces (Mann *et al.,* 2009).

Staphylococci have multiple adherence factors known as microbial surface components recognizing adhesive matrix molecules (MASCRAMM) (Pei and Flock, 2001). The MSCRAMMs that have been found in most strains are SdrG, a fibrinogen-binding protein (Hartford *et al.*, 2001), SdrF, a colagen-binding protein (Arrecubieta *et al.*, 2007), and SdrH an elastin-binding protein (Arrecubieta *et al.*, 2009). However, the most studied is SdrG, that has been described as important to promote S. *epidermidis* adhesion to fibrinogen-coated surfaces (Hartford *et al.*, 2001). Recent studies demonstrated that SrdG is responsible, also, for promoting platelet adhesion and aggregation (Brennan *et al.*, 2009). SdrF has also been demonstrated during ventricular assist device driveline-related infection (Arrecubieta *et al.*, 2007) (Figure 1.2).

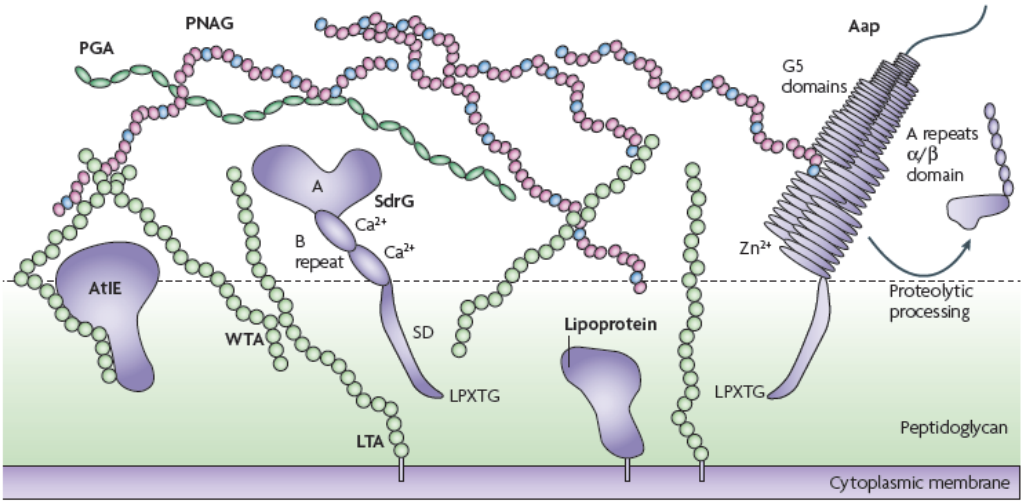


Figure 1.2 - The *S. epidermidis* cell surface (Adapted from Otto, 2009).

1.3.2 Biofilm Maturation

The biofilm maturation is achieved by adhesion proteins, eDNA, techoic acids and polysaccharides, allows the development of the typical three-dimensional structure of the biofilm (Rupp *et al.*, 1999). *S. epidermidis* growing within a biofilm consists, at least, four metabolic states: aerobic growth, anaerobic growth, dormant cells and dead cells (Rani *et al.*, 2007).

One specific operon that is consistentely upregulated within biofilm populations of *S. epidermidis*, in contrast to cells growing in planktonic form, is the arginine deiminase operon (ADI) (Beenken *et al.*, 2004). This pathway is used to catabolize arginine under microaerobic or anaerobic conditions to generate ammonia and ATP (Abdelal, 1979) and under anoxic conditions arginine serve as carbon source (Makhlin *et al.*, 2007). One study report that the generation of ATP through arginine catabolism is an important aspect for biofilm maturation (Diep *et al.*, 2008). The pseudopeptide polymer PGA is very important and synthetized by the gene products of the *cap* locus which seems to be increased during the biofilm mode growth important (Chokr *et al.*, 2007). PGA promotes growth function of *S. epidermidis* at high salt concentrations, in pathogenesis is only known this specie (Fluckiger *et al.*, 2005) and contributes to osmotolerance which indicates a role for *S. epidermidis* colonization (Gerke *et al.*, 1998).

The best studied mechanism of biofilm accumulation is related to the production of β1,6-poli-*N*-acetylglucosamine (PNAG) also referred as polysaccharide intercellular adhesion (PIA), which is produced by many *S. epidermidis* strains (Mack *et al.*, 1996; Wang *et al.*, 2004). The biosynthesis of PNAG is the result of the expression of the genes that constitutes intercellular adhesion (*ica*) *locus*, which compromises *ica*ADBC operation and *ica*R gene (Knobloch *et al.*, 2004) which encodes a regulatory protein (Wang *et al.*, 2007). The *ica*ADBC codifies *ica*A and *ica*D proteins (*N*-acetilglucosamine transferases) responsible by development chain activated of the *N*-acetilglucosamine monomers (GlcNAc) (Gerke *et al.*, 1998). De-acetylation from activated *N*-acetylglucosamine (GlcNAc) monomers by the *ica* gene produces positive charges into the otherwise-neutral polymer which is necessary for surface binding of PNAG. GlcNAc is synthetized by the membrane-located activity (step 1, Figure 1.3a) (Conlon *et al.*, 2004). The PNAG chain is exported by the *ica*C membrane protein (step 2, Figure 1.3a). Then, *ica*B de-acetylase, in cell surface, removes some of the *N*-acetyl groups giving a cationic character which is necessary for surface attachment (step 3, Figure 1.3a) (Conlon *et al.*, 2004). *ica*C responsible for the elongation of monomers and subsequent transport to the bacterial surface (Gerke *et al.*, 1998) and the *ica*B responsible for the partial de-acetylation process (Kristian *et al.*, 2008). This process introduces a positive charge on the polysaccharide, which contributes to the connection of this surface and other biological functions involved in biofilm formation and evasion of the host immune system that will further discussed. The expression of *ica*ADBC operon is regulated by the *ica*A promoter or through expression of *ica*R and both are controlled by a series of regulatory proteins such as SigB, *Sar*A, *Sar*Z, ClpP, tricarboxylic acid (TCA) cycle and luxS (Figure 1.3b) (Handke *et al.*, 2007; Wang

et al., 2007). Both *Sar*A and *Sar*Z are required for *ica*ADBC transcription and subsequent PIA synthesis (Tormo *et al.*, 2005; Wang *et al.*, 2008). Cerca *et al.* (2008), demonstrated that in *S. aureus*, *Sar*A and ∂ᴮ are required for *ica*R expression and *ica*R does not significantly affect its own transcription.

Importantly, some studies demonstrated that PNAG is not essential for biofilm formation, since *S. epidermidis* strains lacking the *ica* gene can form biofilms and the surface proteins, Aap and Bap, may be additionally or exclusively responsible for biofilm formation (Rhode *et al.*, 2005). Colonies that produce PNAG grow as crusty, irregular colonies whereas, PNAG-negative colonies are smooth and creamy (Freeman *et al.*, 1989). Some *ica*-negative *S. epidermidis* strains seems to be mediated by Bap and accumulation associated protein (Aap), which requires proteolytic activation and zinc ions (Zn^{2+}) (Conrady *et al.*, 2008). Zn^{2+} is crucial for the modular association of G5 tandem repeats that may underlie the formation of Aap based fibril-like structures on the bacterial surface (Figure 1.2) and, consequently, mediates the adherence to corneocytes implicating a further role in adherence to skin (Macintosh *et al.*, 2009). Based in some studies, Aap is found approximately 90 % (Sun *et al.*, 2005) and Bap between 15 % - 45 % (Rohde *et al.*, 2007; Bowden *et al.*, 2005) supporting the importance of their presence in biofilm formation.

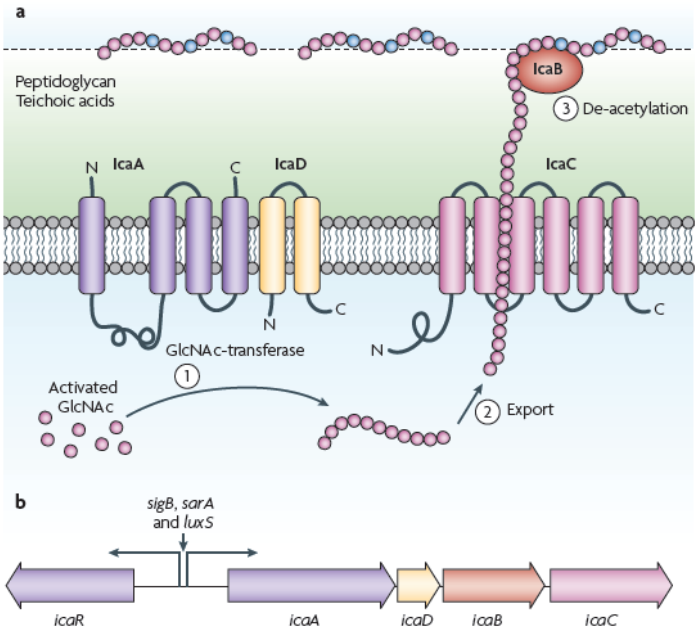


Figure 1.3 - The exopolysaccharide poly-*N*.acetylglucosamine (Adapted from Otto, 2009).

In the detachment process, biofilm release cells in order to maintain the survival of community, promoting the spread of bacteria and leading to the formation of new sites of infection (Voung *et al.*, 2003). Biofilms can be dispersed by many processes such as shedding of daughter cells from actively growing cells (Vuong *et al.*, 2004), as a result of low nutrient level (survival mechanism) (Gross *et al.*, 2001), *quorum sensing* (recognition of nearby cell density) or shearing of biofilm aggregates because of flow effects (Vuong *et al.*, 2003). The mechanisms underlying the release of the cells are poorly understood but many factors can be involved such as mechanical, enzymatic degradation of exopolymers and the rupture of non-covalent interactions by surfactant molecules (phenol soluble modulins - PSM) or ∂-toxins (Otto, 2009).

Little is known regarding dispersal and detachment mechanisms, however, in both *S. aureus* and *S. epidermidis* this process is *agr* dependent (Boles and Horswill, 2008). A model has been proposed for *S. aureus*, which involves *agr* expression which exposes layers of biofilm promoting the detachment of cells clusters, controlling biofilm expansion (Otto *et al.*, 2001). Likewise, in *S. epidermidis agr* activity is limited to the biofilm surface which indicates the existence of a common staphylococcal mechanism of QS-controlled biofilm detachment (Little and Lvins, 1999).

Based in one study, *S. epidermidis agr* mutants showed increased biofilm development and colonization in a rabbit model (Vuong *et al.*, 2004). It is hypothesized that the increased biofilm thickness in *agr* mutants is due to the loss of ∂-toxin and other phenol-soluble modulins (Otto, 2009). These molecules act as surfactants that inhibit noncovalent interactions of bacteria at the surface of biofilm (Vuong *et al.*, 2004). Detachment was related to increased protease activity in biofilm effluent and was related to increased expression of Aur metalloprotease and the *Spl*ABCDEF serine proteases. It is unclear what function these proteases may have in detachment of PNAG/ Aap-Bhp dependent biofilm in *S. epidermidis* (Boles and Horswill, 2008). Therefore, detachment is not just important for promoting genetic diversity, but also for escaping unfavorable habitats aiding in the development of new niches which, consequently, have a very important implication in patients with medical devices (Raad *et al.*, 1992).

1.4 The impact of biofilms in health

S. epidermidis can account for more than 90 % of the resident flora in humans. Despite its generally innocuous nature, in last 20 years emerged a frequent cause of nosocomial infections (Cogen *et al.*, 2008). Up to 60 % of all nosocomial infections are caused by biofilms on medical devices (Donlan and Costerton, 2002). In United States of America, nosocomial infections are responsible for 1.7 million of infections and 99 thousand deaths per year (CDC, 2004). In Portugal, it his estimated than 10 % of all infections are hospital acquired (Pina *et al.*, 2013). It is estimated that biofilms are involved in 65 % of all human infections (Lewis, 2007).

1.4.1 Biofilm infections in indwelling medical devices

The microbial infections associated to the biofilm formation are recognized as etiologic agents of many chronic and persistent infections (Costerton *et al.*, 1999). Probably, due to the very slow growth in the biofilms, these infections are difficult to diagnose and treat with conventional approaches (Howden *et al.*, 2010). With the increase of medical devices, the incidence of these infections also increased the incidence and prophylaxis and limiting the colonization of medical devices the simplest and effective measures to be taken (Rohde *et al.*, 2007).

Medical devices (Figure 1.4) are made out of abiotic materials such as metals, polymers (silicone), but may also contain biological materials such as devitalized bone, blood vessels, muscle fascia from autologous (venous bypass), allogeneic (processed bone), or xenogeneic sources (porcine heart valve) (Darouiche, 2004; Zimmerli and Trampuz, 2011). Interestingly, neither synthetic nor devitalized biological devices are rejected by the body, despite the fact that host reacts to such implants in different ways depending upon the biocompatibility of the device. However, no implant is completely inert after implantation (Anderson *et al.*, 2008; Zimmerli *et al.*, 2004). There are two different type of devices that interact very differently with the host. Whereas, intravascular implants mainly interact with coagulation factors and circulating blood cells, extravascular implants interacting with surrounding tissue, interstitial fluid and attracted phagocytes (De Man *et al.*, 2011). Permanent implants cannot be removed without compromising the replaced function. Therefore, the primary goal is to prevent implant failure due to mechanical reasons or infection (Zimmerli, 2006).

Biofilm infections occur in the hospital environment, especially in very young or old immunocompromised patients (Howden *et al.*, 2010). These infections are usually associated with persistent and relapsing infections such as otitis media (Hall-Stoodley *et al.*, 2006), native valve endocarditis (Martin-Davila *et al.*, 2005), cystic fibrosis (Bjarnsholt *et al.*, 2009), periodontitis and caries (Zjinge *et al.*, 2010), chronic bacterial prostatitis (Mazzoli, 2010), tissue necrosis (Hall-Stoodley, 2008), catheter-related septicaemia and emboli with serious complications to the host (clumps of cells which also contain platelets or erythrocytes) (Costerton *et al.*, 1999). Unfortunately, biomaterial associated infection compromises the quality of life, has a high morbidity and is even associated with mortality. In addition has a high economic impact, since treatment costs more than the primary implantation of the device (Batoni *et al.*, 2010; Ketonis *et al.*, 2010).

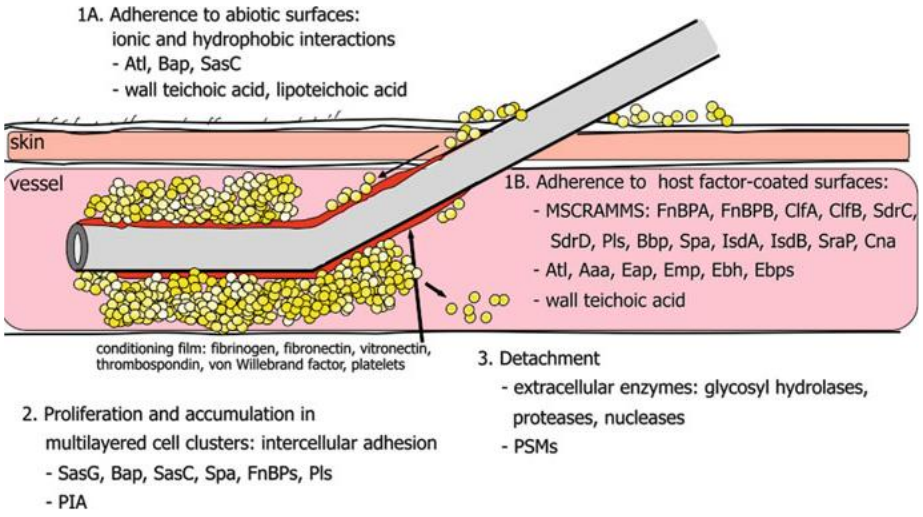


Figure 1.4 - Schematic model of phases involved in *S. epidermidis* biofilm formation on medical devices (Adapted from Heilmann and Götz, 2013).

1.4.2 Immune response and resistance for antibiotics

The most important clinical consequences related to biofilm infections is their higher resistance to antibiotic and to the host immune system (Otto, 2009). The host immune response is a structured system and involves several biological processes that protect the organism against diseases (O´Gara, 2007). It is divided into innate (primary barrier such as skin) and acquired immunity (lymphocytes) as can see in Figure 1.5 (Riken, 2013). *S. epidermidis* has to cope with various mechanisms of host defense during the infection, such as antimicrobial peptides (AMPs), that are present in human skin, or phagocytes, among which neutrophils or polimorphonuclear leucocytes (PMNs) have a proeminent role (Knoblock *et al.*, 2004; Sadyvok and Bayles, 2012).

As mentioned above, PNAG, is involved in evasion of *S. epidermidis* biofilms to the immune system of the host (Wang *et al.*, 2004). PNAG has positive a charge, which is rare in bacterium polysaccharides (Opperman-Sanio and Steinbuchel, 2002). This charge prevents the action of AMPs, also with positive charge, by electrostatic repulsions (Vuong *et al.*, 2004). However, pattern recognition receptors present in bacterium surface by phagocytic cells difficult the binding between antibodies and proteic factors of the complement system (Cheung *et al.*, 2010). Preventing the binding of these last two components of bacteria cell surface limits the process of opsonization and, consequently, phagocytosis of these bacteria by phagocytic cells (O´Gara, 2007).

Besides the protection that PNAG confers to the bacteria, it can also stimulates the production of pro-inflammatory cytokines such as interleukins (IL) 8 and IL-6 by activating toll-like receptor 2 (TLR2) (Cheung *et al.*, 2010). The stimulation of the immune response by biofilms may seem a non-sense in respect of being hostile to pathogens. On the other hand, a continuing inflammatory response causes damage to the surrounding tissue promoting susceptibility to bacterial adhesion and thus biofilm development locations (Sadyvok and Bayles, 2012).

On the other hand, biofilms can be up 1.000 times more tolerant to antibiotics than equivalent planktonic cultures (Hoyle and Costerton, 1992; Mah and O´Tole, 2001). Resistant bacteria can occur during or after an antibiotic treatment (Beiji *et al.*, 2004; Hurford *et al.*, 2012). Whilst biofilm cells may employ a variety of mechanisms to resist the action of antimicrobial agents (Mah *et al.*, 2003), studies have shown that a number of key factors contribute to reduced antimicrobial susceptibility of biofilms (Percival and Bowler, 2004). Several other agents with bactericidal and/or bacteriostatic effects, many of them available in a wide range of household products such as disinfectants, are responsible for resistant bacteria (Levy, 2000; McMurry *et al.*, 1998).

In *S. epidermidis,* specific antibiotic resistance genes are widespread. In many countries, including United States, 75 % - 90 % of all hospital isolates of *S. epidermidis* are resistant to methicillin (Diekema *et al.*, 2001). This resistance is encoded on mobile genetic elements (MGEs), known as the staphylococcal cassette chromosome *mec* (SCC*mec*) (Diep *et al.*, 2008; Wisplinghoff *et al.*, 2003). This cassette chromosome contains the *mec*A gene which encodes a penicillin-binding protein, PBP2a (Chambers *et al.*, 1985). *S. epidermidis* has also acquired resistance also rifamycin, fluoroquinolones, gentamycin, tetracycline, chloramphenicol, erythromycin, clindamycin and sulphonamides (Rogers *et al.*, 2009). Despite widespread resistance to methicillin and other

antibiotics, 80% of catheters infected with *S. epidermidis* can be still treated with antibiotics such as vancomycin and chitosan (Raad *et al.*, 2007; Neoh and Kang, 2009). The frequency of antibiotic resistance reflects the overuse of antibiotics. Furthermore, the ubiquity of *S. epidermidis* as a human commensal microorganism renders this bacterium an optimal carrier and reservoir for antibiotic resistance genes (Miragaia *et al.*, 2005).

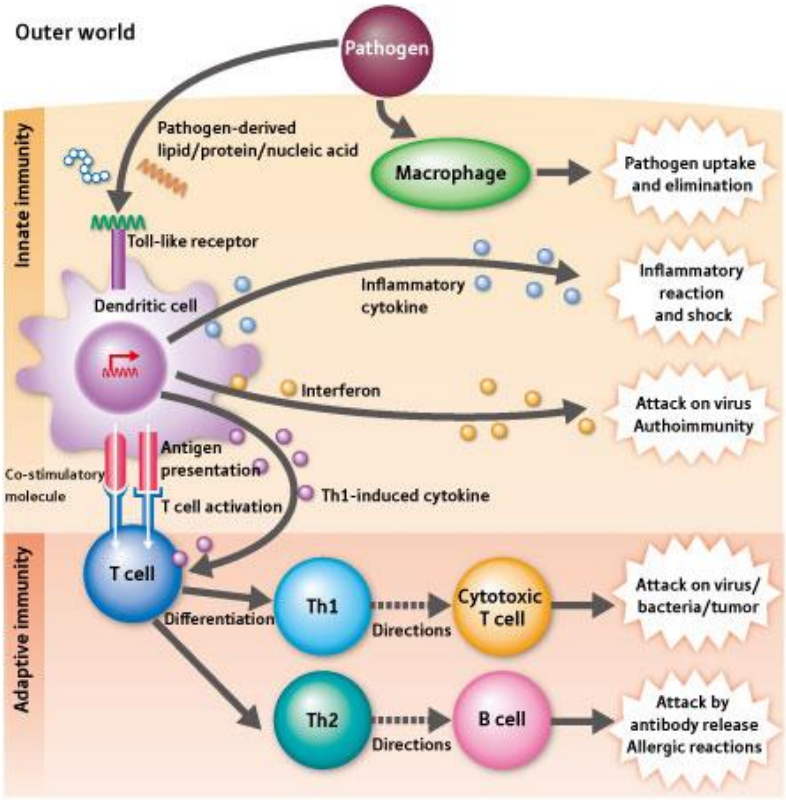


Figure 1. 5 - Innate and Adaptative immunity (Adapted from *http://www.rikenresearch.riken.jp*).

1.5 Biofilm gene expression

The biofilms can quickly adapt to new conditions, phenotypically, genetically and structurally, and changing internal and external conditions, consequently, can result in the resistance to many antibiotics or mechanisms of the host defense. These features provide an important advantages compared with their planktonic existence (Hoyle and Costerton, 1992). *S. epidermidis* has a substantial, genome-wide adaptation to the biofilm mode of growth, including downregulation of basic cell processes as nucleic acid, protein and cell wall biosynthesis (Sadyvok and Bayles, 2012). Therefore, it is very important to study the expression of genes and their influence or contribution in biofilm formation, to better understand the *S. epidermidis* virulence. Since biofilm formation is one of the major virulence factors for these strains (Table 1.1).

Table 1.1 - Main recognized virulence factors of *S. epidermidis* (Adapted from Otto *Nat. Rev. Microbiol.*, 2009, 7(8): 555-67).

Virulence factors	Gene	Function
Biofilm factors		
Primary attachment to abiotic surfaces		
Aae	*Aae*	Autolysin/adhesion
AtlE	*atl*E	Autolysin/adhesin; affects surface hidrophobicity
Teichoic acids	Multiple byosinthetic genes	Affect attachment (only demonstrated in S. aureus)
Primary attachment to matrix proteins (MSCRAMMs)		
AtlE and Aae	*atl*E and *Aae*	Bind to many matrix proteins
Embp	*Embp*	Binds to fibronectin
GehD	*geh*D	Binds to collagen
SrdF	*Srd*F	Binds to collagen
SrdG (Fbe)	*Srd*G (Fbe)	Binds to fibrinogen
SrdH	*Srd*H	Putative binding function
Intercellular aggregation		
Aap	*aap*	Protein intercellular adhesion
Bhp	*bhp*	Protein intercellular adhesion
PNAG (PIA)	*ica*A, *ica*D, *ica*B and *ica*C	Polysaccharide intercellular adhesion
Teichoic acids	Multiple byosinthetic genes	Components of the biofilm matrix
Exoenzymes		
Cysteine proteases SspB and Ecp	*ssp*B	Maybe responsible for tissue damage
Glutamylendopeptidase, GluSE and serine proteases Esp and SspA	*ssp*A	Degrades fibrinogen and complement factor C5
Lipases GehC and GehD	*geh*C and *geh*D	Maybe responsible for the persistence in fatty acid secretions
Metalloprotease/ elastase SepA	*sep*A	Involved in lipase maturation, AMP resistance and protein tissue damage
Protective exopolymers		
PGA	*cap*A, *cap*B, *cap*C and *cap*D	Protects from AMPs and phagocytosis
PIA	*ica*A, *ica*D, *ica*B and *ica*C	Protects from IgG, AMPs, phagocytosis and complement
Resistance to AMPs		
Aps system	*aps*R, *aps*S and *aps*X	Senses AMPs and regulates AMP resistance mechanism
SepA protease	*sep*A	Involved in AMP degradation
Toxins		
PSMs	*psmα, psmϑ, psmε, hld*	Pro-inflammatory cytolysins
Other factors		
SitA, SitB and SitC	*sitA, sitB* and *sitC*	Involved in iron uptake

AMP, antimicrobial peptide; Aap, accumulation-associated protein; Bhp, Bap homologue protein; IgG, immunoglobuline G; MSCRAMM, microbial surface component recognizing adhesive matrix molecules; PGA, poli-γ-glutamic acid; PIA, polysaccharide intercellular adhesin; PSM, phenol soluble modulin.

1.5.1 Techniques used for gene expression determination

In common gene expression studies, there are three fundamental experimental steps before obtaining the final results: RNA extraction, reverse-transcriptase reaction and quantitative polymerase chain reaction (qPCR).

RNA extraction method consists in purify RNA from biological samples. The quality, purity and integrity are an important requirement for any RNA-based analysis (Bustin *et al.*, 2005). The samples have intrinsic properties that can affect yield RNA with different quality (Nolan *et al.*, 2006). Furthermore, the cell wall of gram-positive bacteria´s are constituted by a thicker layer of peptidoglycan compared with gram-negative bacteria´s and, consequently, it´s more difficult to obtain a good quantity and quality of RNA (França *et al.*, 2012) because the lysis of bacterial cell and the high content of proteins and polysaccharides, major component of the biofilm matrix, which is estimated to compromise about 90% of the total biofilm biomass (Flemming, 2010). Due to his, the bacterial cell lysis and nucleic acid purification seems to be difficult in RNA extraction methods, as well as, the purified RNA which still contains inhibitory substances (Juntilla *et al.*, 2009; Santiago-Vásquez, 2006).

Some studies showed that different RNA extraction methods can yield RNA with distinct quality and this can be explained by the complexity of biological samples (França *et al.*, 2011; Schowchow *et al.*, 2012; Shulman *et al.*, 2012). So the type of extraction method to choose is crucial: enzymatic, chemical and/or mechanical. For this case in specific, *S. epidermidis*, the best kits are based on organic extraction and a mechanical lysis step results in a higher yields. However the RNA yield and RNA purity were not a relationship directly in stability of gene expression (França *et al.*, 2011; Sieber *et al.*, 2010). Carvalhais *et al.* (2013), have recently shown that the indicators of quality did not correlate, ever, with an authentic gene expression quantification which has high relevance since the indicators that guarantee RNA quality may not to be enough to determine the authenticity of bacterial gene expression. Nonetheless, the purity and integrity can impact the accuracy of processing or analytical techniques such as complementary DNA (cDNA) synthesis and quantitative real-time PCR (Bustin *et al.*, 2005; Nolan *et al.*, 2006).

The quantification of mRNA has proved to be a useful tool to validate the transcriptional measurements associated with switching to the pathogenic mode of infection (Yao *et al.*, 2005; Beenken *et al.*, 2004; Li *et al.*, 2008; Zhu *et al.*, 2010). Due to the low half-cycle of RNA molecules, a strategy commonly used to quantify the amount of messenger RNA (mRNA) is to

convert those molecules in complementary cDNA. This is achieved by the reverse transcriptase polymerase chain reaction (RT-PCR) (França *et al.*, 2012). Some reports indicated that reverse transcription reaction is very important since during the optimization of cDNA synthesis with commercial kits and revealed high variability in the results by some of the kits tested (França *et al.*, 2012; Sieber *et al.*, 2010). Besides of that, is necessary more studies to a better understand the implication of this step.

The qPCR technique has been widely used in gene expression experiments due to its high sensitivity allowing the accurate quantification of very small amount of starting material. This method is used to amplify and quantify a targeted DNA molecule by fluorescence detection (Didenko *et al.*, 2006). The quantification can be absolute or relative: absolute quantification when the objective is to determine the exact number of copies of mRNA molecules present in the sample and relative quantification expresses the results, not as an absolute amount, but as *n* fold change on expression values (Raajevan *et al.*, 2001; Fang and Cui, 2011).

Sieber *et al.* (2010), also reported there is some variability associated with the qPCR kit used. However, what is not known is how the different experimental steps, influence the observed gene expression variability.

1.6 Study objective

The main goal of this work was to demonstrate and understand the influence in the variability of gene expression quantification, by the individual experimental steps required for gene expression quantification, namely bacterial growth, RNA extraction, reverse transcriptase and real-time quantitative PCR. Additionally, we proposed an optimized protocol to enhance gene expression reproducibility in *S. epidermidis* biofilms.

CHAPTER 2

Genetic expression variability

2.1 Introduction

Transcriptional measurements are often used to study *S. epidermidis* virulence (Batzilla *et al.*, 2006; Dai *et al.*, 2012; Fluckiger *et al.*, 2005; Handke *et al.*, 2004).

The RNA must have high quality for downstream applications like qPCR (Nolan *et al.*, 2006). Normal quality parameters include purity, integrity and yield of RNA (Fleige and Pfaffl, 2006) and, according MIQE guidelines, RNA should be pure, presents high integrity and sufficient quantity (Bustin *et al.*, 2009). All these parameters can influence gene expression. As mentioned before (in Chapter I), different RNA extraction methods (Atshan *et al.*, 2011; França *et al.*, 2011; Pinto *et al.*, 2009) as well as the complexity of biofilm matrix can yield distinct RNA quality (Radstrom *et al.*, 2004; Tichopad *et al.*, 2004). França *et al.* (2012) demonstrated that RNA quality indicators did not, ever, correlate with a reliable gene expression quantification. Furthermore, RNA isolation procedure such as ethanol or phenol may inhibit the PCR process (Bar *et al.*, 2012; Wilson, 1997). Sieber *et al.* (2010) reported that when low concentration of RNA is used, the variability in gene expression is higher. Furthermore, gene expression can also be influenced by other factors, such as contaminants or loss of RNA integrity (Carvalhais *et al.* (2013)). However, what is not known, so far, is the intrinsic contribution, by each individual experimental procedure, to the observed variation in gene expression studies.

The main objective of the work described in this chapter was to understand the origin of variability in gene expression studies, in *S. epidermidis* biofilms. To achieve that, we designed an experimental protocol that would allow us to determine the individual contributions of each of the proposed experimental steps, to the final observed variability, namely the biological variability, from one biofilm to another, the variability from different RNA extraction procedures, the variability from different reverse transcriptase reactions and finally, the variability of the qPCR quantification (Figure 2.1). The genes selected for this study have different functions in the physiology of *S. epidermidis*: *aap* gene is involved in biofilm accumulation and *icaA* gene is involved in biofilm maturation (Vandecasteele *et al*, 2003); *pgi* gene is involved in glycolysis (Gill *et al.*, 2005); *lrgB* gene is thought to be involved in induced cell death, lysis and stress environment response (Sadyvok and Bayles, 2012) and *fmtC* gene is involved in the resistance to antimicrobial peptides (Bao *et al*, 2012) and mechanism of the host´s immune system.

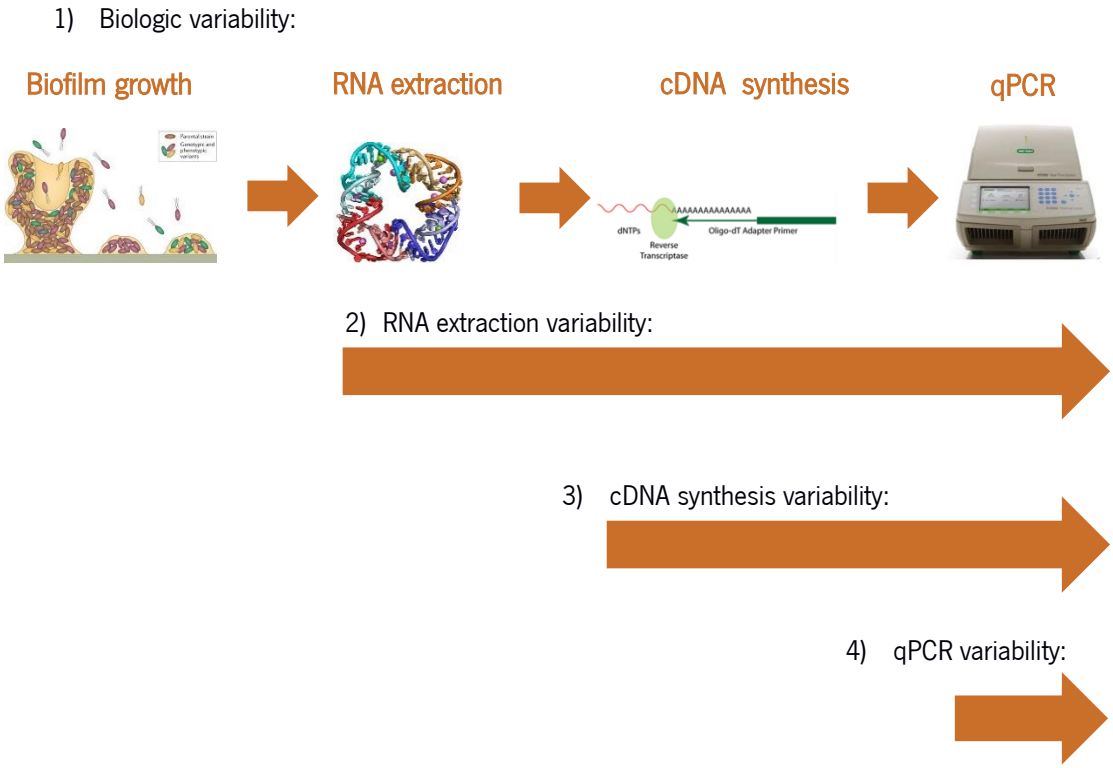


Figure 2.1 - Scheme representation of the experimental design aiming to determine the source of gene expression variability.

2.2 Materials and Methods

2.2.1 Bacterial strain and growth conditions

One well-known biofilm-forming strain was used in this study: S. *epidermidis* RP62A (PubMed accession number: PRJNA57663, ID: 57663) was replicated in petri plates (Frilabo) with Tryptic Soy Agar (TSA, Liofilchem) and were incubated for 48 H at 37° C. For the biofilm assays, two growth media were prepared: Tryptic Soy Broth (TSB, 30 g/L – Oxoid) and TSBG (TSB supplemented with 1% (w/v) of glucose).

2.2.2 Biofilm formation assays

A pre-inoculum was prepared, in plastic tubes of 10 mL (Frilabo), containing 2 mL of TSB. A fresh TSA plate with *S. epidermidis* was used as inoculum. The strain was grown overnight at

37°C with 120 rpm in a shaker-incubator (N-Biotek, NB-205Q). Then, the culture was dilute in TSBG to adjust the O.D. between 0.25-0.30 by spectrophometer reading (Spectronic® 20 Genesis, Spectronic Instruments) with 640 nm filter. After adjusted, 15 µL of the diluted suspension was placed in a 24-well plate (Thermo Scientific) with 1 mL of TSBG and incubated with same conditions for 24 H. The spent media was removed of the 24-well plates and biofilms were washed and resuspended in 1 mL of 0.9 % (w/v) Sodium Chloride (NaCl), to remove planktonic bacteria. The resuspended biofilm (dislodged by scraping) was then transfer to a 1.5 mL RNAse and DNAse free eppendorf tube (Bioplastics, Frilabo).

2.2.3 Customized RNA extraction protocol

The protocol described here combines mechanical and chemical lysis along with silica-membrane RNA isolation (E.Z.N.A.™ Total RNA, VWR, Omega Bio-Tek). Briefly, it consists in suspend bacterial pellet in 500 µL of TRK lysis buffer provided by the kit (supplemented with β-mercaptoethanol, Sigma) plus 500 µL of phenol (AppliChem, Frilabo) after centrifuged at maximum speed for 10 minutes in Centrifuge 5415R (eppendorf) and transferred into a 2 mL safe lock tubes with 0.5 g of glass beads (150-212 µm, MP Biomedicals, IIIKrich, France). This mixture was vortexed (Vortex V-1 Plus, Biosan, Frilabo) for 20 seconds before using the FastPrep® Cell disruptor (BIO 101, ThermoElectron Corporation, Thermo Scientific) with setting 6.5 and 35 seconds. The samples were then cooled on ice for 5 minutes and the beat-beading step repeated twice. Afterwards, samples were centrifuged (Centrifuge ScanSpeed mini blue, Labogene) at 12,000 *g* for 2 minutes and supernatants transferred 700 µL into a 2 mL DNAse/RNAse free tube (Bioplastics) and added equal volume of 70 % ethanol (Fisher Scientific). The samples, 700 µL, were transferred to the RNA isolation column (including any precipitate) and centrifuged (Centrifuge ScanSpeed mini blue, Labogene) at 12,000 *g* for 30 seconds at room temperature (RT). The flow-through was discarded and each column was reinserted into a new collection tube. To wash the columns, 500 µL of wash buffer I (provided by the kit) was added to each column and centrifuged (Centrifuge ScanSpeed mini blue, Labogene) at 10,000 *g* for 30 seconds at RT. The flow-through was discarded and the same collection tube was used. After that, 500 µL of was buffer II (provided by the kit) was added to each column and centrifuged (Centrifuge ScanSpeed mini blue, Labogene) at 10,000 *g* for 30 seconds at RT. The flow-through was discarded. This step was repeated one more time but either the flow-through as collection tube were discarded. Then, was reinserted into

a new collection tube and centrifuged (Centrifuge ScanSpeed mini blue, Labogene) at 16,000 *g* for 3 minutes to remove any trace of the wash buffer II that contains ethanol that is known to influence downstream applications and the columns are transferred to a 1.5 mL DNAse/RNAse free tube (Bioplastics, Frilabo). Finally, RNA elution was achieved by adding 50 µL of DEPC-treated water (provided by the kit) to the center of the membrane and centrifuged at 10,000 *g* for 1 minute.

2.2.4 DNAse Treatment

5 µL of DNase I reaction buffer (Fermentas, Frilabo) and 2 µL of DNase I (Fermentas, Frilabo) were added to 45 µL of the RNA samples, mixed thoroughly by pipetting up and down, and incubated at 37°C for 30 minutes in a thermal block (Alfagene). Then 5 µL of 25 mM EDTA (Fermentas, Frilabo) was added to the mixtures, mixed by pipetting up and down, and incubated at 65°C for 10 minutes, in a thermal block (Alfagene), to inactivate the DNase I enzyme (Fermentas, Frilabo).

2.2.5 RNA Quality Determination

The concentration and purity of the RNA sample was determined with a NanoDrop 1000™ (Thermo Scientific). The purity was achieved based in two absorbance ratios: the absorbance ratio A_{260}/A_{280} is an indicator of protein contamination and the absorbance ratio A_{260}/A_{230} is an indicator of polysaccharide, phenol and/or chaotropic salt contamination (Tavares *et al*, 2011). High quality RNA has both ratios higher than 1.8 (Glasel, 1995). Two independent measurements of each same sample were performed and average. The integrity of the total RNA was assessed by visualization of the 23S/16S banding pattern. RNA samples were analyzed in 1.0 % agarose gel stained with Midori Green DNA (Nippon Genetics Europe GmbH, Germany) at 90V. RNA was stored at -80°C until further use.

2.2.6 cDNA synthesis

The commercial kit used was RevertAaid™ First Strand cDNA Synthesis Kit (Fermentas, Frilabo) and the total RNA was normalized to a 100 µg/µL. The reverse transcriptase reaction was performed accordingly to manufacturer instructions, with small modifications with a final volume of 10 µL, as optimized before (França *et al.*, 2012). Briefly, in one 0.2 mL DNAse/RNAse free tube

(Bioplastics, Frilabo) was added the respective quantity of water, 1 µL random primers (NZYtech, VWR) and RNA in order to obtain a final volume of 10 µL. Then, were heated at 65°C for 5 minutes, in a heat block (Alfagene) and then placed on ice for 5 minutes. This heat-cool process is advisable for RNA templates GC-rich or that contains secondary structures. In a 0.2 mL DNAse/RNAse free tube (Bioplastics, Frilabo), 5 µL of the prepared RNA was added to the 2 µL of buffer (Thermo Scientific, Frilabo), 1 µL of dNTPs (Thermo Scientific, Frilabo), 0.75 µL of reverse transcriptase (Thermo Scientific, Frilabo), 0.25 µL of Ribolock (RNAse inhibitor, Thermo Scientific, Frilabo) and 1 µL of water nuclease free (Thermo Scientific, Frilabo) for each gene. In another tubes 5 µL was added of the previously prepared RNA samples to 5 µL of water (Reverse Transcriptase minus Negative control - NRT). NRT is used to determine the possibility of genomic DNA carry-over with absence of reverse transcriptase enzyme. PCR amplifications were performed using thermal cycler (MJ Mini™ Personal Thermal Cycler, Bio-rad). The following cycle for thermal cycle were 25°C during 5 minutes, 42°C during 60 minutes, 70°C during 10 minutes and 4°C forever. Primers efficiencies were determined by the dilution method.

2.2.7 Gene expression quantification

Biofilm gene expression was determined by qPCR. qPCR analysis was performed using mix iQ™ SYBR Green Supermix (Bio-Rad) in a 10 µL reaction volume. Each reaction contained 2 µL of the diluted cDNA or no-RT control (400 fold dilution of the resulting cDNA template 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 as described before (França *et al.*, 2012). Information about de primers used are listed in Table 2.1.

qPCR run was performed on a CFX 96 (Bio-Rad) with the following cycle: 95°C for 30 seconds, 39 cycles of 95°C for 5 seconds, 60°C for 15 seconds, 68°C for 15 seconds and the products were analyzed by melting curves for unspecific products or primer dimer formation. qPCR products were analyzed by melting curves to confirm that if the desired product was only amplified and all reactions were run in triplicate with two controls namely no-template control (NTC), which is composed by 5 µL of mix and 5 µL of nuclease-free water, for each set primer and NRT as mentioned above were included in each run. The expression of *aap*, *icaA*, *pgi*, *lrgB* and *fmtC* were normalized to the expression of the housekeeping gene *16S* rRNA and was performed by the *Livak*

method and the gene expression quantification was calculated by: ΔCT= CT (housekeeping gene) – CT (target gene) (Livak and Schmittgen, 2001).

Table 2.1 - Primers, amplicons, functions and melting temperature for the primers used in this study.

Target gene	Primer sequence (5´to 3´)	Amplicon size (bp)	Melting Temperature (°C)
16S	FW: GGGCTACACACGTGCTACAA	176	59,79
	RV: GTACAAGACCCGGGAACGTA		59,85
aap	FW: GCACCAGCTGTTGTTGTACC	190	59,22
	RV: GCATGCCTGCTGATAGTTCA		59,98
lrgB	FW: ATATCGCAAGCGCGAAGTAT	165	59,87
	RV: ATTGCTGTCGTTGCAGCTT		59,61
fmtC	FW: CGCCCTCATCATAGCATTG	182	60,19
	RV: CCAATTGGATCACCCAAAAC		60,03
icaA	FW: TGCACTCAATGAGGGAATCA	134	60,20
	RV: TAACTGCGCCTAATTTTGGATT		59,99
pgi	FW: TACTACGACAGAACCAGCAG	170	54,05
	RV: CATCAGGTACAACAAACGTC		53,95

Bp, base pairs; FW, forward; RV, reverse.

2.3 Results and Discussion

Gene expression in *S: epidermidis* biofilm samples has been shown to have a high variability, that can be related to the experimental protocol, as discussed in the introduction of this chapter. Here, we aimed to determine that intrinsic variability of each of the experimental steps required to quantify the expression of five independent genes.

First of all, we determined the integrity of RNA samples and eliminate all that shown significant loss of integrity (such as sample B, in figure 2.2). Then, we determined the efficiency of each primer set. The efficiency was determined at different temperatures, ranging from 50°C to 65°C. The best possible combination of efficiencies was obtained at 60°C (Table 2.2). Furthermore, in all our experiments, all RNA samples extracted were absent of significant genomic DNA, as determined by an average cycle threshold difference of 18 ± 3 which indicates a maximum error of 0.0003 %.

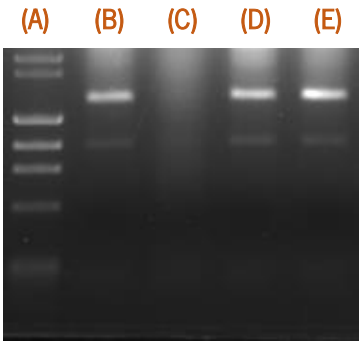


Figure 2.2 – Example of integrity of RNA samples of RP62A: (A) DNA marker, (B to E) independent RNA samples.

Table 2.2 - Efficiency of five genes studied.

Genes	*16S*	*aap*	*lrgB*	*fmtC*	*icaA*	*pgi*
Efficiency (%)	92,5	95,2	90	97,8	90	85

Since external factors can interfere in gene expression measurements, all experimental conditions were strictly maintained, namely the composition of growth medium, agitation, temperature and time of incubation, as well as the material of the 24-well plates. We started by determining the biologic variability observed in four independent 24 H biofilms. Each biofilm was formed in a different day and the RNA was extracted and stored at -80°C until further use. The reverse transcriptase and qPCR reactions were done simultaneously. Table 2.3 represents the gene expression determination for each individual qPCR run.

As it can be observed, in table 2.3, gene expression variability was dependent of the tested gene. Therefore, we calculated the coefficient of variability obtained for each gene. As can be seen in Figure 2.3, gene variability ranged from 27 % (*lrgB*) to 88 % (*icaA*). The overall gene expression variability, as determined by the average of the five tested genes variability, was 61 ± 26 %.

Interestingly, the genes that presented higher variability were *icaA* and *pgi*, both related to the carbon metabolism and subsequent biofilm formation (Cerca, 2012). The use of glucose is often considered fundamental for biofilm formation (Holá *et al.*, 2006); however, excess glucose will induce the medium acidification (Cerca *et al.*, 2011) and this may interfere with the stability of mRNA.

Table 2.3 – Quantification of gene expression normalized to *16S*, using four independent biofilms.

Sample	*aap*	*lrgB*	*fmtC*	*icaA*	*pgi*
Biofilm 1	2,32E-04	5,91E-05	7,32E-05	2,04E-04	8,46E-04
	2,96E-04	5,54E-05	6,84E-05	2,15E-04	1,08E-03
	3,18E-04	6,59E-05	7,08E-05	1,82E-04	8,76E-04
Biofilm 2	5,49E-05	4,88E-05	1,85E-05	2,28E-05	1,39E-04
	6,05E-05	4,74E-05	1,86E-05	2,51E-05	1,15E-04
	6,87E-05	4,73E-05	2,08E-05	2,22E-05	1,45E-04
Biofilm 3	1,35E-04	5,91E-05	3,12E-05	4,70E-05	2,43E-04
	1,18E-04	5,68E-05	3,15E-05	4,73E-05	1,16E-04
	1,33E-04	5,17E-05	3,36E-05	2,77E-05	3,19E-04
Biofilm 4	1,97E-04	8,99E-05	4,25E-05	5,25E-05	2,94E-04
	1,79E-04	9,55E-05	5,10E-05	6,97E-05	2,63E-04
	2,78E-04	8,65E-05	7,41E-05	8,07E-05	2,60E-04

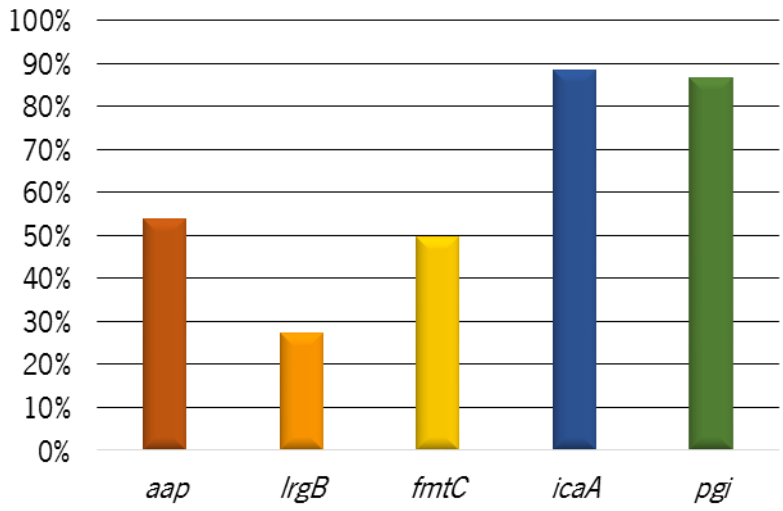


Figure 2.3 - Coefficient of biological variability (in %) of gene expression of *aap*, *lrgB*, *fmtC*, *icaA*, *pgi*.

After assessing the variability associated with independent biological replicates, we proceeded with the next stage of our experimental design. In order to determine the intrinsic variability obtained from the RNA extraction procedure, we performed four different RNA extractions, from the same biological sample, randomly selected from our pool of biofilms. Table 2.4 represents the gene expression found, originated from independent RNA extractions within the same biological sample, therefore eliminating the biological variability.

Table 2.4 – Quantification of gene expression normalized to *16S*, using four different RNA extractions from the same biofilm.

Sample	*aap*	*lrgB*	*fmtC*	*icaA*	*pgi*
RNA 1	2,32E-04	5,91E-05	1,28E-04	2,04E-04	8,46E-04
	2,96E-04	5,54E-05	1,20E-04	2,15E-04	1,08E-03
	3,18E-04	6,59E-05	1,24E-04	1,82E-04	8,76E-04
RNA 2	3,17E-04	4,59E-05	8,39E-05	1,80E-04	2,33E-04
	2,85E-04	4,62E-05	8,33E-05	1,98E-04	3,23E-04
	3,01E-04	4,27E-05	6,59E-05	1,68E-04	5,16E-04
RNA 3	4,91E-04	6,97E-05	1,06E-04	2,44E-04	8,54E-04
	4,98E-04	7,11E-05	1,01E-04	2,02E-04	7,85E-04
	4,66E-04	5,55E-05	8,29E-05	2,27E-04	5,98E-04
RNA 4	4,47E-04	7,26E-05	8,58E-05	2,29E-04	7,79E-04
	4,47E-04	6,22E-05	7,91E-05	2,27E-04	5,61E-04
	4,78E-04	5,82E-05	8,52E-05	2,35E-04	5,24E-04

Not surprisingly, without the biological variability into account, the coefficient of variability observed was smaller, ranging from 12 % (*icaA*) to 37 % (*pgi*), as can be seen in Figure 2.4. The overall gene expression variability was 23 % ± 9.7 %.

In literature, this step is known to influenciate gene expression (França *et al.*, 2012 and Sieber *et al.*, 2010). In this study, the variability was not significant for majority of the genes. However, *pgi* gene had a considerable variability.

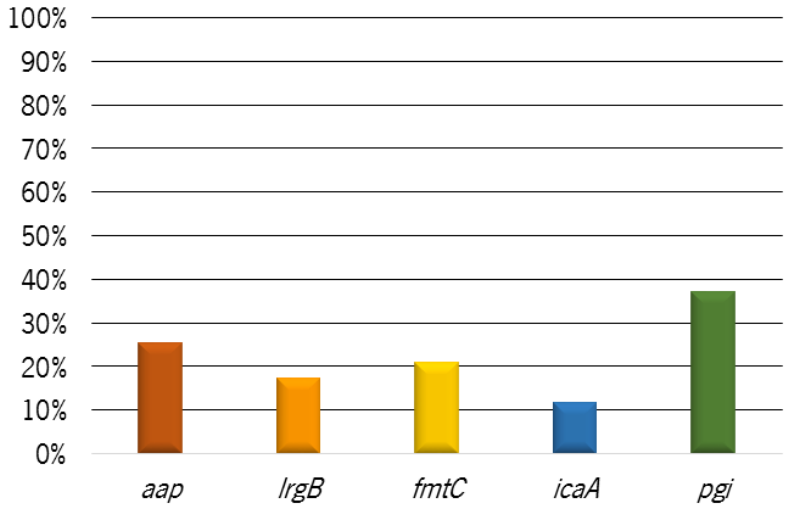


Figure 2.4 - Coefficient of RNA extraction variability (in %) of gene expression of *aap*, *lrgB*, *fmtC*, *icaA*, *pgi*.

Sieber *et al.* (2010), was among the first to address the importance of that transcription reverse procedure in stable gene expression. They determined that gene expression reliability would not be achievable if using low concentrations of starting RNA. However, the intrinsic strength of the process was not determined. After assessing the variability associated with different RNA extractions, we proceeded with cDNA synthesis variability. To achieve that, we performed four independent cDNA synthesis from the same RNA sample, that was randomly selected from pool of samples. Table 2.5 represents gene expression determination from different cDNA obtained from the same RNA sample.

Interestingly, the overall gene expression variability, as determined by the average of each gene variability, was 24 % ± 4.8 %, not much different from the overall gene expression variability observed in the previous experimental step.

Table 2.5 – Quantification of gene expression normalized to *16S*, using four cDNA synthesis obtained from the same RNA sample.

Sample	*aap*	*lrgB*	*fmtC*	*icaA*	*pgi*
cDNA 1	2,32E-04	3,92E-05	8,81E-05	2,04E-04	8,46E-04
	2,96E-04	3,67E-05	8,24E-05	2,15E-04	1,08E-03
	3,18E-04	4,39E-05	8,52E-05	1,82E-04	8,76E-04
cDNA 2	4,80E-04	6,52E-05	1,73E-04	2,12E-04	7,77E-04
	4,90E-04	6,60E-05	1,81E-04	2,30E-04	8,35E-04
	5,43E-04	7,10E-05	1,41E-04	2,42E-04	7,76E-04
cDNA 3	4,75E-04	7,46E-05	1,58E-04	2,32E-04	5,16E-04
	4,47E-04	5,82E-05	1,51E-04	2,78E-04	2,07E-04
	5,27E-04	5,76E-05	1,28E-04	1,49E-04	5,38E-04
cDNA 4	5,89E-04	6,99E-05	1,57E-04	1,91E-04	7,85E-04
	6,06E-04	8,06E-05	1,21E-04	2,69E-04	7,06E-04
	5,38E-04	5,71E-05	1,43E-04	2,61E-04	7,65E-04

The variability was similar between all genes. Although, the gene that presented more variability was *pgi* (30 %) and *icaA* (17 %) with less variability (Figure 2.5). In cDNA synthesis, no discrepancies of expression were seen between different genes.

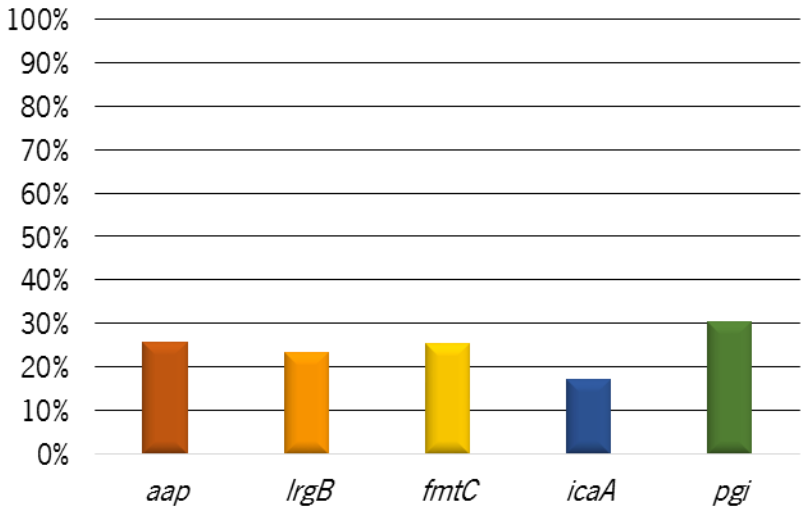


Figure 2.5 - Coefficient of reverse transcriptase variability (in %) of gene expression of *aap*, *lrgB*, *fmtC*, *icaA*, *pgi*.

After determining the gene expression variability from the experimental processes required to obtain and prepare the sample, we then had to quantify the variability of the quantification step of the experiment: the qPCR run. Of note, since this step was always present, in the previous experiments, this last experimental set-up could be considered as the blank of the experiment. We randomly selected one cDNA sample and performed four independent qPCR runs. The results of this experiment are represented in Table 2.6.

Table 2.6 – Quantification of gene expression normalized to *16S*, in four qPCR experiences from the same cDNA.

Sample	*aap*	*lrgB*	*fmtC*	*icaA*	*pgi*
qPCR 1	3,18E-04	7,68E-05	5,03E-05	1,90E-04	1,00E-03
	3,18E-04	6,62E-05	4,63E-05	2,29E-04	1,28E-03
	3,40E-04	6,21E-05	4,99E-05	2,03E-04	1,04E-03
qPCR 2	2,54E-04	5,47E-05	4,24E-05	1,92E-04	1,35E-03
	2,58E-04	5,28E-05	4,81E-05	2,32E-04	1,32E-03
	2,72E-04	5,56E-05	5,31E-05	1,78E-04	9,64E-04
qPCR 3	2,24E-04	6,13E-05	5,51E-05	2,44E-04	1,46E-03
	2,97E-04	5,73E-05	6,36E-05	2,57E-04	1,29E-03
	2,85E-04	5,53E-05	5,74E-05	2,17E-04	7,23E-04
qPCR 4	3,34E-04	5,89E-05	5,81E-05	2,16E-04	8,60E-04
	3,17E-04	4,45E-05	5,73E-05	1,65E-04	1,47E-03
	2,53E-04	4,65E-05	5,72E-05	1,79E-04	1,48E-03

Not surprisingly, the overall gene expression variability, as determined by the average of each gene variability, was the lowest of all the experimental steps: 15 % ± 4.1 %. The gene that presented more variability was *pgi* (22 %) and *fmtC* (11 %) with less variability (Figure 2.6).

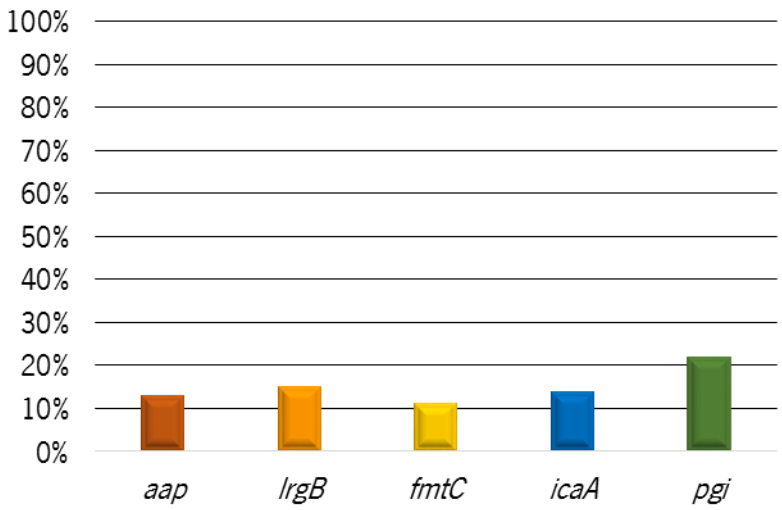


Figure 2.6 - Coefficient of qPCR variability (in %) of gene expression of *aap*, *lrgB*, *fmtC*, *icaA*, *pgi*.

Overall, our study suggests that the observed variability in *S. epidermidis* biofilm gene expression studies is mainly the result of the biologic variability obtained in this complex microbial community.

A major limitation of our study was the use of only five genes. In order to be as representative as possible, we selected genes locate in different positions of the genome and also with distinct functions. Furthermore, since the samples were randomly selected for each experimental step, we can't guarantee that a different selection would result in the same outcome. Ideally, this process should be repeated for each of the samples used. However, due to the costs associated, this was not possible to perform.

CHAPTER 3

Optimization of a protocol to reduce gene expression variability in *S. epidermidis* biofilms

3.1 Introduction

Gene expression studies are an important analysis tool since it allows evaluating and understanding the influence of the genes and respective functions in biofilm formation. This confers virulence to *S. epidermidis* strains and consequently, the incidence of persistent infections (factor (O´Gara and Humphreys, 2001). As well known, biofilm formation depends on some conditions, namely the strains used, growth medium and hydrodinamic conditions (Cerca, N., 2006). Regarding to *S. epidermidis* strains is known that different strains of the same species can be phenotypically and genetically different (Cerca, N., 2006). In this chapter, it will be discussed biofilm formation between some *S. epidermidis* strains and the presence of genes of interest in this study (*aap*, *icaA*, *fmtC*, *lrgB* and *pgi*).

The main objective of the work described in this chapter was to reduce the coefficient of biologic variability in gene expression which presented the majority of variability as discussed in Chapter 2, in *S. epidermidis* biofilms. To achieve that, we proposed an experimental protocol that we hypothesized allow us to diminish the variability from a pool of 20 biofilms (Figure 3.1).

Figure 3.1 - Scheme representation of a proposed experimental protocol aiming to reduce the coefficient of gene expression variability.

3.2 Material and Methods

3.2.1 Bacterial strain and growth conditions

The bacterial strain and growth conditions were performed as described in previous chapter but the strains used were S. *epidermidis* RP62A (PubMed accession number: PRJNA57663, ID: 57663), *S. epidermidis* 9142 (Mack *et al.*, 1994), *S. epidermidis* M129 (Cerca, N., 2006), *S. epidermidis* FJ6 (Cerca, N., 2006), *S. epidermidis* JI6 (Cerca, N., 2006), *S. epidermidis* LE7 (Cerca, N., 2006), *S. epidermidis* IE186 (Cerca, N., 2006) and *S. epidermidis* 1457 (Mack *et al.*, 1992).

3.2.2.1 Biofilm formation disruption

After the respective incubation time and the washing with saline solution, each biofilm was resuspended in 1 mL of 0,9 % (w/v) Sodium Chloride (NaCl) and dislodged by scraping followed by sonication (Cole-Parmer® 750-Watt Ultrasonic Homogenizer, 230 VAC, using a 13 mm microtip) using one cycle: 10 seconds at 30 % of amplitude (3 times each). The 1.5 mL eppendorf tubes (Bioplastics, Frilabo) were kept on ice during sonication (Freitas *et al.*, 2013).

3.2.3 Genomic DNA Extraction and PCR

The protocol described here consists in DNA extraction based on thermal shock (Henriques, A. *et al.*, 2012). Briefly, it consists in 100 µL of ultrapure water plus 10 µL of vial suspension. Then, the samples are incubated 10 min at 100°C, in a thermal block (Alfagene) followed by 5 min on ice. After that, centrifuged at maximum speed 10 min at 4°C. The suspension was removed for a new 1.5 mL eppendorf tubes. For PCR, the mix to each sample are composed by 5 µL of dymezyme (Thermo Scientific, Frilabo), 3 µL of water, 1 µL of primer set and 1 µL of sample. PCR amplifications were performed using thermal cycler (MJ Mini™ Personal Thermal Cycler, Bio-rad). The following cycle for thermal cycle were 94°C during 2 minutes, 94°C during 30 seconds, 58°C during 20 seconds, 72°C during 20 seconds, 72°C during 5 minutes and 4°C forever. 35 cycles were performed between 94°C (30 seconds) to 72°C (5 minutes). DNA samples were analyzed in 1.5 % agarose gel stained with Midori Green DNA (Nippon Genetics Europe GmbH, Germany) at 80 V. DNA samples in the presence of genes of interest have one band with respective size per gene.

3.3 Optimized quantification of gene expression

Gene expression quantification was performed as described in the previous chapter, with a minor modification. Here, a pool of 20 biofilms was resuspended in each 1.5 mL DNase/RNase free tubes, and from each tube two independent RNA extractions were performed.

3.4 Results and Discussion

To validate our findings in a clinical setting, we first started to determine the biofilm formation ability using different *S. epidermidis* strains, selected from a group of commensal and pathogenic isolates (Table 3.1). As expected, we found a high variability of biofilm formation amongst the strains used (Figure 3.2). Comparing all strains used, the clinical isolate (RP62A) formed more biofilm while the commensal isolate (FJ6) less biofilm, in 24 H.

Table 3.1 - Origin of different *S. epidermidis* strains.

Strain	Origin of strain
RP62A	ATCC
9142	Biofilm positive control - clinical isolate (Mack et al., 1994)
M129	Isolated from dialysis-associated peritonitis patients (Cerca, N., 2006)
IE186	Isolated from infective endocarditis patients (Cerca, N., 2006)
1457	Isolated from central venous catheter-associated infection (Mack et al., 1992)
FJ6	Strains isolated from the skin (Cerca, N., 2006)
JI6	
LE7	

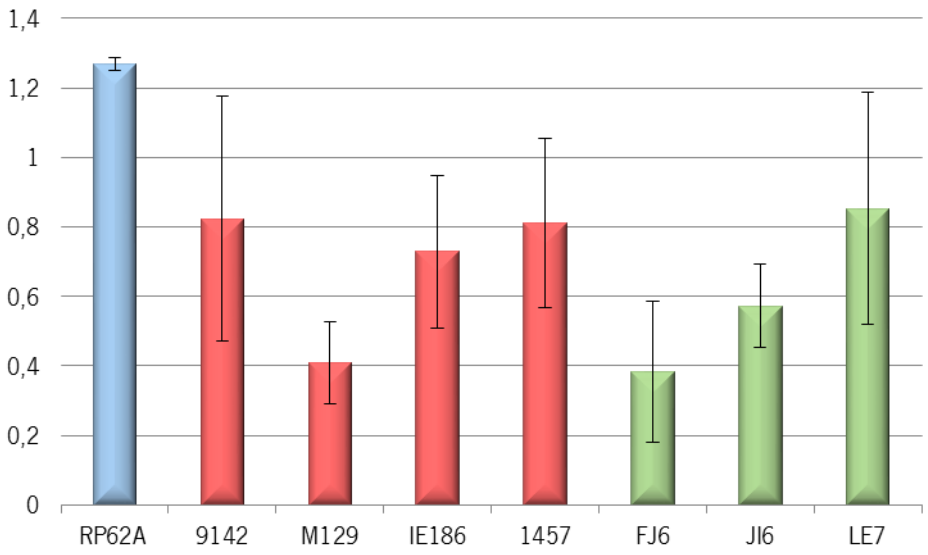


Figure 3.2 - Biofilm formation, at 24 H, was quantified by a spectrophotometry (640 nm filter): ATCC strain at blue color, clinical strains at red color and commensal strains at green color.

After determining the biofilm formation ability, we then wanted to know if the selected strains had the presence of the genes tested in the previous chapter (*aap*, *lrgB, fmtC*, *icaA* and *pgi*). As can be shown in Table 3.2, most *S. epidermidis* had all the five genes but some lacked either *aap*, *icaA* or *pgi*. Taking in account the capacity of biofilm formation, we decided to include strain 9142 in the gene expression studies.

Table 3.2 – Presence of genes of interest by PCR by two independent primer sets.

Strains	Genes				
	aap	*lrgB*	*fmtC*	*icaA*	*pgi*
RP62A	+	+	+	+	+
9142	+	+	+	+	+
M129	+	+	+	-	+
IE186	+	+	+	+	-
1457	+	+	+	+	-
FJ6	+	+	+	+	+
JI6	+	+	+	+	+
LE7	-	+	+	+	+

+, presence of gene; -, absence of gene or primer dimer.

Since biological replicates had more variability, compared with other steps (discussed in Chapter 2), we decided to pool 20 biofilms, in order to diminish the biological variability coefficient. We tested two of the five genes: *aap*, which presented medium coefficient of gene expression variation and *pgi* wich presented an higher coefficient of gene expression variation. The comparison results between biological replicates (Chapter 2) and two optimization assays (two replicates of each) of the *aap* gene can be observed in Figure 3.3 and *pgi* gene in Figure 3.4.

Based on our results, we can observe that our strategy of pooling 20 biofilms, resulted in a reduction of the coefficient of *aap* expression variation from 54 % to 18 % (Figure 3.3). Interestingly, the same was observed for the expression of *pgi* gene (reduction from 87 % to 34 %) (Figure 3.4).

In order to confirm that our findings were valid on clinical isolates, at the same time confirming that they were strain independent, we repeated the same experience using strain 9142. As can be seen in Figure 3.5 (*aap*) and Figure 3.6 (*pgi*), our strategy allowed a reduction of the coefficient of gene expression variation from 94 % to 34 % (*aap*) and from 84 % to 43 % (*pgi*).

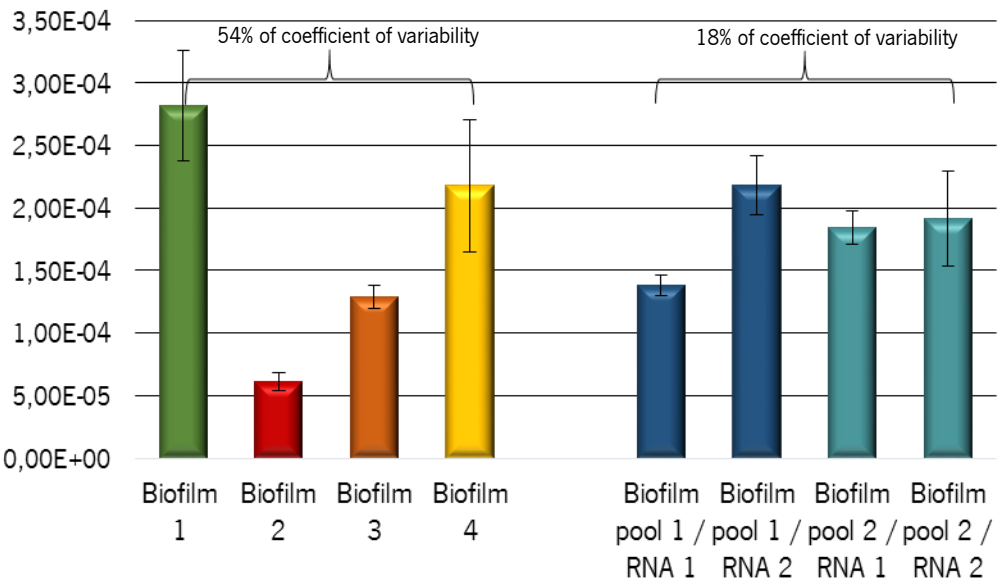


Figure 3.3 - Comparison of biologic variability coefficient, in *aap* gene, without and with a pool of 20 biofilms (RP62A strain).

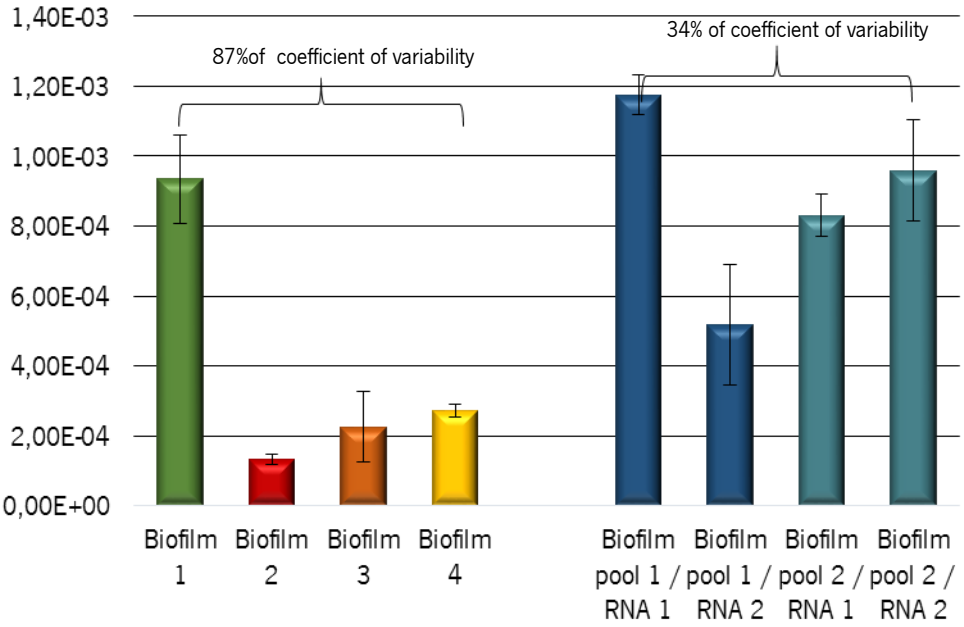


Figure 3.4 - Comparison of biologic variability coefficient, in *pgi* gene, without and with a pool of 20 biofilms (RP62A strain).

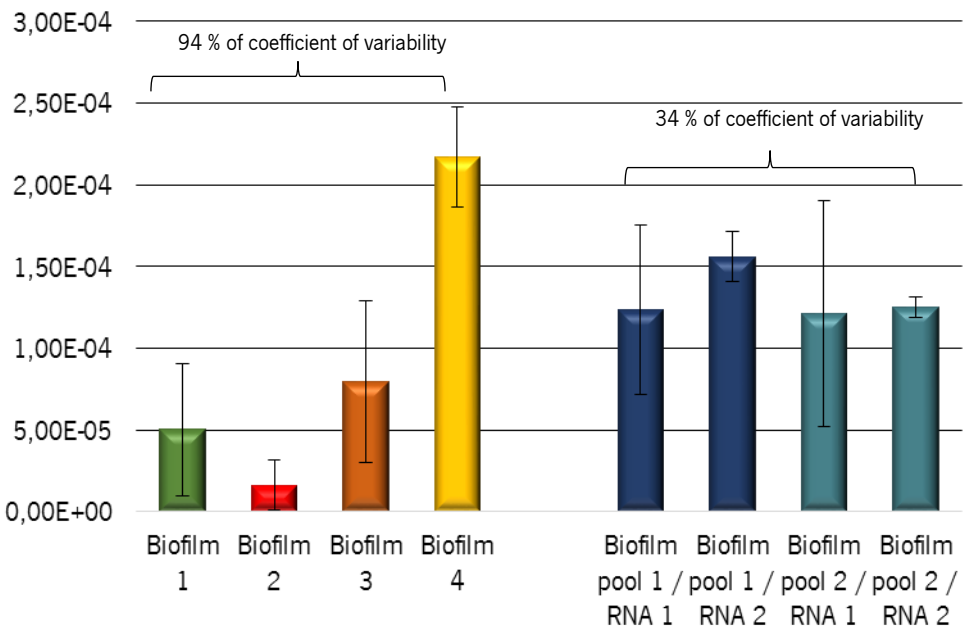


Figure 3.5 - Comparison of biologic variability coefficient, in *aap* gene, without and with a pool of 20 biofilms (9142 strain).

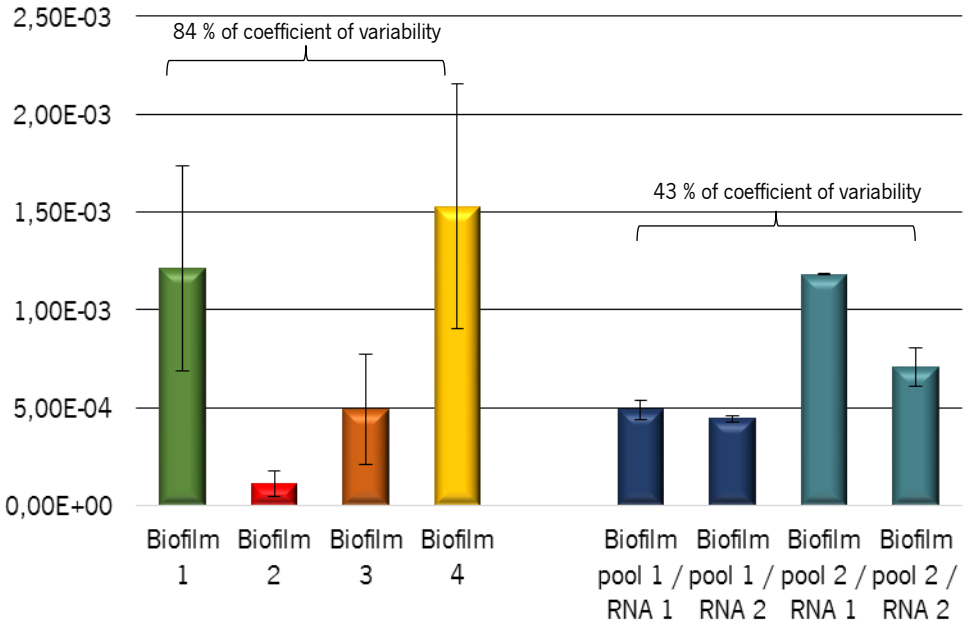


Figure 3.6 - Comparison of biologic variability coefficient, in *pgi* gene, without and with a pool of 20 biofilms (9142 strain).

Overall, our results confirmed that the strategy of pooling 20 biofilms in each RNA extraction, successfully reduced the biological variability in gene expression studies of *S.*

epidermidis biofilms. An interesting question that was not answered was the minimum number of biofilms to be pooling, in order to achieve this reduction in variability. On the other hand, we did not explore if by using a higher number of biofilms, per pool, would further reduced the biological variability. Nevertheless, the principle of pooling the biofilms as a strategy of reducing the variability of the gene expression studies was demonstrated. These results provide an important insight for researchers working with gene expression in biofilms, since our strategy will allow more reproducible and accurate results.

CHAPTER 4

Conclusion remarks and Future perspectives

As described throughout this thesis, *S. epidermidis* is a commensal habitant of human skin but the biofilms formed by this species are responsible for many infections in indwelling medical devices. To better understand how biofilms enhance *S. epidermidis* virulence, it is important to perform gene expression studies with biofilms. However, due to the complex nature of biofilms, RNA extraction can, sometimes, be a problem. One phenomena observed has to do with the high variability of gene expression in biofilms but the origin of this variability was unknown. Therefore, in order to demonstrate and understand the variability of gene expression quantification, we aimed to study the individual experimental steps, namely bacterial growth, RNA extraction, reverse transcriptase reaction and real-time quantitative PCR and its impact on the gene expression variability.

In response to the principal aim of this work, our results revealed that bacterial growth presented more variability, although this percentage differs between genes. The others experimental steps did not revealed a significant variability for the majority of the genes of interest.

Additionally, we proposed an optimized protocol to minimize the variability inherent to biological step and to enhance gene expression reproducibility. We demonstrated that is possible minimize this variability using a pool of 20 biofilms in a single RNA extraction.

A limitation of our study was the use of few genes and strains. In future works, it would be necessary increase the number of strains and test all experimental steps, since we only performed all the tests in RP62A, mainly due the costs associated. Furthermore, the increase of genes could be interesting and clearly, more assays allow comparing the results with a higher deepness. Finally, it would also be interesting to test different quantity of biofilm pools, in order to demonstrate the minimum biofilm pool that may reduce, substantially, the variability. By following this approach, it would be possible to maintain gene expression quantification with more specificity and authenticity.

REFERENCES

Abdelal, A. (1979). Arginine catabolism by microorganisms. *Annu. Rev. Microbiol.*, 33: 139–168.

Anderson, J., McNally, A. (2011). Biocompatibility of implants: lymphocyte/macrophage interactions. *Semin Immunopathol.*, 33: 221–33.

Anderson, J., Rodriguez, A., Chang, D. (2008). Foreign body reaction to biomaterials. *Semin Immunol.*, 20: 86–100.

Arciola, C., Compoccia, C., Speziale, P., Montanaro, M., Costerton, J. (2012). Biofilm formation in *Staphylococcus* implant infections. A review of molecular mechanisms and implications for biofilm-resistant materials. *Biomaterials*, 33: 5967-5982.

Arciola, C., *et al.* (2005). Antibiotic resistance in exopolysaccharide-forming *Staphylococcus epidermidis* clinical isolates from orthopaedic implant infections. *Biomaterials*, 26(33):6530e5.

Arrecubieta, C. *et al.* (2009). *Sdr*F, a *Staphylococcus epidermidis* surface protein, contributes to the initiation of ventricular assist device driveline-related infections. *Plos Pathogen*, 5 (5): e1000411.

Atshan, S., Shamsudin, M., Lung, L., Ling, K., Sekawi, Z., Pei, C., Ghaznavi-Rad, E. (2012). Improved method for the isolation of RNA from bacteria refractory to disruption, including *S. aureus* producing biofilm. *Gene*, 494: 219-224.

Azevedo, N., Cerca, N. (2012). Biofilmes na Saúde, no Ambiente e na Indústria. *Publindústria*, I:3-26.

Baldassarri, L., Donelli, G., Gelosia, A., Simpson, A., Christensen, G. (1997). Expression of slime interferes with in vitro detection of host protein receptors of *Staphylococcus epidermidis*. *Infect. Immun.*, 65: 1522-1526.

Bao, Y. *et al.* (2012). Role of *mrp*F1 and *mrp*F2 in the Pathogenicity of *Enterococcus faecalis*. *Plos One*, 7(6): e38458 Bar, T., Kubista, M., Tichopad, A. (2012). Validation of kinetics similarity in qPCR. *Nucleic Acids Res.*, 40: 1395-1406.

Batoni, G. *et al.* (2010). Use of antimicrobial peptides against microbial biofilms: advantages and limits. *Curr. Med. Chem.*, 18: 256–79.

Batzilla, C., Rachid, S., Engelmann, S., Hecker, M., Hacker, J., Ziebuhr, W. (2006). Impact of the accessory gene regulatory system (*Agr*) on extracellular proteins, codY expression and amino acid metabolism in *Staphylococcus epidermidis*. *Proteomics*, 6: 3602-3613.

Beenken, K. *et al*. (2004) Global gene expression in *Staphylococcus aureus* biofilms. *J. Bacteriol.*, 186: 4665–4684.

Begun, J. *et al.* (2007*). Staphylococcal* biofilm exopolysaccharide protects against *Caenorhabditis elegans* immune defences. *Plos Pathogen,* 3: 57.

Boles, B., Horswill, A. (2008). *Agr*-mediated dispersal of *Staphylococcus aureus* biofilms. *Plos Pathogen*, 4(4): e1000052.

Bowden, M. *et al*. (2005). Identification and preliminar characterization of cell-wall-anchored proteins of *Staphylococcus epidermidis*. *Microbiology*, 151(5): 1453–1464.

Brennan, M. *et al*. (2009). Elucidating the role of *Staphylococcus epidermidis* serine-aspartate repeat protein G in platelet activation. *J. Thromb. Haemost.*, 7(8): 1364–1372.

Bustin, S., Benes, V., Nolan, T., Pfaffl, M. (2005). Quantitative real-time RT-PCR- a perspective. *J. Mol. Endocrinol.*, 34: 597-601.

Bustin, S., Nolan, T. (2004). Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction. *J. Biomol. Tech.*, 15: 155-166.

Carmody, A., Otto, M. (2004). Specificity grouping of the accessory gene regulator quorum-sensing system of *Staphylococcus epidermidis* is linked to infection. *Arch. Microbiol.*, 181: 250–253.

Carvalhais, V., Delgado-Rastrollo, M., Melo, L. and Cerca, N. (2013). Controlled RNA contamination and degradation and its impact on qPCR gene expression in *S. epidermidis* biofilms. *J. Microbial Methods*, 95(2): 195-200.

CDC. National Nosocomial Infections Surveillance (NNIS) system report (2004). *J. Infect. Control*, 32: 470-485.

Cerca, N. (2006). Virulence aspects of *Staphylococcus epidermidis*: Biofilm formation and Poly-*N*-Acetyl Glucosamine production. Dissertation of PhD degree. University of Minho, Braga, Portugal.

Cerca, N. (2011). Biofilm Transcriptomics handbook: Quantifying gene expression from pathogenic bacterial biofilms. I: 13-71.

Cerca, N. (2012). Modulation and analysis of the *Staphylococcus epidermidis* biofilm physiological state. PhD in Biomedical Sciences, ICBAS, University of Porto, Porto, Portugal.

Cerca, N., Brooks, J., Jefferson, K. (2008). Regulation of the intercellular adhesin locus regulator (*ica*R) by *Sar*A, *Sigma*B, and *ica*R in *Staphylococcus aureus*. *Journal of Bacteriology*, 190(19): 6530-3.

Cerca, N. *et al*. (2011). Modulation of poly-*N*-acetylglucosamine accumulation within mature *Staphylococcus epidermidis* biofilms grown in excess glucose. *Microbiol. Immunol*., 55: 673-682.

Cerca, N., Jefferson, K., Oliveira, R., Pier, G., Azeredo, J. (2006). Comparative antibody-mediated phagocytosis of *Staphylococcus epidermidis* cells grown in a biofilm or in the planktonic state. *Infect. Immun*., 74(8): 4849-55.

Cerca, N., Pier, G., Vilanova, M., Oliveira, R., Azeredo, J. (2004). Influence of batch or fed-batch growth on *Staphylococcus epidermidis* biofilm formation. *Letters in Applied Microbiology*, 30: 420-424.

Chambers, H., Hartman, B., Tomasz, A. (1985). Increased amounts of a novel penicillin-binding protein in a strain of methicillin-resistant *Staphylococcus aureus* exposed to nafcillin. *J. Clin. Invest*., 76: 325–331.

Chen, X., Schauder, N., Potier, A., Van Dorsselaer, I., Pelczer, B., Bassler, L., Hughson, F. (2002). Structural identification of a bacterial *quorum sensing* signal containing boron. *Nature*, 415: 545–549.

Cheung, G. *et al*. (2010). *Staphylococcus epidermidis* Strategies to Avoid Killing by Human Neutrophils. *Plos Pathogens*, 6(10): e1001133.

Chokr, A., Leterme, D., Watier, D., Jabbouri, S. (2007). Neither the presence of *ica* locus, nor *in vitro*-biofilm formation ability is a crucial parameter for some *Staphylococcus epidermidis* strains to maintain an infection in a guinea pig tissue cage model. *Microb. Pathog*., 42: 94–97.

Chu, V. *et al.* (2009). Coagulase-negative staphylococcal prosthetic valve endocarditis — a contemporary update based on the International Collaboration on Endocarditis: prospective cohort study. *Heart*, 95: 570–576.

Cockayne, A. *et al*. (1998). Molecular cloning of a 32-kilodalton lipoprotein component of a novel ironregulated *Staphylococcus epidermidis* ABC transporter. *Infect. Immun.*, 66: 3767–3774.

Congen, A., Nizet, V., Gallo, R. (2008). Skin microbiota: a source of disease or defence?. *Br. J. Dermatol.*, 158(3): 442-455.

Conlon, K., Humphreys, H., O'Gara, J. (2004). Inactivations of *rsb*U and *sar*A by IS256 represent novel mechanisms of biofilm phenotypic variation in *Staphylococcus epidermidis*. *J. Bacteriol.*, 186(18): 6208–6219.

Conrady, D., Brescia, C., Horii, K., Weiss, A., Hassett, D., Herr, A. (2008). A zinc-dependent adhesion module is responsible for intercellular adhesion in staphylococcal biofilms. *Proc. Natl Acad. Sci.*, 105(49): 19456–19461.

Costerton, J., Stewart, P., Greenberg, E. (1999) Bacterial biofilms: a common cause of persistent infections. *Science*, 284:1318–1322.

Dai, L. *et al*. (2012). *Staphylococcus epidermidis* recovered from indwelling catheters exhibit enhanced biofilm dispersal and "self-renewal" through down-regulation of *agr*. *BMC Microbiology*, 12: 102.

Darouiche R. (2004). Treatment of infections associated with surgical implants. *N. Engl. J. Med.*, 350: 1422–9.

De Man, F. *et al*. (2011). Infectiological, functional, and radiographic outcome after revision for prosthetic hip infection according to a strict algorithm. *Acta Orthop.*, 82: 27–34.

Didenko, V. (2006). Fluorescent energy transfer nucleic acid probes: designs and protocols. *Human Press*, New Jersey, USA.

Diekema, D. *et al*. (2001). Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997–1999. *Clin. Infect. Dis.*, 32: S114–S132.

Diep, B. *et al*. (2008). The arginine catabolic mobile element and staphylococcal chromosomal cassette *mec* linkage: convergence of virulence and resistance in the USA300 clone of methicillin-resistant *Staphylococcus aureus*. *J. Infect. Dis*., 197: 1523–1530.

Donlan, R., Costerton, J. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.*,15(2): 167-193.

Euzeby, J. (2014). List of Prokaryotic names with Standing in Nomenclature - Genus *Staphylococcus*. Available at http://www.bacterio.cict.fr/s/staphylococcus.html [Acessed 31 January 2014].

Fang, Z., Cui, X. (2011). Design and validation issues in RNA-seq experiments. *Brief Bioinform.*, 12: 280–287.

Fey, P., Olson, M. (2010). Current concepts in biofilm formation of *staphylococcus epidermidis*. *Future microbiol*., 5(6): 917-933.

Fluckiger, U. *et al*. (2005). Biofilm formation, *icaADBC* transcription, and polysaccharide intercelular adhesin synthesis by staphylococci in a device related infection model. *Infect. Immun.*, 73: 1811–1819.

Fluckiger, U., Ulrich, M., Steinhuber, A., Doring, G., Mack, D., Landmann, R., Goerke, C., Wolz, C. (2005). Biofilm formation, *ica*ADBC transcription, and polysaccharide intercellular adhesin synthesis by staphylococci in a device-related infection model. *Infect. Immun*., 73: 1811-1819.

França, A., Bento, J. Cerca, N. (2011). Variability of RNA Quality Extracted from Biofilms of Foodborne Pathogens Using Different Kits Impacts mRNA Quantification by qPCR. *Curr. Microbiol*., 65: 54-59.

França, A., Freitas, A., Henriques, A. And 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(5).

Freeman, D., Falkiner, F., Keane, C. (1989). New method for detection of slime production by coagulase-negative staphylococci. *J. Clin. Pathol*., 42: 872–874.

Freitas, A., 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*, 00: 1-8.

Fuqua, W., Winans, S. and Greenberg, E. (1994). *Quorum sensing* in bacteria: the *Lux*R-*Lux*I family of cell density-responsive transcriptional regulators. *J. Bacteriol.*, 176: 269–275.

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. *J. Biol. Chem.*, 273: 18586–18593.

Gill, S. *et al*. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistance *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistance *Staphylococcus epidermidis* strain. (2005). *Journal of Bacteriology*, 187 (7): 2426-2438.

Glasel, J. (1995). Validity of Nucleic Acid Purities Monitored by A260/A280 Absorbance Ratios. *Biotechniques*, 18: 62-63.

Gross, M., Cramton, S., Gotz, F. and Peschel, A. (2001). Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces. *Infect. Immun.*, 69: 3423-3426.

Handke, L., Conlon, K., Slater, S., Elbaruni, S., Fitzpatrick, F., Humphreys, H., Giles, W., Rupp, M., Fey, P., O'Gara, J. (2004). Genetic and phenotypic analysis of biofilm phenotypic variation in multiple *Staphylococcus epidermidis* isolates. *J. Med. Microbiol.*, 53: 367-374.

Handke, L. *et al*. (2007). ∂^{B} and *Sar*A independently regulate polysaccharide intercellular adhesion production in *Staphylococcus epidermidis*. *Can. J. Microbiol.*, 53(1): 82–91.

Hartford, O., O'Brien, L., Schofield, K., Wells, J., Foster, T. (2001). The *Fbe* (*Sdr*G) protein of *Staphylococcus epidermidis* HB promotes bacterial adherence to fibrinogen. *Microbiology*, 147: 2545–2552.

Heilmann, C. *et al*. (1996). Molecular basis of intercelular adhesion in the biofilm-forming *Staphylococcus epidermidis*. *Mol. Microbiol.*, 20: 1083-1091.

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.

Holá, V., Ruzicka, F., Votava, M. (2006). The dinamics of *Staphylococcus epidermidis* biofilm formation in relation to nutrition, temperature and time. *Scripta Medica (Brno)*, 79(3): 169-174.

Houston, P., Rowe, S., Pozzi, C., Waters, E., O'Gara, J. (2011). Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype. *Infect. Immun.*, 79: 1153e65.

Howden, B., Davies, J., Johnson, P., Stinear, T., Grayson, M. (2010). Reduced vancomycin susceptibility in *Staphylococcus aureus*, including vancomycin –intermediate and heterogenous vancomycin – intermediate strains: resistance mechanisms, laboratory detection, and clinical implications. *Clinical Microbiology Reviews*, 23: 139-199.

Hoyle, B., Alcantara, J., Costerton, J. (1992). *Pseudomonas aeruginosa* biofilm as a diffusion barrier to piperacillin. *Antimicrob. Agents Chemoter.*, 36(9): 2054-6.

Janzon, L., Lofdahl, S., Arvidson, S. (1989). Identification and nucleotide sequence of the delta-lysin gene, *hld*, adjacent to the accessory gene regulator (*agr*) of *Staphylococcus aureus*. *Mol. Gen. Genet.*, 219: 480e5.

Junttila, S., Lim, K., Rudd, S. (2009). Optimization and comparison of different methods for RNA isolation for cDNA library construction from the reindeer lichen *Cladonia rangiferina*. *BMC Res. Notes*, 2: 204.

Kaplan, J. (2009). Therapeutic potential of biofilm-dispersing enzymes. *Int. J. Artif. Organs*, 32(9): 545-554.

Kennedy, S., Oswald, N. (2011). PCR troubleshooting and optimization: the essential guide. *Caister Academic Press*, Norfolk, England.

Ketonis, C. *et al.* (2010). Bacterial colonization of bone allografts: establishment and effects of antibiotics. *Clin. Orthop. Relat. Res.*, 468: 2113–21.

Kloos, W., Musselwhite, M. (1975). Distribution and persistence of *Staphylococcus* and *Micrococcus* species and other aerobic bacteria on human skin. *Appl. Microbiol*, 30: 381–385.

Knobloch, J., Jager, S., Horstkotte, M., Rohde, H., Mack, D. (2004). *Rsb*U-dependent regulation of *Staphylococcus epidermidis* biofilm formation is mediated via the alternative s factor ∂^{B} by repression of the negative regulator gene *ica*R. *Infect. Immun.*, 72(7): 3838–3848.

Kristian, S., Birkenstock, T., Sauder, U., Mack, D., Gotz, F., Landmann, R. (2008). Biofilm formation induces C3a release and protects *Staphylococcus epidermidis* from IgG and complement deposition and from neutrophil dependent killing. *J. Infect. Dis.*, 197(7): 1028–1035.

Li, D., Lundberg, F., Ljungh A. (2000). Binding of von Willebrand factor by coagulase-negative staphylococci. *J. Med. Microbiol*, 49: 217-225.

Li, H. *et al*. (2005). Conversion of *Staphylococcus epidermidis* strains from commensal to invasive by expression of the *ica* locus encoding production of biofilm exopolysaccharide. *Infect. Immun.*, 73(5): 3188–3191.

Li, M., Villaruz, A., Vadyvaloo, V., Sturdevant, D., Otto, M. (2008) AI-2- dependent gene regulation in *Staphylococcus epidermidis*. *BMC Microbiol.*, 8: 4.

Little, S. and Lvins, B. (1999). Molecular pathogenesis of *Bacillus anthracis* infection. *Microbes Infect.*, 1: 131–139.

Livak, K., Schmittgen, T. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2 (-Delta Delta C(T)). *Method. Methods.*, 25: 402-408.

Macintosh, R. *et al*. (2009). The terminal A domain of the fibrillar accumulation-associated protein (*Aap*) of *Staphylococcus epidermidis* mediates adhesion to human corneocytes. *J. Bacteriol.*, 191(22): 7007–7016.

Mack, D., *et al*. (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 adhesion. *Infect. Immun.*, 62: 3244-3253.

Mack, D. *et al*. (1996). The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear β-1,6-linked glucosaminoglycan: purification and structural analysis. *J. Bacteriol.*, 178: 175–183.

Mack, D., Siemssen, N., Laufs, R. (1992). Parallel induction by glucose of adherence and a polysaccharide antigen specific for plastic-adherent *Staphylococcus epidermidis*: evidence for functional relation to intercellular adhesion. *Infect. Immun.*, 60: 2048-2057.

Makhlin, J. *et al*. (2007). *Staphylococcus aureus Arc*R controls expression of the arginine deiminase operon. *J. Bacteriol.*, 189(16): 5976–5986.

Mann, E. *et al*. (2009). Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. *Plos ONE*, 4(6): e5822.

Miragaia, M., Couto, I., de Lencastre, H. (2005). Genetic diversity among methicillin-resistant *Staphylococcus epidermidis* (MRSE). *Microb. Drug Resist.*, 11: 83–93.

Molin, S., Tolker-Nielsen, T. (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr. Opin. Biotechnol.*, 14: 255e61.

Nagarajan, V., Smeltzer, M., Elasri, M. (2009). Genome-scale transcriptional profiling in *Staphylococcus aureus*: bringing order out of chaos. *FEMS Microbiol. Lett.*, 295(2): 204–210.

Neoh, K., Kang, E. (2011). Combating bacterial colonization on metals via polymer coatings: relevance to marine and medical applications. *ACS Appl. Mater. Interfaces*, 3(8): 2808e19.

Nolan, T., Hands, R., Bustin, S. (2006). Quantification of mRNA using real-time RTPCR. *Nat. Protoc.*, 1: 1559-1582.

Novick, R. *et al.* (1993). Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule. *EMBO J.*, 12: 3967–3975.

O'Gara, J. (2007). *ica* and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*. *FEMS Microbiol. Lett.*, 270: 179–188.

O´Gara, J., Humphreys, H. (2001). *Staphylococcus epidermidis* biofilms: importance and implications. *J. Med. Microbiol*, 50: 582-587.

Opitz, L., Salinas-Riester, G., Grade, M., Jung, K., Jo, P., Emons, G., Ghadimi, B., Beissbarth, T., Gaedcke, J. (2010). Impact of RNA degradation on gene expression profiling. *BMC. Med. Genomics.*, 3: 36.

Opperman-Sanio, F., Steinbuchel, A. (2002). Occurrence, functions and biosynthesis of polyamides in microorganisms and biotechnological production. *Naturwissenschaften*, 89: 11-22.

Otto, M. (2001). *Staphylococcus aureus* and *Staphylococcus epidermidis* peptide pheromones produced by the accessory gene regulator *agr* system. *Peptides*, 22(10): 1603e8.

Otto, M. (2009). Review: *Staphylococcus epidermidis* "the accidental-pathogen". *Macmillan Publisher Limited*, 7: 555-567.

Pei, L., Flock, J. (2001). Lack of *fbe*, the gene for a fibrinogen-binding protein from *Staphylococcus epidermidis*, reduces its adherence to fibrinogen coated surfaces. *Microb. Pathog.,* 31(4): 185–193.

Percival, S., Malic, S., Cruz, H., Williams, D. (2011). Introduction to Biofilms. *Spring Series* on Biofilms 6, New York, USA.

Percival, S., Walker, J., Hunter, P. (2000). Microbiological aspects of biofilms and drinking water. *CRC Press*, New York, USA.

Pina, P. *et al.* (2000). An outbreak of *Staphylococcus aureus* strains with reduced susceptibility to glycopeptides in a French general hospital. *Clin. Infect. Dis.,* 31(5): 1306-1308.

Pinto, F., Thapper, A., Sontheim, W., Lindblad, P. (2009). Analysis of current and alternative phenol based RNA extraction methodologies for cyanobacteria. *BMC. Mol. Biol.*, 10: 79.

Qin, Z. *et al*. (2007). Formation and properties of *in vitro* biofilms of *ica*-negative *Staphylococcus epidermidis* clinical isolates. *J. Med. Microbiol.*, 56: 83–93.

Queck, S. *et al*. RNAIII-independent target gene control by the *agr quorum-sensing* system: insight into the evolution of virulence regulation in *Staphylococcus aureus*. *Mol. Cell*, 32: 150–158.

Raad, II., Sabbagh, M., Rand, K., Sherertz, R. (1992). Quantitative tip culture methods and the diagnosis of central venous catheter-related infections. *Diagn. Microbiol. Infect. Dis.*, 15: 13–20.

Radstrom, P., Knutsson, R., Wolffs, P., Lovenklev, M., Lofstrom, C. (2004). Pre-PCR processing: strategies to generate PCR-compatible samples. *Mol. Biotechnol.*, 26: 133-146.

Rajeevan, M., Vernon, S., Taysavang, N., Unger, E. (2001). Validation of arraybased gene expression profiles by real-time (kinetic) RT-PCR. *J. Mol. Diagn.*, 3: 26–31.

Rani, S. *et al*. (2007). Spatial patterns of DNA replication, protein synthesis, and oxygen concentration within bacterial biofilms reveal diverse physiological states. *J. Bacteriol.*, 189(11): 4223–4233.

Rice, K. et al. (2007). The *cidA* murein hydrolase regulator contributes to DNA release and biofilm development in *Staphylococcus aureus*. *Proc. Natl Acad. Sci.*, 104(19): 8113–8118.

Rohde, H. *et al*. (2005). Induction of *Staphylococcus epidermidis* biofilm formation via proteolytic processing of the accumulation-associated protein by staphylococcal and host proteases. *Mol. Microbiol.*, 55(6): 1883–1895.

Rohde, H. *et al*. (2006). Pathogenesis of staphylococcal device-related infections: from basic science to new diagnostic, therapeutic and prophylactic approaches. *Reviews in Medical Microbiology*, 17(2): 45-54.

Rhode, H. *et al*. (2007). Polysaccharide intercelular adhesin or protein factors in biofilm accumulation of *Staphylococcus epidermidis* and *Staphylococcus aureus* isolated from prosthetic hip and knee joint infections. *Biomaterials*, 28(9): 1711-1720.

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. *Eur. J. Cell Biol.*, 89(1): 103–111.

Riken Research. (2007). Elucidating the mechanism behind immunity using dendritic cells. Available at http://www.rikenresearch.riken.jp [Acessed 17 December 2013].

Rogers, K., Fey, P., Rupp, M. (2009). Coagulase-negative staphylococcal infections. *Infect. Dis. Clin. North. Am.*, 23: 73-98.

Rogers, K., Rupp, M., Fey, P. (2008) The presence of *ica*ADBC is detrimental to the colonization of human skin by *Staphylococcus epidermidis*. *Appl. Environ. Microbiol.*, 74(19): 6155–6157.

Rupp, M., Ulphani, J, Fey, P., Bartscht, K., Mack, D. (1999). Characterization of the importance of polysaccharide intercellular adhesin/ hemagglutinin of *Staphylococcus epidermidis* in the pathogenesis of biomaterial-based infection in a mouse foreign body infection model. *Infect. Immun.*, 67(5): 2627–2632.

Sadovskaya, I., Vinogradov, E., Flahaut, S., Kogan, G., Jabbouri, S. (2005). Extracellular carbohydrate–containing polymers of a model biofilm-producing strain, *Staphylococcus epidermidis* RP62A. *Infect. Immun.*, 73: 3007-3017.

Sadykov, M., Bayles, K. (2012). The control of death and lysis in *staphylococcal* biofilms: a coordination of physiological signals. *Curr. Opin. Microbiol*, 15(2): 211-215.

Santiago-Vázquez, L. (2006). Comparison of two total RNA extraction protocols using the marine gorgonian coral *Pseudopterogorgia elisabethae* and its symbiont *Symbiodinium sp*. *Electronic Journal of Biotechnology*, 9: 598-603.

Schoenfelder, S. *et al*. (2010). Success through diversity - how *Staphylococcus epidermidis* establishes as a nosocomial pathogen. *Int. J. Med. Microbiol.*, 300(6): 380-386.

Schowchow, D., Serieys, L., Wayne, R., Thalmann, O. (2012). Efficient recovery of whole blood RNA extraction protocols for high-throughput applications in wildlife species. *BMC Biotechnol.*, 12: 33.

Shulman, L. et al. (2012). Evaluation of four different systems for extraction of RNA from stool suspensions using MS-2 coliphage as an exogenous control for RT-PCR inhibition. *Plos One*, 7(7): e39455.

Sieber, M. *et al*. (2010). Substancial performance discrepancies among commercially available kits for reverse transcription quantitative polymerase chain reaction: a systematic comparative investigator-driven approach*. Anal. Biochem.*, 401: 303-311.

Stoodley, P., Sauer, K., Davies, D., Costerton, J. (2002). Biofilms as complex differentiated communities. *Annu Rev Microbiol* 56: 187-209.

Sun, D., Accavitti, M., Bryers, J. (2005). Inhibition of biofilm formation by monoclonal antibodies against *Staphylococcus epidermidis* RP62A accumulation-associated protein*. Clin. Diagn. Lab. Immunol.*, 12(1): 93–100.

Sung, K., Khan, S., Nawaz, M., Khan, A. (2003). A simple and efficient Triton X-100 boiling and chloroform extraction method of RNA isolation from Gram-positive and Gram-negative bacteria. *FEMS Microbiol. Lett.*, 229: 97-101.

Tichopad, A., Didier, A., Pfaffl, M. (2004). Inhibition of real-time RT-PCR quantification due to tissue specific contaminants*. Mol. Cell Probes.*, 18: 45-50.

Tormo, M., *et al*. (2005). *Sar*A is an essential positive regulator of *Staphylococcus epidermidis* biofilm development. *J. Bacteriol.*, 187(7): 2348–2356.

Vuong, C. *et al*. (2004). Polysaccharide intercellular adhesin (PIA) protects *Staphylococcus epidermidis* against major components of the human innate immune system. *Cell. Microbiol*, 6: 269–275.

Vacheethasanee, K. *et al*. (1998). Bacterial surface properties of clinically isolated *Staphylococcus epidermidis* strains determine adhesion on polyethylene. *J. Biomed. Mater. Res.*, 42: 425–432.

Vuong, C., Durr, M., Carmody, A., Peschel, A., Klebanoff, S., Otto, M. (2004). Regulated expression of pathogen-associated molecular pattern molecules in *Staphylococcus epidermidis*:

quorum-sensing determines proinflammatory capacity and production of phenol-soluble modulins. *Cell. Microbiol.*, 6(8): 753–759.

Vuong, C. *et al.* (2004). A crucial role for exopolysaccharide modification in bacterial biofilm formation, immune evasion, and virulence. *J. Biol. Chem.*, 279: 54881–54886.

Vuong, C., Gerke, C., Somerville, G., Fischer, E., Otto, M. (2003). *Quorum-sensing* control of biofilm factors in *Staphylococcus epidermidis*. *J. Infect. Dis.*, 188: 706–718.

Vuong, C., Kocianova, S., Yao, Y., Carmody, A., Otto, M. (2004). Increased colonization of indwelling medical devices by *quorum-sensing* mutants of *Staphylococcus epidermidis in vivo.,* 190: 1498–1505.

Voung, C., Otto, M. (2002) *Staphylococcus epidermidis* infections. *Microbes and Infection*, 4: 481–489.

Vuong, C., Saenz, H., Götz, F., Otto, M. (2000). Impact of the *agr quorum-sensing* system on adherence to polystyrene in *Staphylococcus aureus*. *J. Infect. Dis.*, 182(6): 1688e93.

Vuogan, C., Gerke, C., Somerville, G., Fischer, E., Otto, M. (2003). *Quorum-sensing* control of biofilm factors in *Staphylococcus epidermidis*. *J. Infect. Dis*, 188: 706-718.

Wang, C. *et al.* (2007). Role of *Clp*P in biofilm formation and virulence of *Staphylococcus epidermidis*. *Microbes Infect.*, 9(11): 1376–1383.

Wang, L., *et al.* (2008). SarZ is a key regulator of biofilm formation and virulence in *Staphylococcus epidermidis*. *J. Infect. Dis.*, 197(9): 1254–1262.

Wang, X. *et al.* (2003). Evaluation of a multilocus sequence typing system for *Staphylococcus epidermidis*. *J. Med. Microbiol.*, 52: 989–998.

Wang, X., Preston, J., Romeo, T. (2004). The *pga*ABCD locus of *Escherichia coli* promotes the synthesis of a polysaccharide adhesin required for biofilm formation. *J. Bacteriol.*, 186: 2724–2734.

Wilson, I. (1997). Inhibition and facilitation of nucleic acid amplification. *Appl. Environ. Microbiol.*, 63: 3741-3751.

Wisplinghoff, H. *et al.* (2003). Related clones containing SCC*mec* type IV predominate among clinically significant *Staphylococcus epidermidis* isolates. Antimicrob. *Agents Chemother.*, 47: 3574–3579.

Woundsinternational (2013). Schematic representation of polymicrobial biofilm formation Available at http://woundsinternational.wordpress.com [Acessed in 18 December, 2014].

Xu, L. *et al*. (2006). Role of the *Lux*S Quorum-Sensing System in Biofilm Formation and Virulence of *Staphylococcus epidermidis*. *Infection and Immunity*, 74 (1): 488-496.

Yao, Y., Sturdevant, D., 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. *J. Infect. Dis.*, 191(2): 289–298.

Yarwood, J., Bartels, D., Volper, E., Greenberg, E. (2004). *Quorum sensing* in *Staphylococcus aureus* biofilms. *J. Bacteriol.*, 186: 1838–1850.

Zhu, T., *et al*. (2010). Impact of the *Staphylococcus epidermidis Lyt*SR two-component regulatory system on murein hydrolase activity, pyruvate utilization and global transcriptional profile. *BMC Microbiol*, 10: 287.

Zimmerli, W. (2006). Infection and musculoskeletal conditions: prosthetic-joint-associated infections. *Best Pract. Res. Clin. Rheumatol.*, 20: 1045–63.

Zimmerli, W., Trampuz, A. (2011). Implant-associated infection. Biofilm infections.*Springer*, (1): 69–90.

Zimmerli, W., Trampuz, A., Ochsner, P. (2004). Prosthetic-joint infections. *N. Engl. J. Med.*, 351: 1645–54.