Escola Superior de Tecnologia da Saúde do Porto
Instituto Politécnico do Porto

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Diversity and characterisation of coagulase-negative staphylococci isolated from healthy individuals in Portugal

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Dissertação submetida à Escola Superior de Tecnologia da Saúde do Porto para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Tecnologia Bioquímica em Saúde, realizada sob a orientação científica do Doutor Nuno Cerca, Investigador Auxiliar do Centro de Engenharia Biológica, Instituto de Biotecnologia e Bioengenharia, Universidade do Minho, e sob a orientação institucional da Professora Doutora Cristina Prudêncio, Professora Coordenadora com Agregação da Escola Superior de Tecnologia da Saúde do Porto, Instituto Politécnico do Porto.

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I dedicate this thesis to my parents

"*O Homem não é uma inutilidade num mundo feito, mas obreiro de um mundo a fazer"*

Leonardo Coimbra

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Abstract

In the past few years the interest in coagulase-negative staphylococci (CoNS) has significantly increased in human medicine. CoNS are common commensal colonisers of the human skin, although now also recognised as major nosocomial pathogens. Over the last decades, several studies have been carried out in order to understand the pathogenicity mechanisms of CoNS. The well known determinants in the pathogenesis of CoNS infections are their ability to form biofilms and an exceptional resistance to several antibiotics. Nevertheless, there is a lack of studies regarding the commensal lifestyle of these microorganisms. Additionally, it is now hypothesised that commensal bacteria might be a reservoir of pathogenic determinants. Therefore, the work described throughout this thesis was aimed to perform a phenotypic and genotypic characterisation of different CoNS species isolated from healthy Portuguese individuals. A total of 61 CoNS isolates, comprising 7 different species, were obtained and characterised at the level of biofilm formation and antibiotic susceptibility profiles. According to the results, biofilm formation ability and presence of biofilm-associated genes were commonly found features, highlighting their pivotal role in the colonising lifestyle of CoNS. This study also addressed the correlation between phenotypic and genotypic characteristics of biofilm formation, corroborating and raising questions about the importance of some genes in this process. Moreover, it was observed a great proportion of isolates with decreased susceptibility and multiple resistances to some important antibiotics. A significant association between antibiotic resistance and biofilm formation was also demonstrated, and some hypotheses about the nature of such association were provided. Lastly, the expression patterns of two biofilm-associated genes at two distinct biofilm developmental stages were determined, confirming their importance in the accumulative stage of biofilm formation. Overall, the results presented in this thesis indicate that staphylococcal skin flora might be an important reservoir of potentially pathogenic bacteria and, simultaneously, bring to light new perceptions about the molecular basis of staphylococcal biofilm formation, and the nature of the association between antibiotic resistance and biofilm formation.

Keywords: antibiotic resistance; biofilm formation; biofilm-associated genes; coagulase negative staphylococci; gene expression; healthy individuals

Resumo

Nos últimos anos, o interese médico em estafilococos coagulase-negativos (ECNs) aumentou significativamente. Os ECNs são colonizadores comuns da pele humana, embora sejam atualmente considerados patogénios nosocomiais importantes. Nas últimas décadas, diversos estudos foram realizados afim de perceber os mecanismos patogénicos destes micro-organismos. Os determinantes mais conhecidos na patogénese de infeções por ECNs são a sua capacidade de formar biofilmes e uma resistência excecional a diversos antibióticos. Todavia, existe uma falta de estudos referente ao estilo de vida comensal destes micro-organismos. Além disso, coloca-se atualmente a hipótese de bactérias comensais constituirem um reservatório de determinantes patogénicos. Desta forma, o trabalho descrito nesta tese visou uma caraterização fenotípica e genotípica de diferentes espécies de ECNs isoladas de indivíduos portugueses saudáveis. No total foram obtidos 61 isolados, englobando 7 espécies distintas, sendo posteriormente caraterizados ao nível de formação de biofilme e de perfis de suscetibilidade a antibióticos. De acordo com os resultados, a capacidade de formação de biofilme e a presença de genes relacionados com este processo foram caracteristicas comummente encontradas, salientando o seu papel preponderante no estilo de vida comensal dos ECNs. Foi também abordada a correlação entre caracteristicas fenotípicas e genotípicas de formação de biofilme, corroborando e levantando questões sobre a importância de alguns genes neste processo. Adicionalmente, uma grande proporção de isolados apresentaram suscetibilidade reduzida e múltiplas resistências a alguns antibióticos importantes. Foi também demonstrada uma associação significativa entre resistência a antibióticos e formação de biofilme, pelo que algumas hipóteses sobre a natureza desta associação foram apresentadas. Por fim, foram determinados os padrões de expressão de dois genes associados com formação de biofilme em duas fases distintas deste processo, confirmando a sua importância na fase de acumulação do biofilme. No geral, os resultados apresentados nesta tese indicam que a flora estafilocócica da pele poderá constituir um importante reservatório de bactérias potencialmente patogénicas e, simultaneamente, apresentam novas perceções sobre a base molecular da formação de biofilme em ECNs e sobre a natureza da associação entre resistência a antibióticos e formação de biofilme.

Palavras-chave: resistência a antibióticos, formação de biofilme, genes associados à formação de biofilme, estafilococos coagulase-negativos, expressão genética, indivíduos saudáveis

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Abbreviations and acronyms

Aap	Accumulation-associated protein
AMP	Antimicrobial peptide
APUA	Alliance for the Prudent Use of Antibiotics
ATCC	American Type Culture Collection
Bap	Biofilm-associated protein
Bhp	Bap homologue protein
CDC	Centers for Disease Control and Prevention
CFU	Colony forming unit
CIP	Ciprofloxacin
CoNS	Coagulase-negative staphylococci
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
ERY	Erythromycin
EUCAST	European Commission for Antimicrobial Susceptibility Testing
GEN	Gentamicin
HAI	Healthcare-associated infection
MDR	Multidrug resistance
MIC	Minimum inhibitory concentration
MSCRAMM	Microbial surface component recognising adhesive matrix molecules
NaCl	Sodium chloride
OD	Optical density
PBP	Penicillin-binding protein
PCR	Polymerase chain reaction
PEN	Penicillin
PGA	Poly-γ-glutamic acid
PIA	Polysaccharide intercellular adhesin
PNAG	Poly-*N*-acetilglucosamine
PSM	Phenol-soluble modulin
qPCR	Quantitative real-time polymerase chain reaction
RIF	Rifampicin
RNA	Ribonucleic acid

ROAR	Reservoirs Of Antibiotic Resistance
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSBg	Tryptic soy broth with 0.4% extra glucose
VAN	Vancomycin

Chapter I

General introduction

1.1. Staphylococci: from health to disease

Taxonomically, coagulase-negative staphylococci (CoNS) represent a group of bacteria belonging to the genus *Staphylococcus*, family Staphylococcaceae. Currently, this genus comprises more than 40 species, and many of them are known to colonise humans (Euzeby, 2013; Euzeby, 1997). Microscopically, staphylococci appear as Gram-positive cocci (0.5-1.5 μm in diameter), occurring singly, in pairs and in irregular grape-like clusters. On a solid culture medium, staphylococcal species exhibit different colony morphologies. While *Staphylococcus aureus* produces large yellow colonies, *Staphylococcus epidermidis* and other CoNS species usually form relatively small white-greyish colonies. In a culture medium supplemented with blood, staphylococcal species could also be classified according to their haemolytic properties. Staphylococci are non-motile, non-spore forming and usually unencapsulated. From a biochemical point of view, most species are facultative anaerobes, catalase positive and oxidase negative. All staphylococci are coagulase-negative, with the exception of *S. aureus* (Götz *et al.*, 2006). However, some strains belonging to *S. intermedius*, *S. hyicus*, and *S. schleiferi* species may also yield positive coagulase reactions. CoNS species can be differentiated into two groups according to novobiocin susceptibility test. Novobiocin-susceptible group includes *S. epidermidis*, *S. haemolyticus*, *S. hominis*, and *S. lugdunensis* among others, which are usually implicated in a wide range of infections. Novobiocin-resistant group includes *S. saprophyticus*, a common cause of urinary tract infections (Venkatesh *et al.*, 2006). From a molecular perspective, the genome size of staphylococci is about 2000-3000 kb, with a GC-content of 30-39% (George and Kloos, 1994; Kloos *et al.*, 1998).

Staphylococci are widely disseminated in the environment, occupying specific ecological niches. They are found as part of the normal microflora of humans and other animals, and usually have a commensal relationship with their host. Staphylococci are also isolated from a wide range of foodstuff such as cheese, milk and meat, and from environmental sources such as air, soil and water (Heikens, 2005; Kloos and Schleifer, 1986; Kloos, 1992; Coton 2010; Schleifer and Bell 2009).

1.1.1. Role of CoNS in the human skin microflora

The human skin microflora is defined as the microorganisms present on the surface and within the deeper layers of the healthy skin, as well as in the hair follicles (Grice *et al.*,

2009). The human skin harbours a wide range of microorganisms, namely bacteria, fungi and viruses (Figure 1.1).

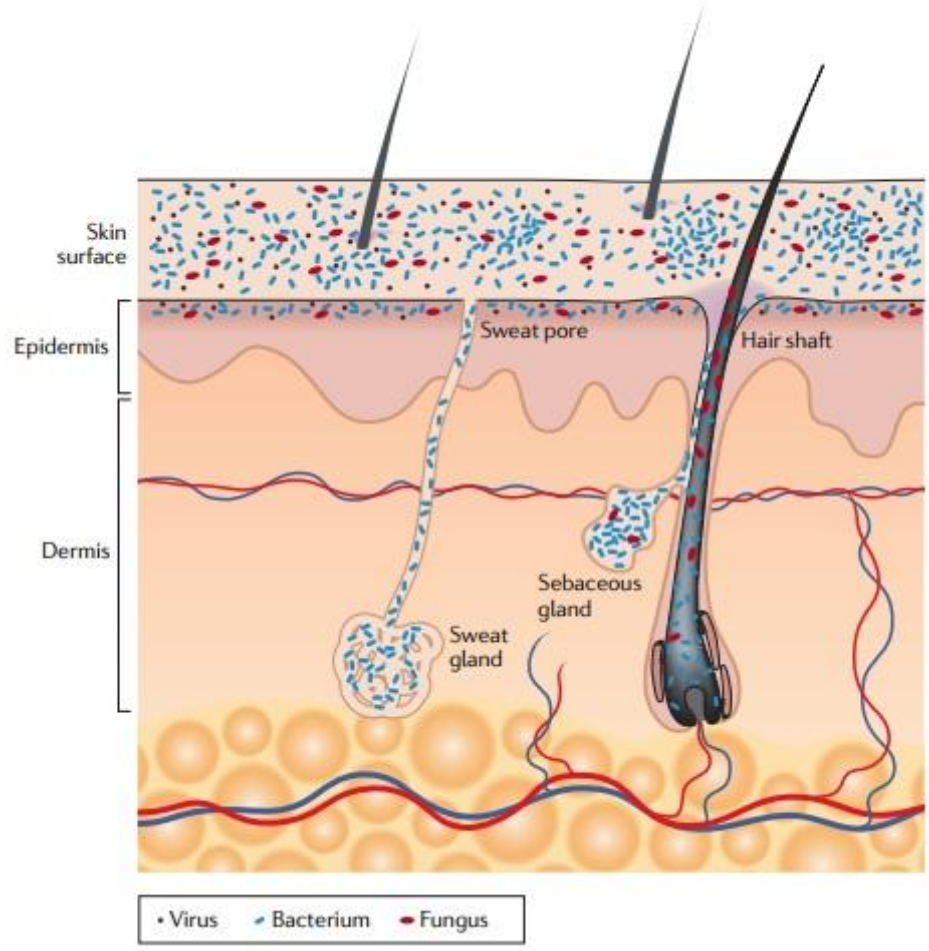

Figure 1.1. Cross-sectional view of human skin and microbial flora associated. Adapted from Nature Rev Microbiol (2011), 9(4):244-53.

Among the most common bacterial colonisers of the human skin are CoNS, which share this environment with a wide range of other bacteria, especially those belonging to *Corynebacterium* and *Propionibacterium* genera. Most skin bacteria, however, cannot be cultured and some recent studies employing culture-independent methodologies have revealed a greater diversity of bacterial species present on the human skin than what was known before (Gao *et al.*, 2007; Grice *et al.*, 2009).

Actually, the human skin represents a fairly harsh environment for microbial growth, mainly due to constant variations in temperature, acidity, and humidity, along with the presence of host antimicrobial peptides (AMPs). Moreover, the self-renewing nature of the skin makes permanent bacteria attachment very difficult (Kong and Segre, 2012).

Although the mechanisms associated with adaptation and colonisation of the skin are not fully understood, analysis of *S. epidermidis* genome revealed the carriage of several genes with predicted function in the protection from the harsh conditions found in the natural habitat of this microorganism (Gill *et al.*, 2005; Zhang *et al.*, 2003). For instance, the presence of sodium pumps and transport systems for osmoprotectants allow the growth of CoNS in environments with high salt concentrations and osmotic pressures (Gill *et al.*, 2005). Moreover, it was demonstrated that *S. epidermidis* and other CoNS species secrete an extracellular polymer, called poly-γ-glutamic acid (PGA), that appears to play an important role in the protection of CoNS from key components of innate host defence such as AMPs, as well as from high salt concentrations (Kocianova *et al.*, 2005).

The colonisation of the skin by CoNS does not usually pose a threat, and may even play a key function in the maintenance of the normal human skin microflora, inhibiting the colonisation by other pathogenic microorganisms (Otto, 2009). Recent studies demonstrated that *S. epidermidis* has an important function in skin immune defence through the production of small peptides, called phenol-soluble modulins (PSMs) (Cogen *et al.*, 2010a; Cogen *et al.*, 2010b). Their results suggested that PSMs interact with AMPs naturally found on the skin, enhancing the killing of some other gram-positive bacteria, such as *S. aureus* and Group A *Streptococcus*, without damaging the normal skin microflora.

1.1.2. Role of CoNS in healthcare-associated infections

According to the Centers for Disease Control and Prevention (CDC), a healthcare-associated infection (HAI) is a localised or systemic condition (1) that results from an adverse reaction to the presence of an infectious agent(s) or its toxin(s), (2) that occurs during a hospital admission, (3) for which there is no evidence that the infection was present or incubating at the time of admission (Horan *et al.*, 2008).

HAIs are a significant cause of morbidity and mortality around the world and represent an increasing problem in modern medicine (Burke, 2003). Klevens *et al.* (2007) attempted to estimate the magnitude of HAIs and associated deaths in United States hospitals during 2002. The estimated number of HAIs obtained was approximately 1.7 million (4.5%) and the estimated deaths caused by or associated with HAIs were approximately 99 000. In the European Union, over 4 million people acquire a HAI every year, resulting in approximately 37 000 deaths directly related with the infection. The

annual costs associated with these infections are estimated at approximately €7 billion, including direct costs only (ECDC, 2012). In developing countries, the situation is even worst, with the proportion of infected patients frequently exceeding 25% (Pittet *et al.*, 2008). In Portugal, the last survey in the field of infection control was conducted in 2012. From a total of 18 258 inpatients, the overall prevalence rate of HAI was 10.6% (Pina *et al.*, 2013). In general, the high prevalence rates of HAI over the world result in prolonged hospital stays, long-term disability, increased resistance of microorganisms to antimicrobials, high costs for patients and their families, and a massive financial burden for health systems (WHO, 2011).

The increasing use of invasive medical devices has been appointed as the major cause for such high frequency of HAIs (Richards *et al.*, 2000; von Eiff *et al.*, 2005). CoNS have been found to be one of the most frequently isolated microorganisms in the context of medical device-related infections (e.g., catheters systems, pacemakers, prosthetic joints and heart valves, and a range of other polymer and metal implants) (Durando *et al.*, 2010; Kollef *et al.*, 2008). As common members of the normal human microflora, CoNS are not usually pathogenic and develop a benign relationship with their host (Otto, 2009). Surprisingly, CoNS are now considered opportunistic pathogens, causing infection especially in immunocompromised individuals, such as premature newborns, patients with malignant diseases, intravenous drug abusers and hospitalised patients with indwelling medical devices (Vadyvaloo and Otto, 2005; Venkatesh *et al.*, 2006). Taking this into account, CoNS have been included in the group of the most important nosocomial pathogens (Wisplinghoff *et al.*, 2003).

CoNS develop from commensal to pathogenic lifestyle only when external barriers, such as skin, are damaged due to wounds or implantation of foreign bodies (e.g. indwelling medical devices) (Longauerova, 2006). The indigenous microflora of the patients is frequently regarded as the main source of infection. The risk of contamination of medical devices by CoNS is high, since they make up a significant proportion of the normal human skin microflora (Otto, 2012). However, there is growing evidence that a large proportion of CoNS infections are also due to specific strains that are able to persist in hospital settings (Blum-Menezes *et al.*, 2009; Miragaia *et al.*, 2007; Widerstrom *et al.*, 2006).

Currently, CoNS are a well known cause of a wide range of human infections (Table 1.1) (Piette and Verschraegen, 2009). Within CoNS group, *S. epidermidis* is by far the

most frequent etiological agent associated with those infections. However, other CoNS species, especially *S. haemolyticus*, *S. lugdunensis* and *S. saprophyticus*, are also associated with severe infectious diseases (Donlan and Costerton, 2002; Fredheim *et al.*, 2009; Shin *et al.*, 2011). Other CoNS, such as *S. capitis*, *S. caprae*, *S. hominis* or *S. warneri*, are implicated in human infection, however, to a lesser extent (John and Harvin, 2007).

Table 1.1. Implication of CoNS in human infection

Clinical conditions	CoNS species	References
Bloodstream infections	CoNS in general	Munoz *et al.* (2004); Wisplinghoff *et al.* (2004)
Endocarditis	*S. epidermidis* (most frequent); *S. hominis*, *S. lugdunensis*, *S. capitis*, *S. caprae*, *S. simulans* (occasionally found)	Chu *et al.* (2004); Wang *et al.* (2007)
Endophtalmitis	CoNS in general	Karacal *et al.* (2007)
Other foreign body-related infections	*S. epidermidis* (most frequent); and other CoNS (occasionally found)	Monsen *et al.* (2000); von Eiff *et al.* (2005)
Surgical site infection	CoNS in general	Cantlon *et al.* (2006); CDC (1996)
Urinary tract infections	*S. saprophyticus* (most frequent); *S. epidermidis* and *S.caprae* (occasionally found)	Raz *et al.* (2005); Widerstrom *et al.* (2007)

1.1.3. Virulence factors of CoNS

The pathogenic potential of a group of bacteria is determined by the combined effect of extracellular factors such as toxins, along with adhesive and invasive determinants mediated by components on the bacterial cell wall (Otto, 2004).

While several virulence factors have been described for *S. aureus*, in CoNS only a few have been properly characterised. CoNS infections are seldom life-threatening as they do not produce many aggressive virulence factors. However, in immunocompromised patients these infections could develop into severe conditions (Goldman *et al.*, 2001; Poutsiaka *et al.*, 2007). Once CoNS reach the epithelial protective layer, they can be highly infectious. Persistence in the host and evasion of the immune machinery seem to be the pathogenic strategies employed by CoNS. This is mainly achieved by their striking ability to form a thick and multilayered biofilm (discussed in more detail in section 1.2) on the surface of indwelling medical devices. It is well established that microorganisms in this

physiological state are highly resistant to antimicrobial treatments and can escape from the mechanisms of innate host defence (Cerca *et al.*, 2006; Cerca *et al.*, 2005; Cheung *et al.*, 2010). Hence, molecules involved in adhesion and biofilm formation are regarded as key virulence determinants of CoNS (Otto, 2009). Some of these determinants, however, also have functions in the commensal lifestyle of CoNS, since they are found on both clinical and commensal isolates (Kocianova *et al.*, 2005; Rohde *et al.*, 2004; Vuong *et al.*, 2004). For this reason, there seems to be an increasing consensus about considering CoNS as "accidental" pathogens. Even though several virulence factors have been characterised, such as toxins (Cogen *et al.*, 2010a; Cogen *et al.*, 2010b), lipases (Longshaw *et al.*, 2000) and proteases (Lai *et al.*, 2007), the most important are definitely those related with biofilm formation (discussed in Chapter III). In Table 1.2 are summarised the most recognised virulence factors of *S. epidermidis* as it is by far the best studied CoNS species.

Table 1.2. Main recognised virulence factors of *S. epidermidis*. Adapted from Nat Rev Microbiol (2009), 7(8):555-67.

Virulence factor	Gene	Function
Biofilm factors		
Primary attachment to abiotic surfaces		
AtlE	*atlE*	Autolysin/ adhesin; affects surface hydrophobicity
Aae	*Aae*	Autolysin/ adhesin
Teichoic acids	Multiple biosynthetic genes	Affect attachment (only demonstrated in *S. aureus*, but with supposed similar function in CoNS)
Primary attachment to matrix proteins (MSCRAMMs)		
SdrF	*sdrF*	Binds to collagen
SdrG (Fbe)	*sdrG (*also known as *fbe)*	Binds to fibrinogen
SdrH	*sdrH*	Putative binding function only
Embp	*Embp*	Binds to fibronectin
AtlE and Aae	*atlE* and *aae*	Bind to several matrix proteins
GehD	*gehD*	Binds to collagen
Intercellular aggregation		
PIA (PNAG)	*icaA, icaD, icaB* and *icaC*	Polysaccharide intercellular adhesion
Bhp	*Bhp*	Protein intercellular adhesion
Aap	*Aap*	Protein intercellular adhesion

Table 1.2. (continuation)

Virulence factor	Gene	Function
Teichoic acids	Multiple biosynthetic genes	Components of the biofilm matrix
Protective exopolymers		
PIA	*icaA, icaD, icaB* and *icaC*	Protects from IgG, AMPs, phagocytosis and complememt
PGA	*capA, capB, capC* and *capD*	Protects from AMPs and phagocytosis
Resistance to AMPs		
SepA protease	*sepA*	Involved in AMP degradation
Aps system	*apsR, apsS* and *apsX*	This system senses AMPs and regulates AMP resistance mechanism
Toxins		
PSMs	*psmα, psmδ, psmε, hld*	Pro-inflammatory cytolysins
Exoenzymes		
Lipases GehC and GehD	*gehC* and *gehD*	Possibly responsible for the persistence in fatty acid secretions
Glutamylendopeptidase GluSE and serine proteases SspA and Esp	*sspA*	Degrades fibrinogen and complement factor C5
Cysteine proteases SspB and Ecp	*sspB*	Possibly responsible for tissue damage
Metalloprotease/ elastase SepA	*sepA*	Involved in lipase maturation, AMP resistance, and potential tissue damage
Other factors		
Staphyloferrins A and B	*sfna* locus	Siderophores (iron acquisition)
SitA, SitB and SitC	*sitA,sitB* and *sitC*	Involved in iron uptake
FAME	Unidentified	Inactivates host-produced bactericidal fatty acids

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

Despite being apparently harmless, several studies have shown that CoNS are able to produce factors that induce inflammatory responses or damage host tissues (Otto, 2004). With regards to toxins, and although not recognized as classical toxin producers, almost all CoNS strains are thought to produce PSMs which appear to have pro-inflamatory effect (Vuong *et al.*, 2004). PSMγ (also known as δ-toxin) has been suggested to have cytolytic properties and several others share high sequence homology to *S. aureus* PSMs which have a prominent ability to lyse human neutrophils (Wang *et al.*, 2007). Moreover, it has been

proposed that PSMs are also involved in the dispersion of bacteria from *S. epidermidis* biofilms (Vadyvaloo and Otto, 2005), which contributes for the persistence of CoNS infections. Nevertheless, their role in human infection is unclear as PSMs also play an important role in the commensal lifestyle of CoNS by competing with other bacteria on the skin, as previously stated in section 1.1.1.

In the following introductory sections, the role of microbial biofilms in human infection is discussed in more detail as well as the issue of antibiotic resistance since these two intimately related fields are the basis for the experimental work described throughout this thesis.

1.2. Microbial biofilms

As previously discussed, biofilm formation is the major recognised virulence factor of CoNS. Nevertheless, this mode of growth is common throughout other bacterial genera and is not necessarily associated with disease. Actually, the majority of bacteria (about 99%) are found naturally organised in biofilms (O'Gara and Humphreys, 2001). In this section a general model of biofilm formation as well as the role of biofilms in infectious diseases are described.

1.2.1. General model of biofilm formation

Although several definitions have been proposed during the last decades, a biofilm is usually referred as a structured community of microorganisms attached to an abiotic or living surface and embedded in a self-produced matrix of extracellular polymeric components, such as polysaccharides, proteins and nucleic acids (Donlan and Costerton, 2002). It is well established that microorganisms in biofilm state have lower growth rate than in planktonic (free-floating) state. It is worth noting that microorganisms rarely live in environments containing high concentrations of nutrients and thus it is more advantageous growing in a biofilm (Cerca and Jefferson, 2012).

There are slightly different models of the biofilm formation process according to the bacterial species involved. Nevertheless, they follow the same basic stepwise process (Figure 1.2): (1) primary attachment of cells to a surface, (2) accumulation of cells in multiple layers, (3) maturation and (4) detachment (Fey and Olson, 2010; Stoodley *et al.*, 2002). Primary attachment of planktonic cells may occur in two distinct ways: (1a) direct

adhesion to the polymer surface, or (1b) adhesion to host matrix proteins that cover the polymer surface as a "conditioning film" (often associated with medical device-related infections) through interaction with specific bacterial binding proteins. This step comprises nonspecific and hydrophobic interactions. After this stage, bacteria multiply forming structures called microcolonies. This stage requires intercellular adhesion, which is achieved by the production of extracellular matrix in which the bacteria become surrounded. Then, maturation of the biofilm structure takes place with formation of channels for water, ion, and nutrient exchange, giving rise to the characteristic three-dimensional appearance of biofilms. Finally, detachment and dispersal of single bacterial cells or large cell clusters occur, which may then initiate a new cycle of biofilm formation elsewhere (Rohde *et al.*, 2006; Schoenfelder *et al.*, 2010).

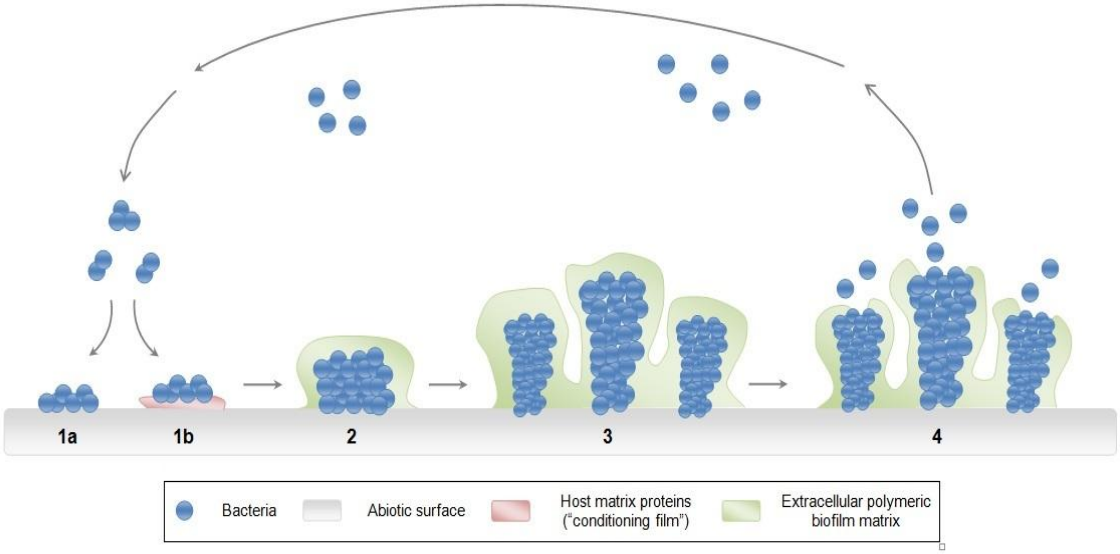

Figure 1.2. General model of biofilm formation showing the distinct phases of this process.

1.2.2. Biofilms and infectious diseases

Today it is recognised the huge impact of microbial biofilms in infectious diseases. It is estimated that biofilms are involved in at least 65% of all human infections (Lewis, 2007). Moreover, the development of biofilms on medical devices accounts for a wide range of persistent infections. Up to 60% of all nosocomial infections are caused by biofilms on medical devices. As bacterial biofilms are highly recalcitrant to antimicrobial therapy, standard antibiotic therapy is often useless. Hence, the removal and replacement of the contaminated medical device is often required, which involves an additional economic and health cost (Donlan and Costerton, 2002).

Although to a lesser extent, microorganisms can also adhere to natural surfaces (i.e. human tissues) and develop a biofilm which may be associated with several 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 (Zijnge *et al.*, 2010), chronic bacterial prostatitis (Mazzoli, 2010), and bacterial vaginosis (Swidsinski *et al.*, 2005).

Currently, it is not completely understood how biofilms provide a protective environment for bacteria in the host, although some important discoveries have been made in the past few years. The extracellular biofilm matrix is thought to play a key role in the protection of bacteria from phagocytosis and other elements of the host immune system. Nevertheless, some important studies have demonstrated that human leukocytes and antibodies can easily penetrate bacterial biofilms, even though they fail to phagocytise and kill the bacteria (Cerca *et al.*, 2006; Leid *et al.*, 2002). These results suggest that the increased resistance of biofilm cells to antibody-mediated phagocytosis may be due to high levels of antigens within the biofilm matrix, which subsequently inhibit antibody-mediated phagocytosis of the bacteria.

The thickness of the biofilm also plays an important role in the protection of bacteria from reactive oxygen species and antibiotics. Very slow growth rates of biofilm cells appear to be responsible for resistance to classes of antibiotics whose efficiency is largely dependent on the growth rate of the cells, particularly cell wall synthesis inhibitors (Evans *et al.*, 1991; Stewart, 1998). In fact, a study conducted by Cerca *et al.* (2005) demonstrated that cells in a biofilm are highly resistant to antibiotics that target cell wall synthesis, although are fairly susceptible to antibiotics that target RNA and protein synthesis. These are very interesting results since RNA and protein synthesis inhibitors are also dependent on the growth rate of the bacterial cells. Therefore, the resistance mechanisms of bacterial biofilms may not rely only on specific features associated with the biofilm phenotype, but also on the mechanism of action of the antibiotic. In fact, the mechanisms of antibiotic resistance in bacterial biofilms are only beginning to be elucidated. Figure 1.3 depicts the four leading hypotheses for mechanisms of antibiotic resistance in biofilms.

As a conclusion, it is now clear that microbial biofilms pose a serious threat to the public health, with increasing importance in a wide range of infectious diseases, especially those related with the use of medical devices.

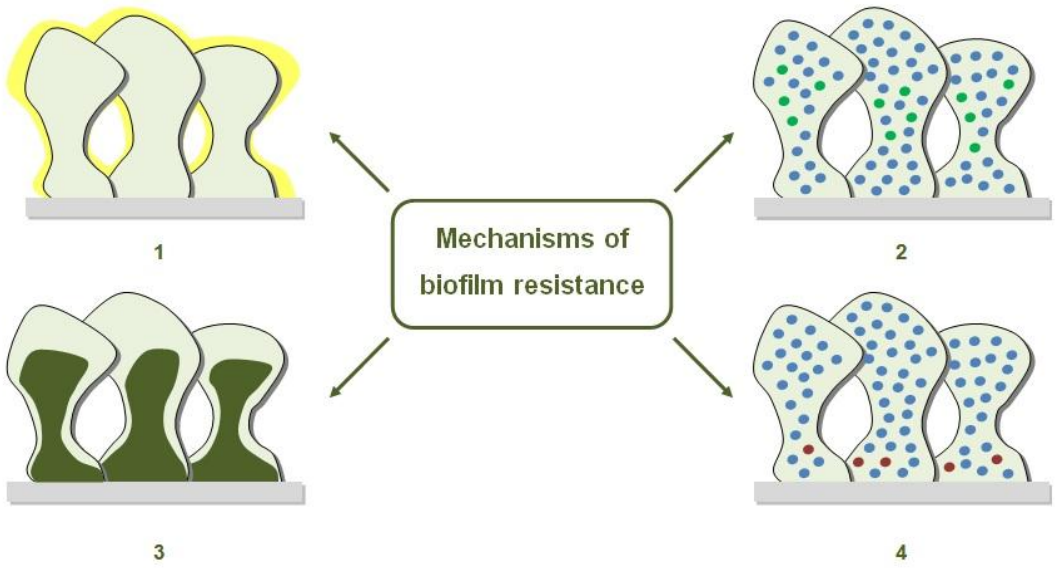

Figure 1.3. Main hypothetical mechanisms of antibiotic resistance in biofilms.

The images represent a cross section of a biofilm with the attachment surface (gray) at the bottom: **(1) Slow penetration** - the antibiotic (yellow) may fail to penetrate in the deeper layers of the biofilm; **(2) Stress response** - some of the bacteria within the biofilm may activate stress response (green dots); **(3) Altered microenvironment** - in zones where there is nutrient depletion or waste product accumulation (dark green), antibiotic action may be antagonised; **(4) Persister cells** - some bacteria may differentiate into a protected phenotype (red dots).

1.3. The issue of antibiotic resistance

The beginning of antibiotic therapy was a successful period allowing the control of a wide range of infectious diseases (Levy, 2002). Since the discovery of penicillin in 1930s, several other antibiotics were discovered and produced in large scale. For this reason, antibiotics became a fundamental piece in the fight against infectious agents. Besides human medicine, other fields took advantage of the use of antibiotics, namely agriculture and livestock (Kummerer, 2004).

Facing the increasing use of antibiotics over the last decades, bacteria have developed several mechanisms to cope with this scenario. Consequently, the emergence and dissemination of antibiotic resistance traits became inevitable and is currently regarded as a major public health issue (Levy and Marshall, 2004).

1.3.1. Factors promoting antibiotic resistance

It is now clear that the massive use of antibiotics is crucial for the selection of resistant bacteria. Selection of resistant bacteria can occur during or after an antibiotic treatment (Beigi *et al.*, 2004; Hurford *et al.*, 2012), and due to the presence and wide dissemination of antibiotic resistance determinants (i.e. antibiotic molecules and resistance genes) into different ecological niches such as air, soil and water (Kummerer, 2003; Martinez, 2008; Wellington *et al.*, 2013). Besides antibiotics, several other agents with bactericidal and/or bacteriostatic effects, many of them available in a wide range of household products (e.g. disinfectants), are also responsible for the selection of resistant bacteria (Levy, 2000; McMurry *et al.*, 1998).

The way antibiotics are available to the population plays an important role in the massive use of antibiotics currently observed and, subsequently, in the emergence of antibiotic resistance. Unfortunately, there are some parts of the world where antibiotics are simply sold on the street, and any person has access to them. On the other hand, in most developed countries a prescription is always required in order to get an antibiotic, yet this procedure seems not enough to prevent abusive consumption of antibiotics. In fact, most physicians are not acquainted or sensitive to the antibiotic resistance issue, and the prescription of antibiotics still remains a common option, even when its use is not necessary or adequate (Levy, 2002).

Besides the massive use of antibiotics and the presence of resistance genes, most of resistance mechanisms employed by bacteria are not capable of destroying or inactivate antibiotic molecules. They rather rely on changing the target of the antibiotic or exporting it out of the bacterial cell (e.g. efflux pumps). Antibiotics are therefore released into the environment in their active form, allowing them to continue to exert their selective pressure in the bacterial populations (Levy and Marshall, 2004).

1.3.2. Mechanisms of antibiotic resistance acquisition

There are two essential factors to have in mind when dealing with the antibiotic resistance phenomenon: (1) the antibiotic, which acts as a selective agent and facilitates the spread of bacteria that possess resistance genes and (2) the resistance genes themselves (Levy, 2002). Since antibiotics and other related organic molecules are, or resemble, natural products, it is not unexpected to find resistance to newly discovered antibiotics.

Actually, bacteria have acquired several resistance mechanisms over their millennia of existence in order to deal with the wide range of molecules that constantly affect their growth (Dancer *et al.*, 1997; Linares *et al.*, 2006).

Antibiotic resistance is normally classified as innate or acquired. Some bacterial species are intrinsically resistant to one or more classes of antibiotics due to physiological and structural features of the bacterial cell (Wright, 2010). Of greater concern are, however, the cases of acquired resistance, where initially susceptible bacterial populations become resistant to antibiotics, proliferating and disseminating under the selective pressure provided by such agents (Tenover, 2006). The process of acquired antibiotic resistance is quite complex, but it generally relies on two distinct mechanisms: genetic mutations and horizontal transfer of genes. The latter mechanism plays an important role in the dissemination of antibiotic resistance traits and may occur between strains of the same species or between different bacterial species or genera. Mechanisms of genetic exchange include conjugation (i.e. sexual transfer of DNA), transduction (i.e. bacteriophage transfer of DNA) and transformation (i.e. acquisition and incorporation of DNA released into the bacterial environment by lysis of other bacteria) (Tenover, 2006). Of the described gene transfer routes, transformation and conjugation appear to occur frequently among bacterial biofilms, although conjugation is possibly the more common mechanism in horizontal gene transfer (Tenover, 2006).

In short, all the previously described mechanisms enable bacterial species to become quickly adapted to the introduction of antibiotics into their environment. In general, mechanisms of bacterial resistance rely on (1) changing or eliminating the binding site of the antibiotic, (2) up-regulating the production of enzymes that inactivate the antibiotic, (3) down-regulating or changing an outer membrane protein channel required by the antibiotic for cell entry, or (4) up-regulating efflux pumps that drive the antibiotic out of the bacterial cell (McManus, 1997).

1.3.3. Reservoirs of antibiotic resistance and the role of commensal bacteria

After use in humans or animals, antibiotics and any of their metabolites are discharged into the sewage system (Daughton and Ternes, 1999; Martinez, 2009). Depending on some physicochemical properties of these compounds (e.g. polarity, water solubility) they may be degraded, associate with sewage sludge, or released into the aquatic environment (e.g. rivers). Sewage sludge is often used as a fertiliser and may allow

the entrance of antibiotics into agricultural systems. Furthermore, antibiotics may also reach agricultural soils trough irrigation with wastewaters (Kinney *et al.*, 2006). The soil ecosystem can subsequently transport these compounds to surface and ground waters allowing them to be cycled within the environment (Blackwell *et al.*, 2007; Topp *et al.*, 2008). On the other hand, humans are constantly exposed to antibiotic resistance determinants by different manners: (1) crops exposed to contaminated sewage sludge, (2) livestock that have accumulated antibiotics and resistant microflora, (3) fish and shellfish exposed intentionally or unintentionally to antibiotic compounds, (4) contaminated surface and groundwater used for drinking water (Kummerer, 2004). Figure 1.4 outlines the dissemination paths of antibiotic resistance determinants and the relationship between different ecological niches.

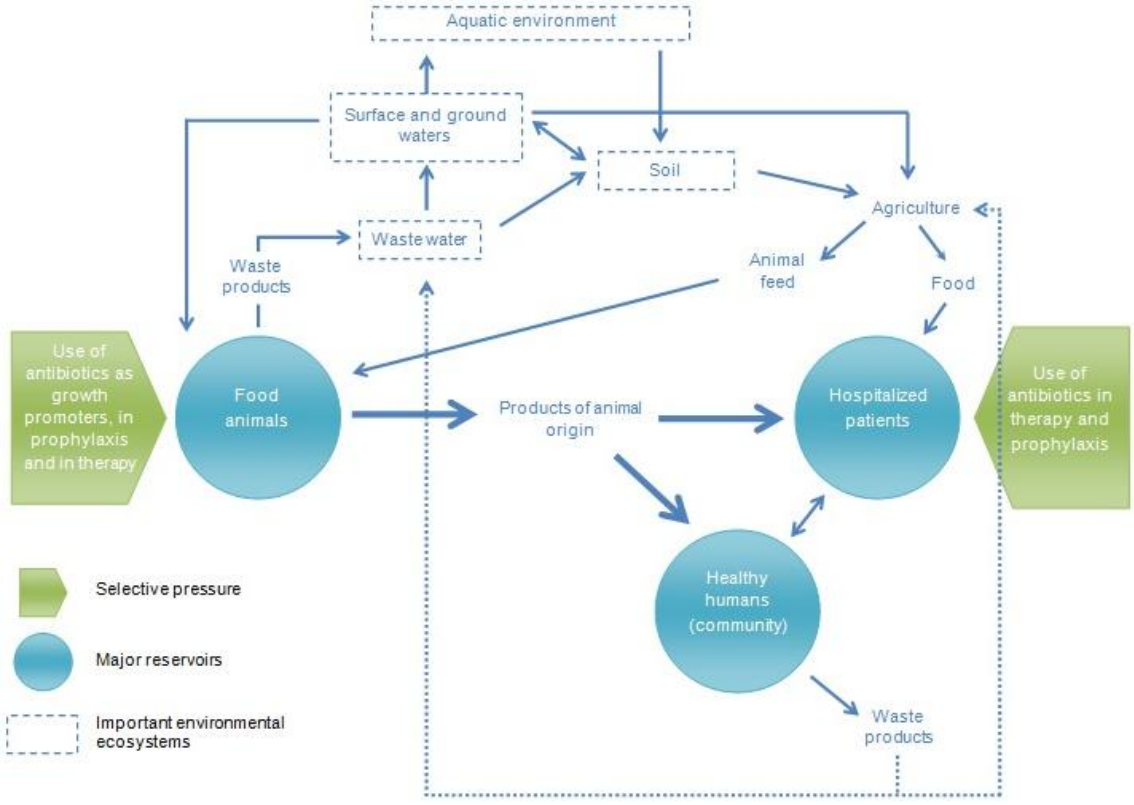

Figure 1.4. The associations between potential environmental reservoirs of antibiotic resistance determinants. Adapted from Int J Antimicrob Agents (2000), 14(4):321-5.

When talking about the phenomenon of antibiotic resistance, it is important to note that both pathogenic bacteria and commensal microflora of humans and animals are affected. For instance, when an antibiotic therapy is applied, commensal bacteria are also affected by the selective pressures of such treatment and may become a major reservoir of

resistant strains and resistance genes that could be horizontally transferred to pathogenic species (Marshall *et al.*, 2009).

Despite the potential role of commensal bacteria in providing a reservoir of antibiotic resistance, these bacterial communities have not been studied extensively. Moreover, information regarding antibiotic resistance in commensal and community-associated bacteria is still scarce. Nevertheless, many commensal bacterial species, previously regarded as relatively harmless residents of the human microflora, have recently emerged as multidrug-resistant microorganisms causing a wide range of infectious diseases in humans (Table 1.3) (Sommer *et al.*, 2009). The increasing number of hospitalised, immunocompromised patients together with the widespread use and misuse of antibiotics have opened a door to the emergence of these "pathogenic" commensals (Jones *et al.*, 2008). Among this group of bacteria are CoNS, but also enterococci, streptococci and other Gram-negative microorganisms.

Recognising the potential role of commensal bacteria as reservoirs of antibiotic resistance determinants, the Alliance for the Prudent Use of Antibiotics (APUA) began to examine this group of bacteria through its Reservoirs Of Antibiotic Resistance (ROAR) project (http://www.roarproject.org/). The aim of this project was, and still is, to encourage identification of commensal (usually non-pathogenic) strains as initial reservoirs of resistance traits before it appears in clinical strains. Since then, some studies on commensal bacteria have been made, and more questions than answers have arisen.

Interestingly, some studies on commensal bacteria have reported that antibiotic resistance might not mirror antibiotic usage (Diaz-Mejia *et al.*, 2002) and may even precede industrial-scale production and distribution of antibiotics (D'Costa *et al.*, 2006). Moreover, it is possible that some molecules present in the environment could somehow mimic current antibiotics and provide a selective pressure in bacterial communities (Slaughter *et al.*, 2001). These overall results may partially explain why antibiotic resistance is widely distributed, even within antibiotic-free environments (Pallecchi *et al.*, 2007). Conversely, a study conducted by Thaller *et al.* (2010) reported residual antibiotic resistance traits among commensal bacteria colonising wild animals from a remote area inhabited by humans and with no antibiotic exposure. Taking these data together, it becomes clear that humans and their activities could be the main factor in the contamination of the environment and subsequent selection of resistant bacteria.

Table 1.3. Emergence of antibiotic resistance in commensal bacteria causing infectious diseases. Adapted from Nature (2008), 451: 990-3.

Species	Disease	Year	Resistance
Acinetobacter baumanii	Nosocomial pneumonia, bacteremia, UTI, meningitis, septicemia	1985	Imipenem
		1993	Gentamicin
		1998	MDR[a]
		2001	Polymixin
Campylobacter jejuni	Campylobacteriosis	1986	Fluoroquinolone
Clostridium difficile	*Clostridium difficile*-associated diarrhea	1989	Clindamycin
Enterococcus faecalis	Endocarditis, bacteremia, catheter-related infection, intra-abdominal and pelvic infections.	1978	Gentamicin
		1986	Vancomycin
		1989	Ampicillin
		1989	Penicillin
		2000	Linezolid
Haemophilus influenzae	Bacteremia, RTI, meningitis, osteoarthritis	1972	Ampicillin
		1975	Chloramphenicol/ Tetracycline
		1975	Chloramphenicol
		1979	MDR
		1979	Chloramphenicol/ Ampicillin
Klebsiella pneumoniae	Pyogenic liver abcess, bacteremia, RTI	1983	Late-generation cephalosporins
Pseudomonas aeruginosa	Opportunistic infections in immunocompromised patients	1985	Ceftazidime
		1987	Fluoroquinolone
		1988	Imipenem
Staphylococcus epidermidis	Infective endocarditis, catheter-related infections, bacteremia, UTI, osteomyelitis, vascular graft infections, prosthetic joint infections	1962	Methicillin
		1994	Rifampicin
Staphylococcus haemolyticus	Septicemia, peritonitis, UTI, infective endocarditis	1984	Vancomycin
		1997	MDR
Staphylococcus aureus	Boils, furunculosis, pneumonia, mastitis, phlebitis, meningitis, UTI, osteomyelitis, endocarditis.	1942	Penicillin
		1960	Methicillin
		1976	MDR
		2000	Vancomycin
Streptococcus pneumoniae	*Streptococcus pneumonia* infection	1967	Tetracycline
		1977	MDR
		1978	Macrolide

MDR, multidrug-resistant; RTI, respiratory tract infections; UTI, urinary tract infections.

Another question that remains to be addressed is whether antibiotic use in agriculture and livestock activities is driving resistance genes from animal-associated bacteria into bacteria that cause human disease. Regarding to agriculture, large amounts of antibiotics are used (Heilig *et al.*, 2002). Most food animals in developed countries are exposed to

antibiotics at some point during their lives. In this respect, antibiotics are used not only to treat or prevent some infectious diseases, but also to function as growth promoters or enhancers of feed efficiency. The main question here is that many antibiotics used in food animal production are the same as, or closely related to, that used in human medicine (McEwen and Fedorka-Cray, 2002). Moreover, the presence of resistance genes in ready-to-eat food stuff may also serve as a source for the acquisition, by humans, of antibiotic-resistant bacteria or associated resistance genes. The European Food Safety Authority (EFSA) recently reported that foodborne bacteria, including known pathogens and commensal bacteria, display an increasing and diverse range of resistance to several antimicrobial agents of human and veterinary importance (EFSA, 2008).

Such diversity of sources for resistant bacteria and/ or resistance genes has an enormous epidemiological importance, as they may contribute to the accumulation of resistance determinants in human microflora. All these facts illustrate the impact of medical progression on antibiotic resistance and, most importantly, how usually harmless commensal bacteria are becoming into a truly threat to human health.

1.4. Outline and objectives of this thesis

S. epidermidis and other CoNS species are now regarded as potential pathogenic microorganism. In addition, the morbidity and mortality rates as well as the costs associated with CoNS infections have reached frightening numbers during the past few years. Therefore, studies about this group of bacteria are urgently need and of extreme importance. On the other hand, the recognition of commensal bacteria as a reservoir of antibiotic resistance determinants is relatively recent. Hence, studies on commensal bacteria from different ecological niches are just now emerging. In fact, information about physiologic and genetic features of CoNS colonising healthy individuals in the community is scarce, and there is a serious lack of studies regarding CoNS isolated in Portugal.

Taking into account all these factors, the main goal of this thesis was to perform a phenotypic and genotypic characterisation of CoNS isolated from healthy Portuguese individuals. This goal was achieved through the following tasks:

1. Isolation of several CoNS species from healthy individuals (Chapter II);
2. Evaluation of biofilm formation and correlation with the presence of biofilm-associated genes (Chapter III);
3. Evaluation of antibiotic susceptibility profiles and correlation with biofilm formation ability (Chapter IV);
4. Analysis of biofilm-associated genes expression at different stages of *S. epidermidis* biofilm development (Chapter V).

Lastly, this thesis is concluded by Chapter VI that describes the main conclusions drafted from this study, as well as possible future directions that should be addressed in order to answer some of the main questions that this work has raised.

Chapter II

Isolation and distribution of CoNS species on the skin of healthy individuals

2.1. Introduction

Staphylococcus spp. are usually identified based on colony morphology after culture onto solid medium. In contrast to other gram-positive cocci, such as enterococci and streptococci, all staphylococci are catalase positive. The optimum growth temperature is 30ºC - 37ºC. Another important feature of staphylococci is that they usually thrive in media with high concentrations of sodium chloride (about 10%). However, not all catalase-positive, Gram-positive cocci belong to the genus *Staphylococcus*. *Micrococcus* spp., also common colonisers of the human skin, are also catalase positive, and their cell shape and colony morphology usually resemble those of staphylococci (Engelkirk and Duben-Engelkirk, 2008). There are currently some methods that allow the differentiation between staphylococci and micrococci, for instance the modified oxidase test. While all micrococci possess the enzyme cytochrome c oxidase, most staphylococci, with few exceptions, lack this enzyme (Faller and Schleifer, 1981). Another important clue that may help in the identification of micrococci is their characteristic arrangement in tetrads after Gram staining. Furthermore, micrococci cells are usually larger than those of staphylococci (Engelkirk and Duben-Engelkirk, 2008)

After the initial isolation procedure, the following step is the identification of presumptive staphylococci at the species level. Phenotypic methods are the most commonly used for CoNS species differentiation. These methods are based on biochemical reactions and, in most cases, easy to perform and cost-effectiveness. However, phenotypic identification of CoNS seems to be unsatisfactory and not completely reliable and reproducible. The main drawback of these methods is that there is some degree of variability in the expression of phenotypic features by isolates belonging to the same species (Grant *et al.*, 1994; Heikens *et al.*, 2005; Renneberg *et al.*, 1995).

To overcome the problems revealed by phenotypic methods, several molecular methods have been developed for CoNS species differentiation. DNA sequence-based methods targeting different housekeeping genes, such as *16S rRNA* (Becker *et al.*, 2004), *dnaJ* (Shah *et al.*, 2007), *gap* (Ghebremedhin *et al.*, 2008; Yugueros *et al.*, 2000), *hsp60* (Goh *et al.*, 1996), *rpoB* (Drancourt and Raoult, 2002; Mellmann *et al.*, 2006), *sodA* (Poyart *et al.*, 2001), and *tuf* (Hwang *et al.*, 2011), are currently used for CoNS species identification. Comparatively with phenotypic methods, genotypic methods have higher discriminatory power, are independent of microbial growth, faster, and less laborious (Layer *et al.*, 2006). Moreover, there is currently a large reference sequence database,

which makes genotypic identification of CoNS the most accurate method available (Zadoks and Watts, 2009).

In routine clinical microbiology laboratories, CoNS are seldom identified to the species level, even when they are isolated from usually sterile biological specimens (e.g. blood or cerebrospinal fluid) (Weinstein *et al.*, 1998). However, and taking into account the increasing importance of CoNS as nosocomial pathogens, it is crucial to study the epidemiology and the pathogenic determinants of each species. Moreover, there is evidence of species-related variation in the pathogenic potential, with some species being more pathogenic than others (Tan *et al.*, 2006).

2.1.1. Aims

As discussed throughout the Chapter I, many of the recognised virulence factors of CoNS, especially those related with biofilm formation, are thought to play an important role in the commensal lifestyle of these microorganisms. Moreover, the recognition of commensal bacteria as reservoirs of antibiotic resistance determinants is relatively recent. To address these questions it is essential to gather a significant number of isolates so that we can thorough characterise them and draw significant conclusions.

This chapter describes the isolation of commensal CoNS from the skin of Portuguese healthy individuals, and their identification at the species level using a DNA sequence-based method targeting *rpoB* gene.

2.2. Materials and methods

2.2.1. Study area and population

The present study was carried out in different cities of the Northern region of Portugal. The study was focused on healthy volunteers, preferentially with no contact with hospital environment. Participants were asked to provide skin swab samples and to complete a questionnaire recording demographic data (age, gender, occupation, and area of residence), hospitalisation history and antibiotic use (in the preceding 12 months), contact with hospital environment and/ or healthcare personnel, active smoking habits, allergic diseases and daily wash habits (Appendix A). All volunteers gave written informed consent (Appendix B). This study was approved by the Ethics Sub-commission for Health and Life Sciences of the University of Minho, Portugal (process SECVS 002/2013).

2.2.2. Sample collection and isolation procedures

Skin swab samples from each participant were obtained by rubbing a sterile cotton-tip transport swab with Amies transport medium and charcoal (VWR, Portugal) on the palm and between fingers of both hands. The swabs were inoculated on Tryptic Soy Agar (TSA) plates (Liofilchem, Teramo, Italy) supplemented with 7% sodium chloride (NaCl) (Sigma-Aldrich, USA) for selective growth of *Staphylococcus* spp.. After incubation for 72h at 37ºC, colony morphology (including colour, shape, margin, elevation, and pigment characteristics) of all colonies were recorded. Colonies resembling CoNS were selected and streaked in fresh TSA plates until pure cultures were obtained.

Preliminary identification of the isolates was achieved by means of classical microbiological methods: Gram staining, oxidase and catalase testing (Appendix C). Gram-positive cocci in clusters, catalase positive and oxidase negative were regarded as presumptive staphylococci and further studied. These isolates were stored at -80°C in TSB containing 20% (v/v) glycerol. Only one isolate *per* participant was included in the study, except for two subjects from whom two different CoNS species were isolated.

2.2.3. Molecular identification of CoNS species

DNA templates for PCR reactions were obtained by suspending five colonies of an overnight culture on TSA plates in 500 µl of sterile Mili-Q water (Milipore Corporation, MA, USA). The suspension was boiled at 100ºC for 20 minutes in thermal block, and then centrifuged at 15000 *g* for 5 minutes in order to pellet cells and insoluble debris. The supernatants were transferred to new tubes and stored at -20ºC until further use.

Isolates were identified at the species level by partial *rpoB* gene sequencing, as previously described (Mellmann *et al.*, 2006). PCR reactions were performed in a final volume of 60 µl according to the following conditions: 4 µl of DNA template, 30 µl of DyNAzyme II PCR Master Mix 2x (Finnenzymes, Thermo Scientific, Finland) 4 µl of primer mixture (10 µM of each forward and reverse primers) and 22 µl of nuclease-free water. PCR amplifications were performed using the MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). Information about primers and amplification conditions is shown in Table 2.1. In each PCR run, a positive control (*S. epidermidis* ATCC 35984), and a negative control (water) were also included. Amplified products were analysed in 1% agarose gel stained with Midori Green DNA stain (Nippon Genetics Europe GmbH, Germany). The PCR products were purified using GRS PCR & Gel Band Purification Kit

(Grisp, Portugal) according to the instructions of the manufacturer. Sequencing of the purified PCR products was performed by the EUROFINS service (Eurofins MWG GmbH, Ebersberg, Germany) using the *rpoB* reverse primer.

Table 2.1. Primers and amplification conditions for *rpoB* gene

Target gene	Oligonucleotide primers sequence (5' to 3')	Amplicon size (bp)	PCR conditions
rpoB	Fw: CAA TTC ATG GAC CAA GC Rv: CCG TCC CAT GTC ATG AAA C	899	5 min at 94 °C; 35 cycles of 45 s at 94 °C, 60 s at 52 °C and 90 s at 72 °C 10 min at 72 °C

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

2.2.4. Sequence alignments and phylogenetic analysis

The nucleotide sequences of each isolate were analysed using two different web-based alignment tools: (1) BLAST analysis (http://blast.ncbi.nlm.nih.gov/blast.cgi), and (2) BiBi Bio-informatic Bacterial identification analysis (http://umr5558-sud-str1.univ-lyon1.fr/lebibi/lebibi.cgi). Identification at the species level was based on sequence similarity with the type strain of ≥97%. Phylogenetic trees were constructed based on *rpoB* sequences of all isolates. The nucleotide sequences were aligned using the ClustalX software, version 2.0 (Larkin *et al.*, 2007). Phylogenetic trees were inferred with PHYLIP software package, version 3.69 (Felsenstein, 1989), using the maximum likelihood method. Tree figures were visualised with the FigTree software, version 1.4.0 (Rambaut, 2012). The DNA sequence similarity analysis was performed with BioEdit, version 7.2.0 (Hall, 1999).

2.3. Results and discussion

2.3.1. Characterisation of study population

A total of 59 subjects were included in the study with mean age of 23 (range 10-79) years. The age range with more volunteers was between 19-30 years (56%), indicating that a large proportion of the study population was composed by young people. Regarding the medical history collected, 37% reported having contact with medical staff and 5% were medical care employees, 12% were hospitalised in the preceding 12 months, and 25% were

under treatment with antibiotics during the same period. A more detailed characterisation of the study population is present in Table 2.2.

Table 2.2. General characteristics of study population

Characteristic	*n* (%)	Characteristic	*n* (%)
Socio-demographic characteristics		*Antibiotic use in the last year*	15 (25)
Age, years		*Contact with medical staff*	22 (37)
0-18	8 (14)	*Medical care employees*	3 (5)
19-30	33 (56)	*On medication*	15 (25)
31-50	10 (17)	*Allergic diseases*	12 (20)
51-79	8 (14)	**Daily habits**	
Gender		*Hand washing with frequency*	58 (98)
Male	29 (49)	*Shower everyday*	49 (83)
Female	30 (51)	*Smoker/ exposure to tobacco smoke*	22 (37)
Medical characteristics		*Tattoos/ acupuncture in the last year*	2 (3)
Hospitalisations	11 (19)	*Gym/ pool/ sauna users*	20 (34)
In the last year	7 (12)		

2.3.2. Identification and distribution of CoNS species

From the 59 swab samples included in this study, a group of 61 presumptive staphylococci was initially isolated. Identification at the species level based on partial *rpoB* gene sequence was successfully performed for all isolates, representing 7 different CoNS species.

Sequence based analysis of *rpoB*, a conserved gene that encodes the β-subunit of the bacterial RNA polymerase, seems to have more discriminatory power than other target genes, especially *16S rRNA*, in the identification of staphylococcal isolates at the species and subspecies level (Mellmann *et al.*, 2006). Although it was not our aim to compare the discriminatory power between *rpoB* and other genes, our results demonstrate the suitability of this gene in the identification of the different staphylococcal species. As a result, *rpoB*

sequence-based identification may supplement, or even replace, phenotypic methods in the identification of staphylococcal species. Nevertheless, its effective use, especially in medical laboratories, requires the elaboration of a representative, publicly accessible reference database for sequence comparisons, as well as a clearly defined sequence similarity threshold for the correct identification of different species and subspecies (Hellmark *et al.*, 2009).

Regarding to the CoNS species found in this study, among the 61 CoNS isolates, 31 (50.8%) were identified as *S. epidermidis*. Besides *S. epidermidis*, other CoNS species were isolated: *S. capitis* (*n* = 9, 14.8%), *S. hominis* (*n* = 8, 13.1%), *S. warneri* (*n* = 6, 9.8%), *S. haemolyticus* (*n* = 4, 6.6%), *S. equorum* (*n* = 2, 3.3%) and *S. pettenkoferi* (*n* = 1, 1.6%).

It is well established that *S. epidermidis* is the predominant human staphylococcal species, comprising about 65-90% of all staphylococci isolated from human sources (Kloos and Musselwhite, 1975). Nevertheless, we found in this study a slightly lower proportion of *S. epidermidis*, despite representing more than half of all isolates. A similar study on community-associated CoNS isolates conducted by Widerstrom *et al.* (2011) reported the same rate. As previously stated in Chapter I, *S. epidermidis* has been identified as a major cause of HAIs, causing a wide range of infections, especially in patients with predisposing factors (e.g. indwelling or implanted foreign polymer bodies) (Piette and Verschraegen, 2009). Other frequent human skin colonisers include *S. capitis* and *S. hominis*. *S. capitis* is mostly found on the head and is more frequently isolated during puberty, whereas *S. hominis* predominates on dry, glabrous skin (Kloos and Schleifer, 1975). Hence, the relatively high proportion of *S. capitis* and *S. hominis* isolates found in the present study is not surprising. Nevertheless, *S. capitis* was not only isolated from subjects within the puberty period, but also in older individuals. From a clinical point of view, *S. capitis* has been isolated from cases of endocarditis (Takano *et al.*, 2011), and septicaemia (Ng *et al.*, 2006). With regards to *S. hominis*, and in contrast with *S. epidermidis*, a study using an animal infection model has shown that this species has low pathogenic potential (Lambe *et al.*, 1990). Nevertheless, there are some studies reporting the involvement of *S. hominis* in severe clinical conditions, such as septicaemia (Chaves *et al.*, 2005; Lindsay *et al.*, 1993) or endocarditis (Cunha *et al.*, 2007; Sunbul *et al.*, 2006). Therefore, and taking into account all these aspects, care must be taken in the decision of clinical significance of these two CoNS species.

In the present study, *S. warneri* and *S. haemolyticus* were found to be less prevalent. According to previous studies, *S. warneri* and *S. haemolyticus* are less frequent colonisers of the human skin (Kloos and Schleifer, 1975; Schleifer and Kloos, 1975). Over the past decades, *S. warneri* has emerged as a harmful pathogen, able of causing infections associated, or not, with implant materials (Campoccia *et al.*, 2010), and even in immunocompetent patients (Stollberger *et al.*, 2006). Moreover, *S. warneri* has been occasionally involved in cases of meningitis (Incani *et al.*, 2010), ventricular shunt infections (Martinez-Lage *et al.*, 2010), and endocarditis (Kini *et al.*, 2010). On the other hand, *S. haemolyticus* is an important multiresistant opportunistic pathogen in HAIs related to indwelling medical devices, representing the second most frequently isolated microorganism in infections due to CoNS (second only to *S. epidermidis*) (de Allori *et al.*, 2006; Viale and Stefani, 2006). Similarly to most CoNS species, *S. haemolyticus* is recognised by its ability to form biofilms, although it appears that these species also exhibits higher resistance rates to several classes of antibiotics (de Allori *et al.*, 2006; Fredheim *et al.*, 2009).

S. equorum was originally isolated from the skin of healthy horses (Schleifer *et al.*, 1984). Only a few *S. equorum* strains were found in relevant human infections (Marsou *et al.*, 2001; Nováková *et al.*, 2006). However, it is known that humans may be transiently colonised by species that normally colonise other animals (Otto, 2010), which was also verified in the present study.

Lastly, one isolate found in this study was identified as *S. pettenkoferi*, a newly recognised member of CoNS (Trulzsch *et al.*, 2007). Although information about *S. pettenkoferi* is still limited, there are some reports of human infections caused by this microorganism, namely bloodstream infections (Mihaila *et al.*, 2012; Song *et al.*, 2009) and osteomyelitis (Loiez *et al.*, 2007).

2.3.3. Phylogenetic relationship between CoNS isolates

The similarity of the *rpoB* sequences ranged from 64.9 to 99.7%, which was demonstrative of the discriminative power of the *rpoB* gene in CoNS identification. According to the phylogenetic analysis by maximum-likelihood method, the CoNS species were divided into three clusters (Figure 2.1): the first contained the *S. epidermidis* group; the second cluster comprised the groups of *S. hominis*, *S. warneri*, *S. equorum* and *S. pettenkoferi*; and the third cluster contained the groups of *S. haemolyticus* and *S. capitis*. It

is important to take into account that the phylogenetic relationships among CoNS species we derived in our study were limited, since a restricted number of different species was available for study. Besides, some of the *rpoB* sequence-based relationships we found were in accordance with those previously published (Drancourt and Raoult, 2002; Ghebremedhin *et al.*, 2008; Hellmark *et al.*, 2009). Of upmost importance, the relationship between *S. capitis* and *S. haemolyticus*, formerly regarded as incorrect by Drancourt and Raoult (2002), was indeed reported later by Ghebremedhin *et al.* (2008), and confirmed by our results.

2.4. Conclusions

The results described in this chapter show that sequence-based analysis of *rpoB* gene is a simple, rapid, and reliable method for the identification of different CoNS species. Moreover, the great diversity of CoNS species found in this study emphasises the need for studying each species as a separate entity and with its own features.

In the following chapters, the strains isolated and described herein will be further characterised, namely at the level of biofilm formation and antibiotic susceptibility profiles.

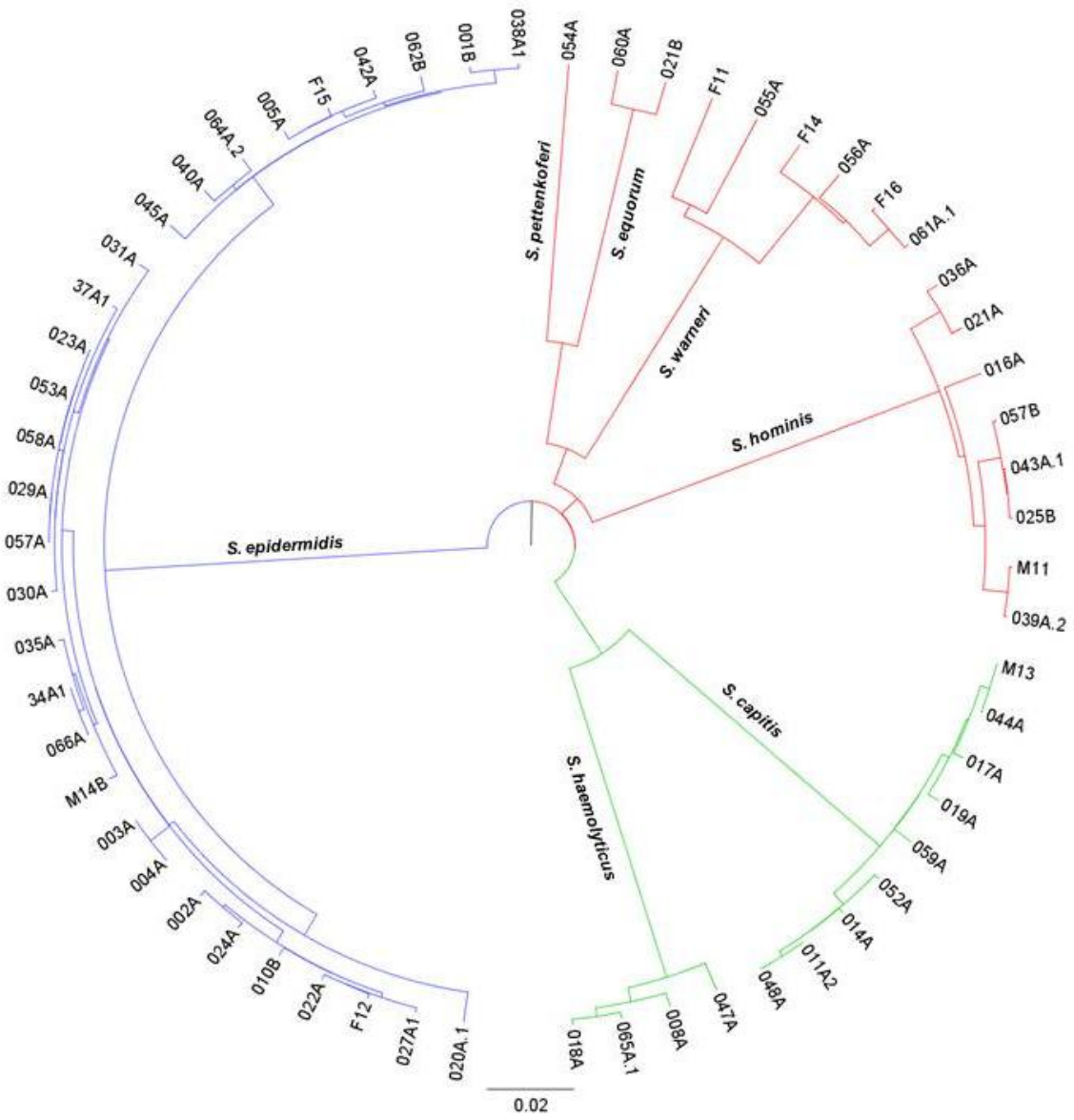

Figure 2.1. Maximum likelihood tree based on partial *rpoB* gene sequences showing the phylogenetic relationships among 61 CoNS isolates representing 7 different species. The scale bar indicates the evolutionary distance between sequences.

Chapter III

Carriage of biofilm-associated genes among commensal CoNS and its impact on biofilm formation

3.1. Introduction

The genotypic basis of CoNS biofilm formation and its complex regulation have been studied most extensively in *S. epidermidis*. On the other hand, the biofilms of a few other CoNS species associated with human infections, such as *S. haemolyticus* and S. *hominis*, have been studied to a lesser extent (Fredheim *et al.*, 2009; Mendoza-Olazaran *et al.*, 2013). With regards to *S. epidermidis*, several biofilm-associated factors have been described with different roles in the biofilm formation process (Otto, 2004; Otto, 2009) (Figure 3.1).

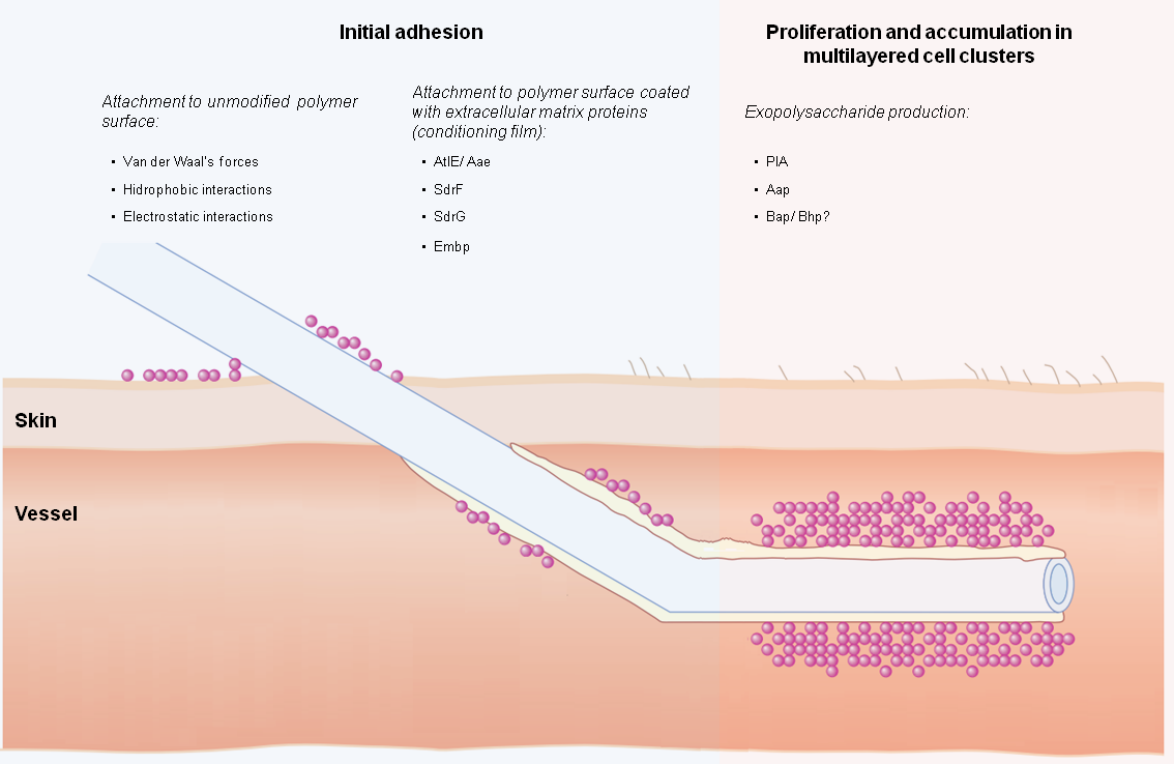

Figure 3.1. Schematic model of the phases involved in *S. epidermidis* biofilm formation on medical devices. Adapted from Lancet Infect Dis 2002; 2(11):677-85.

The first step of biofilm formation, i.e. adhesion, depends on the bacterial cell surface characteristics and on the nature of the attachment surface (von Eiff *et al.*, 2002). Several proteins such as the autolysins/ adhesins AtlE and Aae (Heilmann *et al.*, 1997; Heilmann *et al.*, 2003), and cell wall-associated adhesins that interact with host extracellular matrix components (also called Microbial Surface Components Recognising Adhesive Matrix Molecules, MSCRAMMs) have been associated with this step (Arrecubieta *et al.*, 2007; Nilsson *et al.*, 1998; Williams *et al.*, 2002). In the following step, i.e. accumulation, the production of an extracellular matrix mainly composed by

polysaccharides, proteins and nucleic acids occurs. This polymeric matrix plays a crucial role in the stabilisation of the biofilm architecture. One of the first described factors mediating biofilm accumulation in staphylococci was the polysaccharide intercellular adhesin (PIA), also known as poly-*N*-acetilglucosamine (PNAG), which is synthesised by *icaADBC* operon-encoded proteins (Maira-Litran *et al.*, 2002; Rohde *et al.*, 2007; Ziebuhr *et al.*, 1997). Although the *icaADBC* gene is frequently found in clinically significant CoNS isolates, some studies have demonstrated that biofilm formation can also occur in *ica*-negative isolates (Dice *et al.*, 2009; Esteban *et al.*, 2010; Qin *et al.*, 2007). These data have suggested the existence of PIA-independent mechanisms of biofilm accumulation. Since then, other factors mediating intercellular adhesion and biofilm accumulation were identified such as the accumulation-associated protein (Aap) (Rohde *et al.*, 2005) and the Bap homologue protein (Bhp) (Tormo *et al.*, 2005). The last step of biofilm formation is the less well characterised. It is thought that biofilm dispersal is controlled by the major quorum sensing system, *agr*, which controls a range of PSMs and proteases (Vuong *et al.*, 2004; Yao *et al.*, 2005).

3.1.1. Role of PIA, Aap and Bhp in biofilm formation

PIA is a partially de-acetylated β1-6-linked *N*-acetylglucosamine (GlcNAc) homopolymer that surrounds and connects staphylococcal cells within a biofilm (Mack *et al.*, 1996). The biosynthetic operon responsible for PIA synthesis is constituted by 4 synthetic genes, *icaA, icaD, icaB* and *IcaC* (Gerke *et al.*, 1998; Heilmann *et al.*, 1996), and an upstream negative regulator gene, *icaR* (Conlon *et al.*, 2002a; Conlon *et al.*, 2002b) (Figure 3.2). It is known that the membrane-located GlcNAc transferase IcaA and the accessory IcaD membrane protein are responsible for the synthesis of PIA chains from activated GlcNAc monomers (Gerke *et al.*, 1998). The transmembrane protein IcaC has predicted functions in the externalisation and elongation of the growing polysaccharide chain (Gerke *et al.*, 1998) After externalisation, the cell surface-located enzyme IcaB partially de-acetylates the GlcNAc residues, introducing positive charge into the otherwise neutral polymer that is essential for the binding of PIA to bacterial cell surface (Vuong *et al.*, 2004).

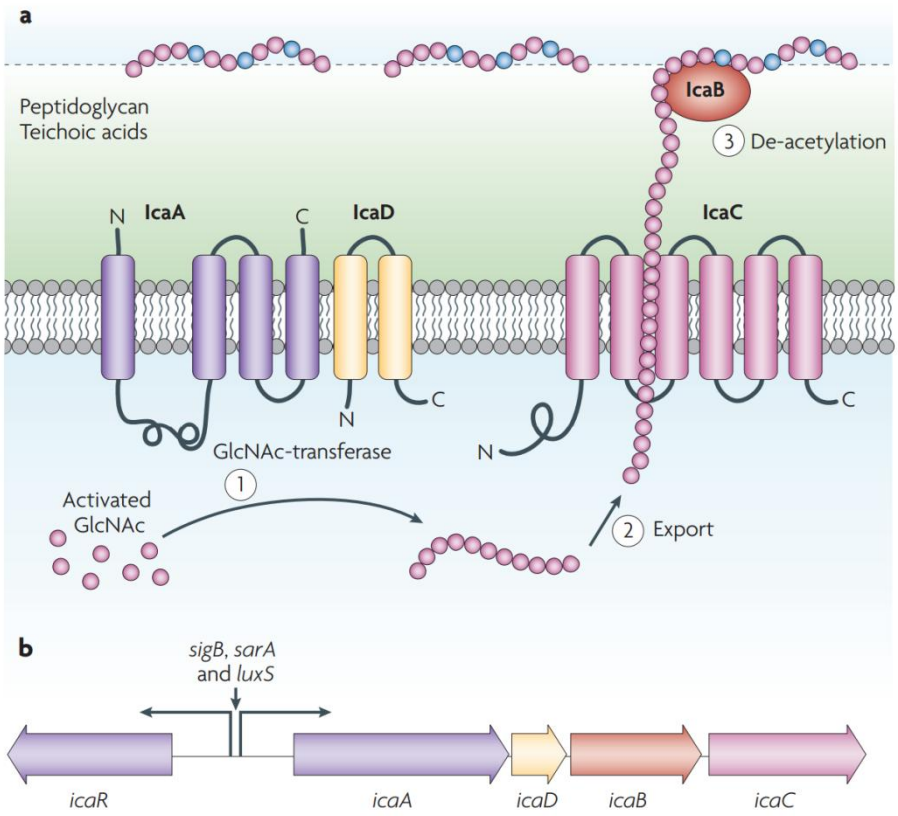

Figure 3.2. The polysaccharide intercellular adhesin (PIA): **(a)** schematic representation of the *icaADBC*-encoded proteins and their function during PIA synthesis; **(b)** organisation of the *icaADBC* operon. Green shading represents negative charge and blue shading represents positive charge; C, carboxyl; N, amino. Adapted from Nat Rev Microbiol (2009), 7(8):555-67.

The expression of the *ica* operon is very complex. First, it can be regulated either directly at the *icaA* promoter or through expression of IcaR protein, and is independent of the quorum sensing[1] system *agr* (Vuong *et al.*, 2003). Nevertheless, another staphylococcal quorum sensing system called *luxS* is known to repress *ica* transcription and decrease biofilm formation (Xu *et al.*, 2006). The global regulatory proteins SarA e SigB (Knobloch *et al.*, 2004; Tormo *et al.*, 2005) both up-regulate *ica* transcription. Another possible mechanism of regulation is the insertion and excision of the bacterial insertion sequence IS*256* at various locations of the operon, which can impair the production of PIA and, subsequently, biofilm formation (Ziebuhr *et al.*, 1999).

PIA has been regarded as one of the most important molecules in biofilm formation (Rohde *et al.*, 2010). In the last decade, several epidemiological studies were carried out in

[1] A method of cell density-dependent gene regulation in bacteria

order to investigate the distribution of the *icaADBC* gene and PIA production in CoNS clinical isolates (Arciola *et al.*, 2005; Bradford *et al.*, 2006; Chokr *et al.*, 2006; Frebourg *et al.*, 2000; Rohde *et al.*, 2007; Vandecasteele *et al.*, 2003). Interestingly, many of these studies found PIA production very common among this bacterial population, highlighting the importance of PIA during CoNS infections. In line with this assumption, the contribution of PIA to the virulence of CoNS species (especially *S. epidermidis*) was investigated in several animal models of infection (Li *et al.*, 2005; Rupp *et al.*, 2001; Rupp *et al.*, 1999a; Rupp *et al.*, 1999b), demonstrating a link between PIA synthesis and virulence. At the same time, some other studies on animal models of *S. epidermidis* infections raised some questions about the general and exclusive importance of *icaADBC* and PIA for pathogenesis (Chokr *et al.*, 2007; Francois *et al.*, 2003). Hence, while it has been thought for a long time that PIA was essential for biofilm formation, it is now known that this process may be additionally or exclusively mediated by specific surface proteins.

Aap is a cell wall-anchored protein encoded by the *aap* gene and comprises two major domains, A and B. Aap is known to interact with PIA, forming a protein-polysaccharide biofilm network (Bateman *et al.*, 2005). In fact, Aap was initially assigned to have a role in anchoring PIA to the bacterial cell surface (Hussain *et al.*, 1997). However, Aap was later found to promote biofilm formation in a PIA-independent manner (Rohde *et al.*, 2005). In *S. epidermidis* removal of Aap domain A leads to exposure of domain B, which in turn provides this protein intercellular adhesive properties essential in the accumulation stage of biofilm formation (Rohde *et al.*, 2005). Interestingly, Aap also seems to play an essential role in the colonisation of the skin (Macintosh *et al.*, 2009), which makes this protein a bifunctional molecule important for both commensal and pathogenic lifestyles of *S. epidermidis*. The regulatory mechanisms that control transcription of *aap* gene are not yet clearly understood. Nevertheless, a study on *S. epidermidis* biofilms at different developmental stages found this gene to be down-regulated in the late stage of biofilm formation (Yao *et al.*, 2005). This result suggested that Aap may play a role in the early stages of biofilm development rather than in the maintenance of *S. epidermidis* biofilms. Similarly to *icaADBC*, *aap* gene is common among clinical isolates (Rohde *et al.*, 2007; Stevens *et al.*, 2008; Vandecasteele *et al.*, 2003; Vuong *et al.*, 2004). Additionally, Aap-dependent biofilm formation was also directly demonstrated in clinical isolates (Rohde *et al.*, 2007; Rohde *et al.*, 2005), indicating that Aap is, like PIA, an important virulence factor.

Besides Aap, another surface protein called Bhp is also thought to be involved in CoNS biofilm formation (Tormo *et al.*, 2005). This protein is homologue to the biofilm-associated protein (Bap) of *S. aureus*, which was first identified in strong biofilm-forming *S. aureus* strains from chronic bovine mastitis (Cucarella *et al.*, 2001). It seems that Bap has different roles during biofilm formation, namely in primary attachment (interaction with abiotic surfaces and host extracellular matrix proteins) and in the step of bacterial accumulation, possibly in cooperation with PIA (Cucarella *et al.*, 2001). Besides *S. aureus*, Bap is also found (usually in a small proportion) among different CoNS strains isolated from animal infections, although it was initially thought to be absent in human-related strains (Tormo *et al.*, 2005). However, a recent study reported a small proportion of human *S. epidermidis* isolates carrying the *bap* gene (Potter *et al.*, 2009). Interestingly, its homologue Bhp was also found to be present in human strains of *S. epidermidis* and, taking into account the sequence and protein similarities with Bap, Bhp was assumed to have similar functions (Tormo *et al.*, 2005). Bhp is encoded by the *bhp* gene, which is found in approximately 10-45% of *S. epidermidis* isolated from human infections, depending on the study (Bowden *et al.*, 2005; Rohde *et al.*, 2007; Rohde *et al.*, 2004). Therefore, the significance of Bhp for *S. epidermidis* and other CoNS biofilm formation and pathogenesis remains to be determined.

In summary, it becomes clear that different molecules are involved in CoNS biofilm formation, with both proteins and polysaccharides having similar importance (Schommer *et al.*, 2011).

3.1.2. CoNS: a life between commensalism and pathogenicity

The ubiquitous nature of CoNS, as commensal microorganisms of the skin and mucous membranes, makes the differentiation between clinical significant (invasive) and commensal (contaminating) isolates a difficult task. Thus, a reliable method to correctly identify invasive isolates is vital to clinical decision making (Gu *et al.*, 2005). Biofilm phenotype has been appointed as a marker to differentiate invasive from commensal isolates, though results are not very consistent (Cho *et al.*, 2002; de Silva *et al.*, 2002; Galdbart *et al.*, 2000; Jain and Agarwal, 2009; Nayak *et al.*, 2011; Vogel *et al.*, 2000; Ziebuhr *et al.*, 1997). These controversial results are mainly due to the lack of a standardised assay for evaluation of biofilm formation, environmental factors and phenotypic variation of biofilm formation (Fitzpatrick *et al.*, 2002; Gu *et al.*, 2005).

Furthermore, genes that influence biofilm formation have also been studied as molecular markers for invasive CoNS isolates (e.g. *atlE*, *ica*, *aap* and *bhp*) (Frebourg *et al.*, 2000; Galdbart *et al.*, 2000; Pei *et al.*, 1999; Vandecasteele *et al.*, 2003; Zhang *et al.*, 2003). The *ica* gene locus is by far the most investigated in this area, and was previously suggested as a molecular marker of pathogenic isolates (Arciola *et al.*, 2002; Frebourg *et al.*, 2000). Nevertheless, some studies have revealed a relatively high prevalence of *ica* gene among commensal isolates (Frebourg *et al.*, 2000; Koskela *et al.*, 2009; Rohde *et al.*, 2004; Vandecasteele *et al.*, 2003). Consequently, this fact may leads to a low specificity when the presence of this gene is used to differentiate pathogenic from commensal isolates (Vandecasteele *et al.*, 2003).

As previously discussed in Chapter I, most virulence factors of CoNS also play important roles in the commensal lifestyle of these species. Therefore, it is now hypothesised that these so called virulence factors are in fact essential mechanisms for skin colonisation, and CoNS are merely "accidental pathogens" that use the "weapons" they possess to defend themselves from the host immune effectors (Otto, 2009).

3.1.3. Aims

To clarify the hypothesis stated above, we studied the biofilm formation ability in a population of CoNS isolated from healthy individuals. In addition, we analysed the relationship between biofilm formation and the detection of *icaA*, *aap* and *bhp* genes in *S. epidermidis* isolates in order to assign the contribution of each gene in this process.

3.2. Materials and methods

3.2.1. Bacterial isolates

The group of 61 CoNS previously isolated (Chapter II) were tested for biofilm formation. The 31 isolates belonging to *S. epidermidis* species were additionally screened for the presence of the biofilm-associated genes *icaA*, *aap* and *bhp*.

3.2.2. Quantification of biofilm formation

Quantitative determination of biofilm formation was performed by using a microtiter-plate assay as previously described (Stepanovic *et al.*, 2007), with some modifications. Briefly, bacteria were inoculated into tryptic soy broth (TSB, Liofilchem, Teramo, Italy) and incubated overnight at 37°C with shaking at 120 rpm. Overnight

cultures were adjusted to an optical density (OD) at 640 nm of 0.3 (approximately 2 x 10^8 CFU/ml) and diluted 1:100 in TSB supplemented with 0.4% (w/v) glucose (TSBg). Subsequently, 200 µl of each bacterial suspension were placed into a 96-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium), and incubated at 37°C with shaking at 120 rpm for 24 (±2) h. After incubation, the bacterial cells in suspension were removed carefully, and each well was washed twice with 200 µl of 0.9% NaCl, air dried and stained by crystal violet technique. In brief, 100 µl of 99.9% methanol (Fisher Scientific, Fair Lawn, NJ, USA) were added to each well and allowed to react for 15 minutes. Afterwards, methanol was removed and the plate was briefly air-dried. Next, 200 µl of 1% (v/v) crystal violet (Merck, Darmstadt, Germany) were added and allowed to stain for 5 minutes. Then, the staining solution was carefully removed, the plate was washed twice with distilled water, and finally 160 µl of 33% (v/v) glacial acetic acid (Fisher Scientific, Fair Lawn, NJ, USA) were added. Absorbance was measured at 570 nm using a microplate reader.

S. epidermidis 9142 and its isogenic strain *S. epidermidis* 9142-M10 were used as positive and negative controls, respectively. *S. epidermidis* 9142 is a strong biofilm producer that produces PIA, and strain 9142-M10 has a transposon inserted into the *ica* locus and thus does not produce a PIA-based biofilm (Cerca *et al.*, 2004). All isolates were tested in eight wells in duplicate, and these experiments were repeated as part of three to five independent assays. Additionally, wells with sterile TSB alone served as controls to check sterility and non-specific binding of media. The mean of the absorbance of non-inoculated wells was calculated and subtracted to all test values. Finally, the results of each isolate were averaged. The optical density of each isolate (OD_S) was compared with the mean absorbance of negative control (OD_{NC}). Therefore, isolates were classified as follows: non-biofilm producer ($OD_S \leq O_{NC}$), weak biofilm producer ($OD_{NC} < OD_S \leq 2xOD_{NC}$), moderate biofilm producer ($2xOD_{NC} < OD_S \leq 4xOD_{NC}$) and strong biofilm producer ($OD_S > 4xOD_{NC}$).

3.2.3. Detection of biofilm-associated genes

DNA templates were obtained as described in Chapter II. PCR reactions were performed in a final volume of 10 µl according to the following conditions: 2 µl of DNA template, 5 µl of DyNAzyme II PCR Master Mix 2x (Finnenzymes, Thermo Scientific, Finland), 1 µl of primer mixture (10 µM of each forward and reverse primer) and 2 µl of nuclease-free water, as described before (França *et al.*, 2012). PCR amplifications were

performed using the MJ Mini thermal cycler (Bio-Rad, Hercules, CA, USA). Information about primers and amplification conditions is shown in Table 3.1. In each PCR run, a positive control (*S. epidermidis* ATCC 35984), and a negative control (water) were also included. Amplified products were analysed in 1.5% agarose gel stained with Midori Green DNA stain (Nippon Genetics Europe GmbH, Germany).

Table 3.1. Primers and amplification conditions for *icaA*, *aap* and *bhp* genes

Target gene	Oligonucleotide primers sequence (5' to 3')	Amplicon size (bp)	PCR conditions
icaA	Fw TGC ACT CAA TGA GGG AAT CA Rv TCA GGC ACT AAC ATC CAG CA	417	5 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 56 °C and 45 s at 72 °C 10 min at 72 °C
aap	Fw GCT CTC ATA ACG CCA CTT GC Rv GGA CAG CCA CCT GGT ACA AC	617	
bhp	Fw TGG ACT CGT AGC TTC GTC CT Rv TCT GCA GAT ACC CAG ACA ACC	213	

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

3.2.4. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.02 (La Jolla, CA, USA). Chi-square or Fisher's exact test were used to examine the relationship between categorical variables. A $p<0.05$ was considered statistically significant.

3.3. Results and discussion

3.3.1. Biofilm formation

The study of biofilm formation revealed that 35 (57%) isolates were able to produce biofilm. Of the 61 isolates, 21 (34%) were classified as weak, 6 (10%) as moderate, and 8 (13%) as strong biofilm producers (Table 3.2). Similar results have been reported by other researchers, who observed a significant number of commensal *S. epidermidis* isolates able of forming biofilm (de Araujo *et al.*, 2006; Hellmark *et al.*, 2013). In fact, and with the exception of *S. epidermidis*, information on the biofilm formation of other CoNS species has been very limited, probably due to the lack of genome information. Interestingly, all

CoNS species tested exhibited ability to form biofilms, in different degrees. In respect to this, none of the *S. capitis* and *S. warneri* isolates were able to exhibit a strong biofilm phenotype. Nevertheless, and taking the data together, we found no statistically significant difference in biofilm formation between *S. epidermidis* and other CoNS species ($p = 0.30$). This finding supports the idea that biofilm formation is a common feature among several CoNS species (and not only in *S. epidermidis*), and also plays an important role in an environment like the skin, where they are exposed to harsh conditions and extensive mechanical stress. Conversely, Klingenberg *et al.* (2005) observed that the percentage of biofilm formation within a group of neonatal sepsis isolates was higher among *S. epidermidis* than in other CoNS species. One possible explanation for these controversial findings is that this and our study focus on two distinct CoNS populations (pathogenic and commensal CoNS, respectively).

Table 3.2. Biofilm formation ability among 61 commensal CoNS species from healthy individuals

	CoNS species						
	S. epidermidis	***S. capitis***	***S. homins***	***S. warneri***	***S. haemolyticus***	**Other CoNS**	**Total, number (%)**
Number (%) of isolates	31 (51)	9 (15)	8 (13)	6 (10)	4 (6)	3 (5)	**61 (100)**
Biofilm formation ability							
Non-biofilm producer	11 (35)	5 (56)	4 (50)	4 (67)	1 (25)	1 (33)	**26 (43)**
Weak biofilm producer	11 (35)	3 (33)	2 (25)	2 (33)	2 (50)	1 (33)	**21 (34)**
Moderate biofilm producer	5 (16)	1 (11)	0 (0)	0 (0)	0 (0)	0 (0)	**6 (10)**
Strong biofilm producer	4 (13)	0 (0)	2 (25)	0 (0)	1 (25)	1 (33)	**8 (13)**

3.3.2. Detection of *icaA*, *aap* and *bhp* genes and its relationship with biofilm formation

Due to the lack of genome information regarding to other CoNS species, we decided to investigate the presence of *icaA*, *aap* and *bhp* only in *S. epidermidis* isolates. Of the 31 *S. epidermidis* isolates, 12 (39%) were positive for *icaA*, 18 (58%) for *aap*, and 6 (19%) for *bhp*. The same primers were also used to evaluate whether amplification would occur from DNA isolated from some isolates belonging to the six other CoNS species found in this

study. However, no products were obtained, thus it was considered these sets of primers were not suitable for the screening of these genes in non-*S. epidermidis* species. This could be partially explained by the fact that primer design was only based on the sequences of the *S. epidermidis* published genomes.

The majority of the *S. epidermidis* isolates (23, 74%) were positive for at least 1 of the 3 genes, and only 8 isolates (26%) were negative for all 3 genes. A great proportion of isolates (*n* = 12, 39%) exhibited only one of the three genes. Nevertheless, 11 isolates (35%) had at least 2 genes, 9 of those isolates (29%) had a 2-gene combination, and 2 isolates (6%) had all 3 genes. The combination most frequently observed was *icaA-aap* (*n* = 8, 26%).

Other studies that addressed the distribution of these genes (except *bhp*), among commensal *S. epidermidis* isolates, also reported very similar results (de Araujo *et al.*, 2006; Hellmark *et al.*, 2013). The high rate of isolates carrying the *aap* gene suggests that Aap is of extreme importance in the commensal stage of this bacterium. Recently, Macintosh *et al.* (2009) found that this protein can promote adhesion to corneocytes (dead cells present in the outermost layer of the human epidermis) and is likely to be an important adhesin in S. *epidermidis* skin colonisation. Additionally, intercellular adhesion provided by PIA and biofilm-related proteins is assumed to be crucial in an ecological niche like the skin (Otto, 2009). On the other hand, Rogers *et al.* (2008) suggested that the presence of *icaADBC* is detrimental to the colonisation of human skin by *S. epidermidis*, which may partially explain why the carriage rate of *icaA* is lower than that observed for the *aap* gene in our population. Finally, only a minority of isolates was found to carry the *bhp* gene, a result already reported by Rohde *et al.* (Rohde *et al.*, 2004), which suggest a less important role of Bhp in the commensal lifestyle of *S. epidermidis*.

Analysing the distribution of each gene, we observed that *icaA* and *aap* were more frequently detected among biofilm formers comparatively to non-biofilm former isolates (Figure 3.3). Nevertheless, a small fraction of non-biofilm formers also carried *icaA* or *aap* genes. Regulation of these two genes appears to be extremely complex and not completely understood, as described above (Arciola *et al.*, 2004; Bowden *et al.*, 2005; Conlon *et al.*, 2002a; Fitzpatrick *et al.*, 2002; Yao *et al.*, 2005; Ziebuhr *et al.*, 1997), and may be involved in *S. epidermidis* phase-variation which appears to improve bacterial survival and growth under changing environmental conditions *in vivo* (Ziebuhr *et al.*, 1999). Therefore, down-regulation of the expression of *icaA* and *aap* genes might be responsible for the

observed biofilm-negative phenotype in those isolates. These findings emphasise the complexity associated with the biofilm structure and its expression.

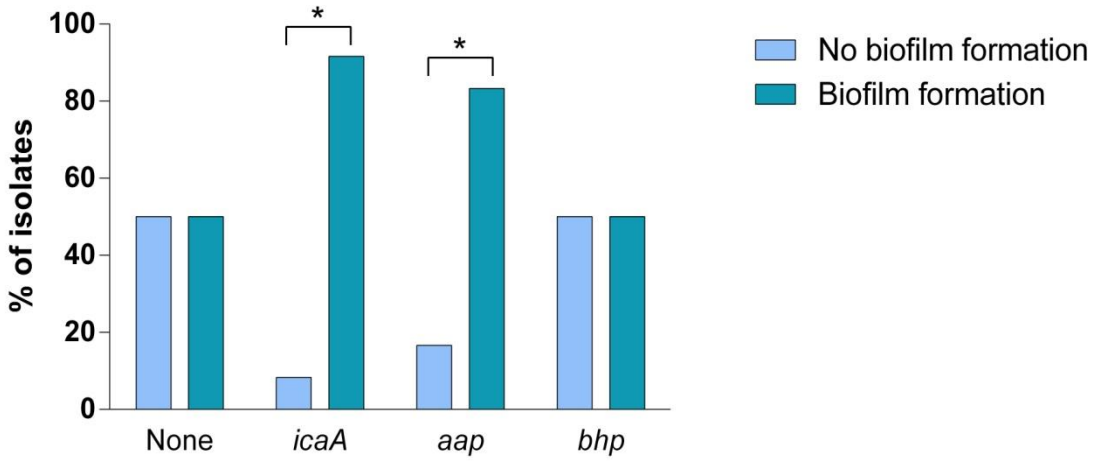

Figure 3.3. Distribution of *icaA*, *aap* and *bhp* genes between biofilm and non-biofilm formers; * statistically significant difference ($p < 0.05$).

On the other hand, the *bhp* gene was equally distributed between biofilm and non-biofilm formers. In fact, the assumption that Bhp could be involved in biofilm accumulation was based solely on its remarkable structural similarity with Bap from *S. aureus* (Tormo *et al.*, 2005), but no experimental evidence to support this hypothesis has been obtained to date. The few studies on this matter have reported inconsistent results on the distribution of the *bhp* gene among *S. epidermidis* isolates from human infections (Bowden *et al.*, 2005; Rohde *et al.*, 2007; Rohde *et al.*, 2004), which have hindered the elucidation of the role of this protein in biofilm formation. Although only a minority of isolates analysed in this study carried the *bhp* gene, we observed that the presence of this gene is associated with moderate/ strong biofilm formation only in the cases in which *icaA* or *icaA+aap* were also detected. Most importantly, our results represent a little step forward in the elucidation of Bhp in *S. epidermidis* biofilm formation, and challenge the hypothesis that this protein plays a significant role in the accumulation stage of this process.

Interestingly, in 4 isolates classified as weak-biofilm producers, none of the 3 genes was detected (Figure 3.4). These isolates may suggest divergence at the primer binding site (false negative PCR result) or represent a distinct mechanism of biofilm formation independent of *ica, aap* or *bhp*. A similar study on commensal *S. epidermidis* isolates from healthy individuals (de Araujo *et al.*, 2006) also reported a biofilm-producing isolate that

lacked both *icaA* and *aap* genes, although the presence of the *bhp* gene was not investigated. Recently, attention has been given to the extracellular matrix binding protein (Embp). Previously found to mediate binding to fibronectin (Williams *et al.*, 2002), Embp was recently demonstrated to be sufficient for biofilm formation in *icaADBC*- and *aap*-negative *S. epidermidis* isolates (Christner *et al.*, 2010; Linnes *et al.*, 2013) Thus, it would be interesting to investigate the presence of *embp* gene in those isolates.

It is noteworthy that all isolates exhibiting a strong biofilm (100%), and 60% with a moderate biofilm phenotype harboured at least two of the three genes studies. On the other hand, only 36% of the weak biofilm-producers carried two genes (*icaA* and *aap*). The statistical analysis demonstrated a significant association (p = 0.03) between the detection of these genes and biofilm formation, indicating a strong correlation between strong biofilm formation and the simultaneous presence of two or three biofilm-associated genes (Figure 3.4).

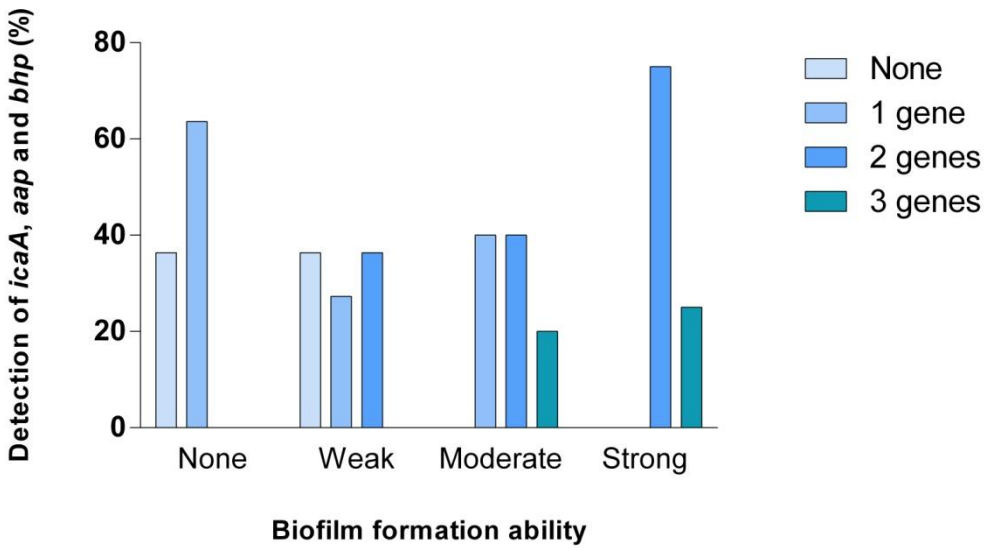

Figure 3.4. Association between the detection of *icaA*, *aap* and *bhp* genes and biofilm formation in 31 commensal *S. epidermidis* isolates obtained from healthy individuals.

Another important finding is that all isolates in which the *bhp* gene was detected alone (3, 10%) no biofilm formation was observed. This finding put in question again the role of Bhp in biofilm formation, and suggests that this protein alone may not be able to form a mature, multilayered biofilm. Nevertheless, it would be interesting to assess whether this gene is being expressed or not in such isolates. In contrast, some isolates in

which only *aap* gene was detected were able to produce biofilm, including moderate biofilm formation.

Overall, our observations emphasise the importance of these genes, especially *ica* and *aap*, in *S. epidermidis* biofilm formation. In addition, the data obtained here suggest that the simultaneous presence of two or three of the genes studied has an important impact in the biofilm formation, highlighting the real complexity of this highly regulated process.

3.4. Conclusions

In conclusion, this study provides evidence that biofilm formation ability is a transversal feature among commensal, community-associated CoNS, and not exclusive of clinical isolates causing infection as some authors have argued (Galdbart *et al.*, 2000; Ziebuhr *et al.*, 1997). Our results provide evidence that biofilm formation and biofilm-related factors also have an important role in the commensal lifestyle of these microorganisms, and may partially explain why CoNS are becoming important nosocomial pathogens, despite their lack of recognised virulence factors other than biofilm formation.

Additionally, our experiments addressed the contribution of some biofilm-related factors in biofilm formation, confirming the extreme importance of PIA and Aap, and raising some questions about the real importance of Bhp in this process.

Chapter IV

Antibiotic resistance among community-associated CoNS and association with biofilm formation

4.1. Introduction

Currently, there are a wide range of antibiotics that can be used in the treatment of staphylococcal infections. Some of them are naturally found substances (e.g. penicillin), while others are synthetically developed (e.g. ciprofloxacin). Nevertheless, staphylococci, and CoNS in a particular manner, have been recognised by their striking ability to rapidly developing resistance to antibiotics (Lentino *et al.*, 2008).

The key for the successful establishment of staphylococci as nosocomial pathogens comes partially from their extraordinary ability to deal with selective pressures, as well as developing or acquiring antibiotic resistance (Schoenfelder *et al.*, 2010). One of the most representative examples of this situation is the case of the β-lactam antibiotics. Penicillin, introduced in the 1940s, was the first effective and non-toxic antibiotic with activity against staphylococcal infections. However, and only a few years later, penicillin-resistant strains emerged, a resistance due to the production of an enzyme called β-lactamase (Livermore, 2000). As a result, a β-lactamase stable penicillin called methicillin was introduced in 1960. Unexpectedly, resistance to this antibiotic took no more than two years to emerge (Enright *et al.*, 2002).

Similar developments have occurred for almost all the other classes of antibiotics (see table 4.1), which have drastically reduced the treatment options for staphylococcal infections (Livermore, 2000). Glycopeptides, particularly vancomycin, are currently regarded as one of the last resorts for the treatment of infections caused by methicillin-resistant *S. aureus* and CoNS. Nevertheless, an increasing number of studies reporting both decreased susceptibility and resistance to vancomycin among staphylococcal clinical isolates, (Center *et al.*, 2003; Dunne *et al.*, 2001; Garrett *et al.*, 1999; Juarez-Verdayes *et al.*, 2006; Nunes *et al.*, 2006; Pina *et al.*, 2000; Schwalbe *et al.*, 1987) has evidenced a serious threat to the efficacy of this antibiotic in a near future.

However, it must be noted that antibiotic resistance is not an exclusive feature of invasive, clinically significant isolates. In fact, most surveys on staphylococcal antibiotic resistance have focused on this group of isolates (Claesson *et al.*, 2007; Ghadiri *et al.*, 2012; Monsen *et al.*, 2005; Sharma *et al.*, 2010), but when it comes to the prevalence of resistance among staphylococcal commensal flora from healthy population, we only find

Table 4.1. Classes of antibiotics with activity against staphylococci, and their respective mechanisms of action and resistance (Fluit *et al.*, 2001)

Class	Main antibiotics	Mechanism of action	Mechanisms of resistance
β-lactams	Penicillins Methicillin[1] Oxacillin Amoxicillin	Interference with the synthesis of the bacterial cell wall: β-lactam antibiotics bind to specific target enzymes called penicillin-binding proteins (PBPs) that are essential for cell wall peptidoglycan synthesis	Inactivation of the antibiotic molecule by enzymatic hydrolysis of the β-lactam ring (β-lactamase production)
			Acquisition of the *mecA* gene that encodes an alternative penicillin-binding protein (PBP2a) with low affinity for most β-lactam antibiotics
Glycopeptides	Vancomycin	Interference with the synthesis of the bacterial cell wall	Acquired genetic resistance involving the *vanA* gene
			Thickness of the bacterial cell wall (less understood mechanism)
Fluoroquinolones	Ciprofloxacin	Interference with the bacterial DNA replication: inhibition of the enzymes DNA gyrase and topoisomerase IV, which are involved in the folding and supercoiling of the DNA after replication	Mutational alterations in the target genes
			Over expression of efflux pumps
Aminoglycosides	Gentamicin	Inhibition of protein synthesis by binding to the bacterial 30S ribosomal subunit	Inactivation of the antibiotic molecule by aminoglycoside-modifying enzymes
			Mutational alterations in the target genes
Macrolides	Erythromycin	Inhibition of protein synthesis by binding to the bacterial ribosomal 50S subunit	Mutational alterations in the target genes
Rifamycins	Rifampicin	Inhibition of protein synthesis by binding to the β-subunit of the RNA polymerase (encoded by the *rpoB* gene)	Mutational alterations in the target genes (mainly point mutations - single nucleotide polymorphism (SNP) - in the *rpoB* gene

[1]Methicillin is no longer in clinical use

a residual number of published studies (Begovic *et al.*, 2013; de Araujo *et al.*, 2006; Dominguez *et al.*, 2002). Additionally, it is important to take into account that human indigenous commensal flora is also exposed to some selective pressures, including those from antibiotic use, that it may act as a reservoir of resistance determinants (Marshall *et al.*, 2009). Hence, a larger number of studies on commensal bacteria are required in order to assess their role in the development and spread of antibiotic resistance.

4.1.1. Aims

Since commensal bacteria are now regarded as a reservoir for antibiotic resistance determinants, the main aim of the work described in this chapter was to investigate the frequency of antibiotic resistance among commensal, community-associated CoNS isolates obtained from Portuguese healthy individuals. According to the questionnaire data, we also attempted to determine whether there is medical and/ social factors that could affect colonisation by antibiotic-resistant isolates.

In addition, we aimed to correlate antibiotic resistance with biofilm formation, as well as with the presence of biofilm-associated genes (data previously described in Chapter III) in order to provide new insights into this controversial issue.

4.2. Materials and methods

4.2.1. Bacterial isolates

The group of 61 CoNS previously isolated (Chapter II) was tested for antibiotic susceptibility.

4.2.2. Assessment of antibiotic susceptibility profiles

Antibiotic susceptibility was tested using the broth microdilution method according to the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (EUCAST-ESCMID, 2003). The following antibiotics and dilution ranges (in $\mu g.ml^{-1}$) were tested: ciprofloxacin (CIP, 0.125-8), erythromycin (ERY, 0.125-8), gentamicin (GEN, 0.125-8), penicillin (PEN, 0.125-8), rifampicin (RIF, 0.015-2), and vancomycin (VAN, 0.125-8) (Sigma-Aldrich, USA).

Before performing the antibiotic susceptibility assay, several dilutions of each antibiotic in TSB were prepared. Every well was inoculated with 95 µl of TSB

supplemented with antibiotic plus 5 µl of an overnight culture previously diluted 1:100 in 0.9% NaCl. This procedure led to a final bacterial concentration in each well of approximately 5 x 10^5 CFU/ml as assessed by CFU plating (Appendix D). The plates were incubated at 37°C for 16-20 h (24h for vancomycin) and in the following day the wells were analysed in terms of presence or absence of cell growth. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of an antibiotic that inhibited the visible growth of a microorganism. Each isolate was tested at least in two independent occasions in duplicate. *Staphylococcus aureus* ATCC 29213 was used as a control strain. All isolates were classified into susceptible, intermediate, or resistant based in the most recent MIC breakpoints for *Staphylococcus* spp. established by EUCAST (EUCAST, 2013). Multidrug resistance was defined as resistance to three or more classes of the antimicrobial agents tested.

4.2.3. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.02 (La Jolla, CA, USA). The differences in the distribution of antibiotic resistant isolates were analysed by Chi-square or Fisher's exact test. A $p<0.05$ was considered statistically significant.

4.3. Results and discussion

4.3.1. Overall antibiotic resistance

The results of antimicrobial susceptibility are summarised in Table 4.2. The highest rates of resistance were detected for erythromycin (n = 27, 44%), penicillin (n = 23, 38%) and gentamicin (n = 15, 25%) while the lowest rate was exhibited for ciprofloxacin (n = 4, 7%). All isolates were susceptible to rifampicin and vancomycin. Overall, 75% (n = 46) of the isolates were resistant to at least one class of antibiotics tested, and 39% (n = 24) exhibited resistance towards more than one antimicrobial class. Multidrug resistance was observed in 15% (n = 9) of CoNS isolates. Interestingly, the frequency of resistance for gentamicin and penicillin was significantly higher in *S. epidermidis* than in other CoNS.

Antibiotic resistance is a multifactorial phenomenon, and high rates of antibiotic consumption, along with their misuse, are pivotal factors that have created this serious public health issue (Steinke and Davey, 2001). Moreover, it is currently known that antibiotic molecules are widely disseminated in a broad range of environmental sources (Martinez, 2009). Hence, their presence in different ecological niches may also account for

Table 4.2. Antimicrobial resistance profiles to six antimicrobial agents (μg.ml^{-1}) in 61 CoNS isolates isolated from the skin of healthy individuals

Antimicrobial agent	*S. epidermidis* (*n* = 31)		Others CoNS[2] (*n* = 30)		Overall % resistance[1] (*n* = 61)	*p*-value[3]
	MIC range	% Resistance[1]	MIC range	% Resistance[1]		
Ciprofloxacin	0.25 - 8	6	≤0.125 – 2	7	7	1.000
Erythromycin	≤0.125 - >8	48	≤0.125 - >8	40	44	0.610
Gentamicin	0.25 - 2	42	≤0.125 – 2	7	25	0.002*
Penicillin	≤0.125 - >8	52	≤0.125 - >8	23	38	0.030*
Rifampicin	≤0.015 - 0.03	0	≤0.015 - 0.06	0	0	n/a
Vancomycin	1 - 4	0	≤0.125 – 4	0	0	n/a

[1] isolates classified as intermediate-resistant are included here

[2] *S. capitis, n = 9; S. hominis, n = 8; S. warneri, n = 6; S. haemolyticus, n = 4; S. equorum, n = 2; S. pettenkoferi, n = 1*

[3] Fisher's exact test

* Statistically significant difference

n/a - not applicable

the local selection of resistant bacteria. Recent data from a European surveillance program (EUCAST, 2013) reported that Portugal was one of the countries with higher rates of antibiotic consumption outside hospitals. Penicillins and macrolides were the most prescribed drugs in the last years, which may partially explain the high levels of resistance for these classes of antibiotics found in our study. In contrast to the very few studies on CoNS isolated from healthy individuals (Cove *et al.*, 1990; Widerstrom *et al.*, 2011), we observed a higher resistance rate to gentamicin.

It is also noteworthy that a considerable proportion of isolates (n = 42, 69%) exhibited a decreased susceptibility to vancomycin (MIC $\geq$ 2 $\mu g.ml^{-1}$). Of these isolates, 71% (n = 30) were *S. epidermidis*. Although resistance to vancomycin has not become widespread to date, some researchers have reported increasing vancomycin MICs both in clinical (Center *et al.*, 2003; Garrett *et al.*, 1999; Pina *et al.*, 2000) and in commensal (Palazzo *et al.*, 2005) staphylococcal isolates.

Additionally, we found no significant statistic difference in the distribution of resistant isolates among the different groups of subjects (Table 4.3). Most importantly, it appears that the frequency of resistance to individual antibiotics does not seem to be affected by the use of antibiotics in the community. Nevertheless, one cannot neglect the effect of the increased antibiotic use in the accumulation of antibiotic-resistance determinants.

In general, these are worrisome results since it appears that resistance and/ or decreased susceptibility to currently available drugs for most staphylococcal infections are not confined to the hospital settings but are also becoming spread in the community. Moreover, the widespread of multiresistant CoNS observed among our study population, colonising even subjects without contact with hospital environment and/ or antibiotic use in recent past, is also a matter of concern. However, the origin of such resistance remains unclear.

4.3.2. Association between antibiotic resistance and biofilm formation

In an attempt to identify whether there is a relationship between antibiotic resistance and biofilm formation, we performed a comparison between the data obtained on antibiotic susceptibility assays and the data obtained on biofilm formation (previously described in Chapter III).

Table 4.3. Distribution of resistant isolates among the 59 healthy individuals

Characteristic	Colonised with antibiotic resistant isolate		Relative risk (95% CI[1])	*p*-value[2]
	Yes (*n* = 40)	No (*n* = 19)		
Socio-demographic characteristics				
Age (years)				0.275[3]
0-18	6	2		
19-30	19	14		
31-50	8	2		
51-79	7	1		
Gender				1.000
Male	20	9		
Female	20	10		
Medical characteristics				
Hospitalisation in the last year	6	1	1.311 (0.9133 - 1.882)	0.411
Antibiotic use in the last year	13	2	1.412 (1.039 - 1.920)	0.101
Contact with medical staff/ medical care employee	15	7	1.036 (0.7186 - 1.495)	1.000
On medication	13	2	1.412 (1.039 - 1.920)	0.101
Allergic diseases	10	2	1.306 (0.9365 - 1.820)	0.303
Daily habits				
Hand washing with frequency	39	19	0.6724 (0,5618 - 0.8048)	1.000
Shower everyday	32	17	0.8163 (0.5632 - 1.183)	0.476
Smoker/ exposure to tobacco smoke	15	7	1.036 (0.7186 - 1.495)	1.000
Tattoos/ acupuncture in the last year	1	1	0.7308 (0.1807 - 0.2956)	0.544
Gym/ pool/ sauna users	15	5	1.170 (0.8284 - 1.653)	0.558

[1]Confidence interval; [2]Fisher's exact test; [3]Chi-square test

From this analysis, we observed that the frequency of antibiotic resistance was always higher in biofilm formers than in non-biofilm formers for all antibiotics, with exception for ciprofloxacin (Figure 4.1). This analysis did not include rifampicin and vancomycin since no resistance was observed for these two antibiotics. In general, we also found a significant higher frequency of antibiotic resistance in biofilm formers than in non-biofilm formers (p = 0.02) (Figure 4.1). Moreover, we also found a significant higher proportion of multidrug-resistant isolates among biofilm formers comparing with non-biofilm formers (p = 0.03). Overall, our findings demonstrate a clear tendency of isolates with biofilm formation ability to be resistant to two or more antibiotics simultaneously.

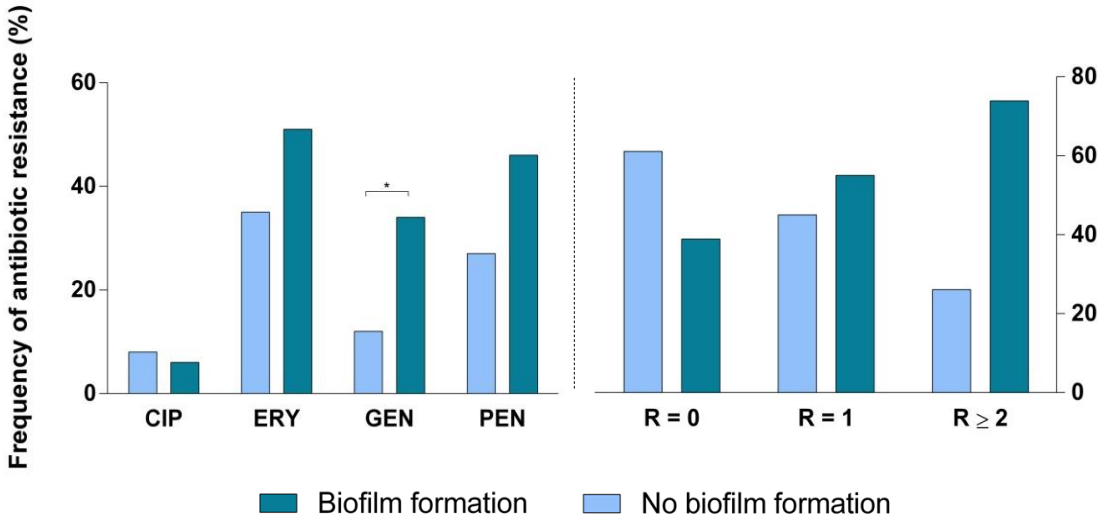

Figure 4.1. Association between antibiotic resistance and biofilm formation in 61 CoNS isolates from healthy individuals. * represents a statistically significant difference (p<0.05); CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; PEN, penicillin; R represents the number of antibiotics to which the isolates were found to be resistant.

In order to elucidate the nature of the relationship between antibiotic resistance and biofilm formation, we also compared the data obtained on antibiotic susceptibility assays with the detection of *icaA* and *aap* genes. However, and in this particular case, the analysis was limited to 31 isolates, since the presence of such genes was only assessed for *S. epidermidis* isolates, as discussed in the previous chapter.

In general, we also observed a tendency of isolates that carry the *icaA* and/ or *aap* genes to be resistant to two or more antibiotics simultaneously (Figure 4.2). However this association was not statistically significant, perhaps due to the reduced sample size.

Interestingly, and when analysing the frequency of resistance for each of the antibiotics tested, the results were slightly different. It may be noticed that, even though generally present, the association between the presence of *icaA* gene and antibiotic resistance was particularly evident for gentamicin, with a *p*-value much close to the significance level (p = 0.07) (Figure 4.3). However, and with regards to *aap* gene, the same association was not observed. In this case, only resistance to ciprofloxacin and gentamicin was more frequent among *aap*+ isolates, although no statistically significant differences were found.

The association between antibiotic resistance and biofilm formation in staphylococci is not recent and not even a consensual concept. While some researchers did not find such

association (Kotilainen *et al.*, 1991), several others have been reported it in both clinical (Arciola *et al.*, 2005; Montanaro *et al.*, 2007) and commensal CoNS isolates (Agarwal and Jain, 2012; de Araujo *et al.*, 2006).

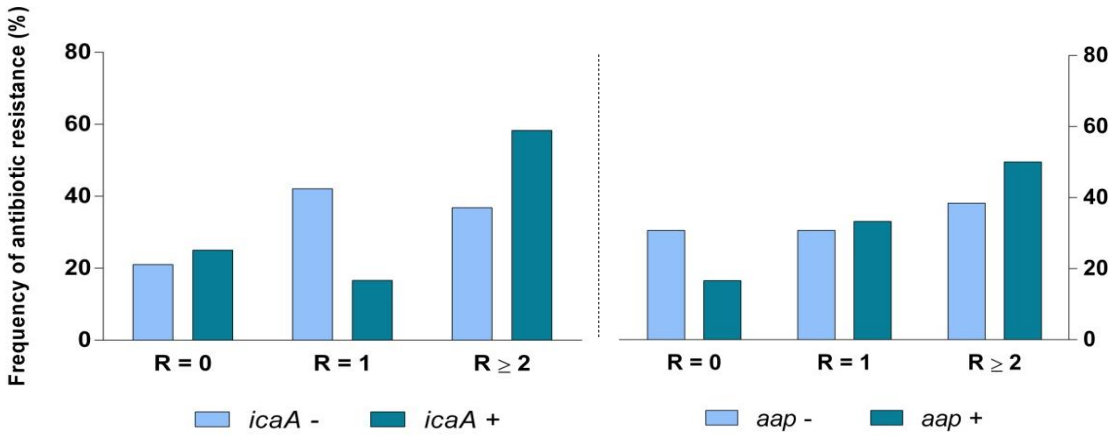

Figure 4.2. Association between antibiotic resistance and detection of biofilm-associated genes in 31 *S. epidermidis* isolates from healthy individuals. R represents the number of antibiotics to which the isolates were found to be resistant.

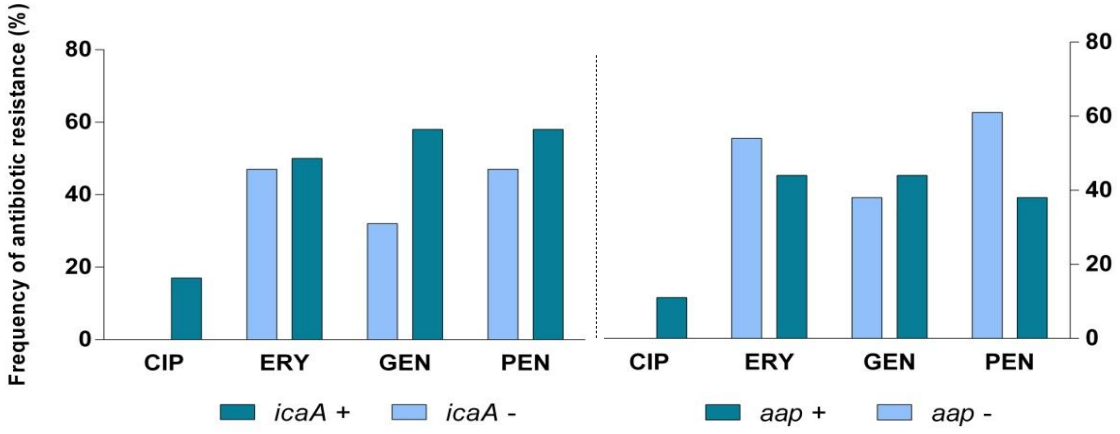

Figure 4.3. Association between resistance and detection of biofilm-associated genes for each antibiotic tested in 31 *S. epidermidis* isolates from healthy individuals; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; PEN, penicillin.

The two important questions that remain to be elucidated on this matter is the nature of such association and if it is observed in different classes of antibiotics. Overall, our findings in commensal isolates from healthy individuals support the existence of an association between antibiotic resistance and biofilm formation. Most importantly, this association was more pronounced for gentamicin, a finding also previously reported in

clinical *S. epidermidis* isolates (Kozitskaya *et al.*, 2004; Montanaro *et al.*, 2007). Some authors have suggested that this association could be mediated by the presence of transposons (Kozitskaya *et al.*, 2004; Ziebuhr *et al.*, 2000). It is currently known that antibiotic resistance genes are often associated with these mobile genetic elements, and some of them are flanked by insertion sequences, such as IS*256*. *Tn4001*, a transposon carrying a gene that confers resistance to a wide range of aminoglycosides, including gentamicin (Culebras and Martinez, 1999), is usually flanked by the IS*256* (Kozitskaya *et al.*, 2004). On the other hand, the association of IS*256* with biofilm formation, and specifically with the *icaADBC* operon, is well documented, as was already discussed in Chapter III. This may in turn explain the association we observed between carriage of *icaA* gene and resistance to the different antibiotics tested, and why the same association was not observed for *aap* gene.

It is tempting to conclude from our results that this association might be restricted to some classes of antibiotics, especially aminoglycosides, and that it may be primarily associated with *icaADBC* operon and not with the biofilm phenotype *per se*. However, and in order to prove this hypothesis, further investigations with a much larger number of isolates as well as antibiotic classes is mandatory.

4.4. Conclusions

In general, we concluded that the rate of antibiotic resistance in CoNS isolated from the skin of healthy individuals in Portugal is relatively high, providing evidence that resistant CoNS strains are indeed disseminated in the community, supporting the hypothesis that staphylococcal skin flora represents a reservoir of potentially pathogenic bacteria. Moreover, the reduced susceptibility of these microorganisms to commonly used antibiotics might promote their persistence in the healthy community and long-term colonisation. Then, and most important of all, our results emphasise the urgency in the establishment of more strict policies regarding to antibiotic consumption since the availability and misuse of antibiotics appear to select and/or maintain antibiotic-resistance, even in healthy, non-treated individuals. Additionally, multidrug-resistance is one of the most feared aspects of antibiotic resistance and an increasing challenge for medical community. Hence, the development of novel anti-staphylococcal agents is of paramount importance.

On the other hand, our results enabled us to observe a clear association between antibiotic resistance and biofilm formation. To our knowledge, this is the first study on community-associated CoNS isolates (and not only *S. epidermidis*), reporting such association. Nevertheless, and taking into account the results of other studies, our findings lead us to challenge the hypothesis that this association is common throughout all antibiotic classes. We rather hypothesise that this association is confined to some classes of antibiotics and is primarily associated with the presence of the *icaADBC* operon.

Chapter V

Expression of *icaA* and *aap* at different stages of *S. epidermidis* biofilm development

5.1. Introduction

Biofilm formation is a complex process that seems to be controlled by several factors. In the last decade, several research groups have been focused on the molecular mechanisms behind biofilm formation, aiming to identify different gene expression patterns in different bacterial species, and at different biofilm developmental stages (Beloin and Ghigo, 2005).

Essentially, genetic analyses have revealed a wide range of genetic determinants involved in biofilm formation but, most importantly, they have uncovered the concept that there are multiple pathways to form a biofilm (O'Toole, 2003). In contrast, several studies have reported that only a small proportion of the bacterial genome undergoes a significant change in expression when bacteria shift from a planktonic, free-floating, to a biofilm mode of growth (Ren *et al.*, 2004; Stanley *et al.*, 2003; Whiteley *et al.*, 2001).

With regards to *S. epidermidis*, there is also a broad knowledge on the molecular basis of biofilm formation. The function of important biofilm-associated genes, such as *icaADBC* and *aap*, in the mediation of the accumulative phase of biofilm development was already established, as described throughout Chapter III. However, studies that follow gene expression from early to late stages of biofilm formation are lacking. Hence, it is not currently know the specific contribution of each biofilm-associated gene in distinct phases of *S. epidermidis* biofilm development.

5.1.1. Aims

The aim of this study was to determine whether two important *S. epidermidis* biofilm-associated genes, *icaA* and *aap*, are differentially expressed at two distinct biofilm developmental stages. For this purpose, gene expression levels of *S. epidermidis* biofilms grown *in vitro* were assessed at early (12h) and mature (54h) phases of biofilm growth.

5.2. Materials and methods

5.2.1. Bacterial isolates

Two well-characterised (in Chapters III and IV) biofilm-forming *S. epidermidis* isolates from healthy individuals were used in this work: 004A (moderate-biofilm producer, *icaA*+, *aap*+, and *bhp*-) and 023A (weak-biofilm producer, *icaA*-, *aap*+, and *bhp*-).

5.2.2. Biofilm formation

Biofilms were grown as previously described (França *et al.*, 2012). Briefly, bacteria was inoculated into TSB and incubated overnight at 37°C with shaking at 120 rpm. Overnight cultures were adjusted to an OD_{640} of 0.3 (approximately 2 x 10^8 CFU/ml) and diluted 1:100 in fresh TSBg. Subsequently, 1 ml of the diluted bacterial suspension was placed into a 24-well microtiter plate (Orange Scientific, Braine-l'Alleud, Belgium), incubated at 37°C with shaking at 120 rpm, and allowed to grow for 12 or 54 h. In biofilms grown for 54 h, the TSBg medium containing suspended bacterial cells was removed and an equal volume of fresh TSBg was added every 24 h.

5.2.3. RNA extraction

RNA extraction from *S. epidermidis* biofilms was performed using a previously optimised protocol (França *et al.*, 2012). This method combines chemical (phenol) and mechanical (glass beads) lysis of bacterial cells along with silica membrane-based RNA isolation (E.Z.N.A. Total RNA Kit, Omega Bio-Tek, GA, USA). Bacterial biofilms were thoroughly washed, suspended in 1 ml of 0.9% NaCl, and immediately placed on ice. Suspended biofilm cells were centrifuged at 16000 *g* for 10 min at 4ºC and the supernatants were discarded. Then, the bacterial pellets were suspended in 500 µl of TRK lysis buffer (provided by the kit) supplemented with 2% β-mercaptoethanol, plus 500 µl of 90% phenol solution (Applichem, Darmstadt, Germany). The resulting mixture was transferred into 2 ml safe lock tubes containing 0.5 g of acid-washed 150-212 mm silica beads (Sigma, USA). The tubes were then placed into FastPrep cell disruptor (BIO 101, ThermoElectron Corporation, USA) and beat for 35 s at 6.5 $m.s^{-1}$. The samples were immediately placed on ice for 5 min and the beat-beading step was repeated twice. Afterwards, samples were centrifuged at 16000 *g* for 1.5 min at 4ºC, the supernatants transferred into 2 ml DNase/ RNase free tubes and mixed with an equal volume of 70% ethanol. The samples (including any remaining precipitate) were transferred to the silica columns and centrifuged at 10000 *g* for 30 s at room temperature (RT). The flow-through was discarded and each column was placed into a new collection tube. To wash the columns, 500 µl of Wash Buffer I were added to each column and centrifuged at 10000 *g* for 30 s at RT. The flow-through was discarded and each column was placed into the same collection tube. After that, 500 µl of Wash Buffer II were added to each column and centrifuged at 10000 *g* for 30 s at RT. This step was repeated once more, the flow-through

was discarded, and the columns were placed into new collection tubes for a new centrifugation at 16000 *g* for 2 min. The collection tubes were discarded and each column was then placed into 1.5 ml DNase/ RNase free tubes. Lastly, RNA elution was achieved by adding 50 µl of DEPC-treated water into the centre of the column, incubated for 1 min at RT, and centrifuging at 16000 *g* for 2 min.

5.2.4. DNase treatment

Genomic DNA was digested with DNase I (Fermentas, Ontario, Canada). 2 µl of DNase I and 5 µl of DNase buffer were added to each RNA sample and incubated at 37ºC for 30 min. Then, to inactivate the DNase I activity, 5 µl of 25 mM EDTA were added to the mixture and incubated at 65ºC for 10 min.

5.2.5. RNA quality determination

The concentration and purity of the total RNA was spectrometrically determined using a NanoDrop 1000 (Thermo Scientific, USA). Three independent measurements of the same sample were performed and averaged. The absorbance ratios A_{260}/A_{280} and A_{260}/A_{230} were used as indicators of protein and polysaccharide/ phenol/ chaotropic salts contamination, respectively. The integrity of the total RNA was assessed by visualisation of the 23S/16S banding pattern. RNA samples were analysed in 1.5% agarose gel stained with Midori Green DNA stain (Nippon Genetics Europe GmbH, Germany). RNA was stored at -20ºC until further use.

5.2.6. cDNA synthesis

cDNA synthesis was performed using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Ontario, Canada) following the manufacturer's instructions. The same amount of total RNA (1 µg) from each sample was reverse transcribed in a 10 µl reaction volume. To determine the possibility of genomic DNA carry-over, control reactions were performed under the same conditions but lacking the reverse transcriptase enzyme (no-RT control). All RNA samples extracted were absent of significant genomic DNA, as determined by an average cycle threshold difference of 18.39 ± 3.10, equivalent to a maximum quantification error of 0.0003%.

5.2.7. Gene expression quantification

Biofilm gene expression was determined by quantitative real-time PCR (qPCR). qPCR analysis was performed using 2x iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a 10 µl reaction volume. Each PCR reaction contained 2 µl diluted cDNA or no-RT control (1:200 in DEPC-treated water), 5 µl of master mix, 1 µl of primer mixture (10 µM of each forward and reverse primers), and 2 µl of nuclease-free water. Information about the primers used is listed in Table 5.1. The efficiency of each primer set was previously determined (França *et al.*, 2012). qPCR runs were performed on a CFX 96 (Bio-Rad, Hercules, CA, USA) with the following cycle parameters: 95ºC for 3 min, 49 cycles of 95ºC for 10 s, 56ºC for 15 s, and 72ºC for 20 s. qPCR products were analysed by melting curves to confirm that only the desired product was amplified. All reactions were run in triplicate and a no template control (nuclease-free water) for each primer pair was included in each run. The expression of *icaA* and *aap* was normalised to the expression of the housekeeping gene *16S rRNA* using the $2^{\Delta Ct}$ method, where 2 stands for 100% PCR efficiency and $\Delta Ct = Ct_{16S\ rRNA} - Ct_{target\ gene}$.

Table 5.1. List of the primers used in qPCR experiments

Target gene	Oligonucleotide primers sequence (5' to 3')		Amplicon size (bp)
16S rRNA	Fw	GGG CTA CAC ACG TGC TAC AA	176
	Rv	GTA CAA GAC CCG GGA ACG TA	
icaA	Fw	TGC ACT CAA TGA GGG AAT CA	134
	Rv	TAA CTG CGC CTA ATT TTG GAT T	
aap	Fw	TAA CTG CGC CTA ATT TTG GAT T	190
	Rv	GCA TGC CTG CTG ATA GTT CA	

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

5.2.8. Statistical analysis

All statistical analyses were performed using GraphPad Prism version 6.02 (La Jolla, CA, USA). The mean normalised gene expression was compared among the different

conditions assayed by applying student's t test. A $p<0.05$ was considered statistically significant.

5.3. Results and discussion

5.3.1. *icaA* and *aap* expression during biofilm development

In order to determine the expression profile of *icaA* and *aap* genes in different stages of *S. epidermidis* biofilm formation, we analysed the gene expression levels of both genes in two independent biofilm-forming *S. epidermidis* isolates at early (12 h) and late (54 h) phases of growth. By comparing the expression levels, we were able to identify an up-regulation of both genes in 54 h comparatively with 12 h biofilms (Figure 5.1). This up-regulation was however more evident for *icaA* gene, in strain 004A, with an approximately 5-fold expression increase.

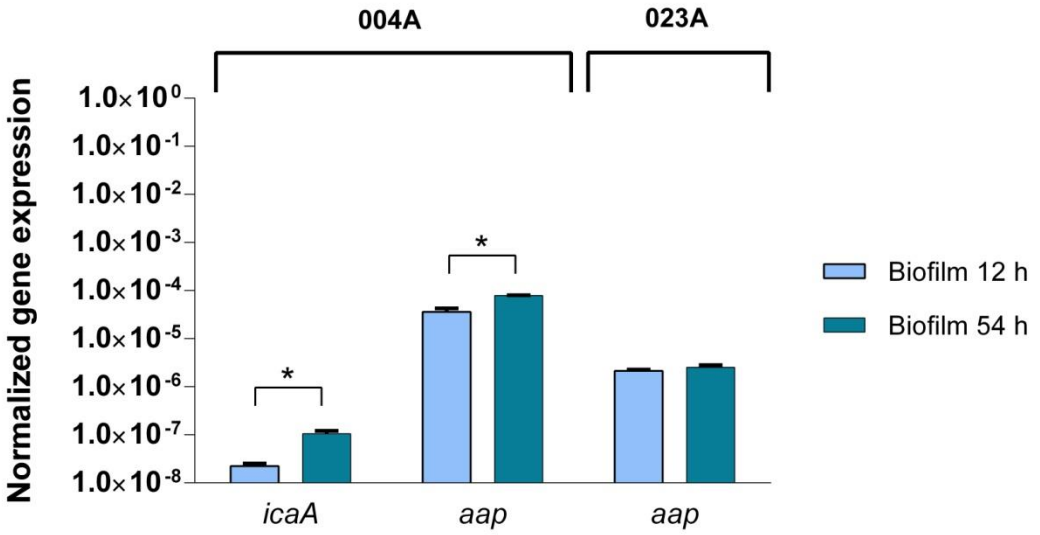

Figure 5.1. Variation of *icaA* and *aap* expression levels in two different developmental stages of biofilm formation; results are expressed as mean ± standard deviation; * represents statistically significant differences ($p<0.05$).

The role of PIA, encoded by the *ica* locus, in intercellular adhesion and bacterial aggregation was already previously discussed. *In vitro* studies have demonstrated that *S. epidermidis* mutants lacking PIA are not able to accumulate into multilayered biofilms (Dobinsky *et al.*, 2002; Mack *et al.*, 1994). However, studies about gene expression in *S. epidermidis* during biofilm development are still scarce, which makes difficult the

comparison of results. A study conducted by Patel *et al*. (Patel *et al.*, 2012) found an approximately 10-fold increase in the expression of *icaA* gene from 12 to 48 h, with the largest increase in expression occurring between 12 and 24 h. In fact, we also observed the same trend, i.e., the expression of *icaA* gene increases over the time, although we have studied the gene expression at a later time point (54 h). Although it is well established that the *ica* locus is conserved between *S. epidermidis* and *S. aureus* (Cramton *et al.*, 1999), some studies on *S. aureus* biofilms found an inverse trend in the expression profile of *ica* genes (Beenken *et al.*, 2004; Resch *et al.*, 2005). These studies found *ica* genes to be highly expressed after 8 h of growth, gradually decreasing at later growth stages (up to 48 h). On the other hand, another study reported that *ica* transcription, as well as PIA synthesis, appear to be markedly higher in *S. epidermidis* than in *S. aureus* strains, both *in vitro* and *in vivo* (Fluckiger *et al.*, 2005), which partially might explain the controversial results between these two staphylococcal species.

With regards to *aap* gene, and despite being more expressed at a later stage in both isolates, it is noteworthy that its expression was much higher at both phases in comparison with *icaA* expression. This finding may indicate that *aap* has a higher contribution than *icaA* at both earlier and later phases of *S. epidermidis* biofilm formation, at least in strain 004A. Nevertheless, studies on more strains are mandatory to confirm this result. Interestingly, the *aap* expression level was higher in strain 004A than that observed in strain 023A in both growth phases, which appears to be a reasonable explanation for the different biofilm phenotypes exhibited by each one, i.e., moderate and weak biofilm formation, respectively. Similarly to PIA, the importance of Aap in biofilm formation was previously demonstrated (Hussain *et al.*, 1997; Rohde *et al.*, 2005), and our results appear to corroborate its function.

5.4. Conclusions

Overall, our findings confirm the role of PIA and Aap in the accumulative stage of biofilm formation, since we found both *icaA* and *aap* genes up-regulated at 54 h comparatively with 12 h of growth. Moreover, our results apparently indicate that *icaA* is of more importance in a later stage of biofilm formation, which might lead to a biofilm with a stronger architecture.

In order to better understand the roles of *icaA* and *aap* in biofilm accumulation, it would be interesting to evaluate the expression of these genes at several stages of biofilm development (e.g., 6, 12, 24, 48, and 60 h), as well as the quantification of biofilm formation, viable cells, and polysaccharide and protein concentrations at these time points.

Chapter VI

Main conclusions and future directions

6.1. Main conclusions

Research on CoNS species has been mainly focused on clinical isolates, which are known to be well equipped with a range of pathogenic traits, especially an exceptional ability to form biofilms and high resistance rates to multiple classes of antibiotics. However, it is not still clear whether these two determinants are also well disseminated among commensal, community-associated CoNS isolates. In fact, research on commensal bacteria in general has been scarce, perhaps because this subset of microorganisms is widely regarded as harmless. Nevertheless, some researchers dedicated to the study of commensals have hypothesised that these bacteria might be an underappreciated reservoir of pathogenic determinants.

Recognising this hypothetical role of commensal bacteria, and in order to fulfil the lack of studies in this neglected research field, we aimed to perform a phenotypic and genotypic characterisation of different CoNS species isolated from healthy Portuguese individuals.

The first task of this work, i.e., the isolation and identification procedures, revealed a great diversity of CoNS species colonising the human skin, which emphasised the need for studying each species as a separate entity and with its own features.

Throughout the following tasks, a thorough characterisation of all isolates was performed. Our initial results provided evidence that biofilm formation ability and the presence of biofilm-associated genes were common features among commensal, community-associated CoNS. It was then hypothesised that, besides being regarded as virulence factors, these features might also be essential pieces in the colonising lifestyle of these microoganisms. Additionally, our experiments also addressed the contribution of some biofilm-associated genes in biofilm formation, confirming the extreme importance of PIA and Aap, and raising some questions about the real importance of Bhp in this process. With regards to antibiotic susceptibility testing, our results showed a great proportion of isolates with decreased susceptibility and multiple resistances to some of the current available drugs for most staphylococcal infections. Moreover, we observed a significant association between antibiotic resistance and biofilm formation, although our results led us to hypothesise that such association might be confined to some classes of antibiotics and primarily associated with the *icaADBC* operon.

In the last task, we aimed to determine the expression patterns of two biofilm-associated genes (*icaA* and *aap*) at two distinct biofilm developmental stages. With respect to this, our findings corroborated the role of both genes in the accumulative stage of biofilm formation.

Overall, we are able to conclude that staphylococcal skin flora might be regarded as a reservoir of biofilm-forming and antibiotic resistant microorganisms, or in other words, a reservoir of potentially pathogenic bacteria. Simultaneously, our experiments also bring to light new perceptions about the molecular basis of staphylococcal biofilm formation, as well as the nature of the association between antibiotic resistance and biofilm formation.

6.2. Future directions

The conclusions drawn from the work described in this thesis highlight the urgency in the application of more strict policies regarding to antibiotic consumption, as well as in the development of novel anti-staphylococcal agents. In respect to this, suitable alternatives to antibiotic therapy should be a priority.

On the other hand, our findings also raised some important questions that need to be elucidated. Most importantly, the experiments on biofilm formation and antibiotic susceptibility should be reproduced in a larger number of isolates, so that our conclusions can acquire more significance. With regards to biofilm formation, further studies are required to elucidate the role of Bhp in *S. epidermidis* biofilm formation. Additionally, the presence of biofilm-associated genes in non-*S. epidermidis* CoNS species should be investigated, in order to understand whether the staphylococcal biofilm formation process is transversal among CoNS or each species have their own mechanism. Moreover, other molecules known to be involved in biofilm formation should be studied in depth, such as the case of Embp. Furthermore, the association between antibiotic resistance and biofilm formation should also be further investigated in order definitely clarify its nature.

Lastly, it would be interesting to evaluate the expression of a wide range of genes at several stages of biofilm development and growth conditions, possibly by using microarray technology. By following this approach it would be possible to assign the specific contribution of each gene during biofilm formation.

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Supplementary information

Appendix A - Questionnaire

INQUÉRITO

Inquérito para estudo sobre a colonização da pele pela bactéria *Staphylococcus epidermidis*

ID Amostra:	Idade:	Sexo: M ☐ F ☐
Localidade:		
Profissão:		

1. Alguma vez esteve hospitalizado(a) nos últimos dois anos?

   a) Sim b) Não

   1.1. Em caso afirmativo, especifique há quanto tempo ocorreu a hospitalização.

   _____

2. Durante o último ano esteve sob tratamento com antibióticos?

   a) Sim b) Não

   2.1. Em caso afirmativo, especifique o antibiótico (ou a finalidade do tratamento).

   _____

3. Tem algum tipo de contacto com profissionais de saúde e/ ou pessoas que frequentem um ambiente hospitalar?

   a) Sim b) Não

4. Actualmente está a tomar algum tipo de medicação?

   a) Sim b) Não

   4.1. Em caso afirmativo, especifique o(s) medicamento(s) (ou a sua finalidade).

   _____

5. Lava as mãos com frequência?

   a) Sim b) Não

6. Toma banho todos os dias?

   a) Sim b) Não

7. Fuma com regularidade, ou está exposto(a) a ambientes com fumo?

   a) Sim b) Não

8. Apresenta alguma alergia, nomeadamente associada à pele?

   a) Sim b) Não

   8.1. Em caso afirmativo, especifique o tipo de alergia.

   _____

9. Efectuou alguma tatuagem/ tratamento com acupunctura no último ano?

   a) Sim b) Não

10. Frequenta com regularidade locais públicos como piscinas, ginásios, saunas ou similar?

    a) Sim b) Não

Agradecemos a sua atenção!

Appendix B - Informed consent

Universidade do Minho

Campus de Gualtar
4710-057 Braga

UNIVERSIDADE DO MINHO
ESCOLA DE ENGENHARIA

Departamento de
Engenharia Biológica

Assunto: **CONSENTIMENTO INFORMADO, LIVRE E ESCLARECIDO PARA PARTICIPAÇÃO EM INVESTIGAÇÃO de acordo com a Declaração de Helsinquia e a Convenção de Oviedo**

Título do estudo: "Interações moleculares entre biofilms de *S. epidermidis* e células do sistema imune de murganhos"

Enquadramento: Tradicionalmente visto como um microrganismo comensal, *Staphylococcus epidermidis* é agora visto como um importante agente patogénico oportunista sendo o mais comum agente causador de infeções hospitalares. A nível mundial têm-se vindo a dar também alguma importância à presença de *S. epidermidis* resistentes a antibióticos na população geral, isto é, fora do âmbito hospitalar. Estudos neste sentido, em Portugal, não são muito comuns, sendo clara a necessidade de se obter informação sobre a presença, ou ausência, de estirpes resistentes e/ou multirresistentes na população geral. Dessa forma, os objetivos deste estudo são o isolamento e identificação de *S. epidermidis* e outros estafilococos coagulase negativos (CoNS), provenientes da epiderme de voluntários saudáveis, assim como a determinação da capacidade de virulência desses microorganismos, a determinar pela sua resistência a antibióticos e a capacidade de formação de estruturas microbianas conhecidas como biofilmes.

Explicação do estudo: A colaboração por parte dos voluntários saudáveis prende-se na recolha de uma amostra epitelial e a resposta a um questionário anónimo. Para recolher a amostra, deverá ser utilizado a zaragatoa que é fornecida. A extremidade semelhante a um cotonete de algodão deverá ser raspada no verso da mão, abundantemente, e posteriormente colocada no recipiente fornecido, enroscando-o de forma a ficar bem selado. Juntamente com este processo, o voluntário deverá responder ao questionário enviado em anexo. A amostra e questionário deverão ser colocados no ponto de entrega mais próximo. Do questionário serão tiradas conclusões que poderão explicar a ocorrência de bactérias resistentes a antibióticos na população geral. A caracterização microbiológica será efetuada posteriormente no Departamento de Engenharia Biológica, da Universidade do Minho.

Condições e financiamento: Projeto com financiamento da Fundação para a Ciência e Tecnologia (fundos nacionais, referência PTDC/BIA-MIC/113450/2009, e fundos europeus, programa Compete, referência FCOMP-01-0124-FEDER-014309). A participação é voluntária não havendo contrapartidas diretas para o participante. Este estudo mereceu o parecer favorável da Subcomissão de Ética para as Ciências da Vida e da Saúde da Universidade do Minho.

Confidencialidade e anonimato: Todo o estudo será anónimo, não sendo requerido nenhum dado que possa eventualmente identificar o voluntário. Os dados recolhidos serão utilizados exclusivamente no âmbito deste projeto.

Investigador responsável: O investigador responsável por este projeto é Nuno Miguel Dias Cerca, Investigador Auxiliar no Centro de Engenharia Biológica, Universidade do Minho. Poderá ser contactado através do email nunocerca@ceb.uminho.pt ou do telefone 253604423. A equipa de investigação agradece a vossa colaboração.

Assinatura do investigador responsável:: … … … … … … … … … … … … … …… … … … … …

Declaro ter lido e compreendido este documento, bem como as informações verbais que me foram fornecidas pela/s pessoa/s que acima assina/m. Foi-me garantida a possibilidade de, em qualquer altura, recusar participar neste estudo sem qualquer tipo de consequências. Desta forma, aceito participar neste estudo e permito a utilização dos dados que de forma voluntária forneço, confiando em que apenas serão utilizados para esta investigação e nas garantias de confidencialidade e anonimato que me são dadas pelo/a investigador/a.

Nome: … … … … … … … …… … … … …… … … … … … … … … … … … …
Assinatura: … … … … … … … …… … … … … … … … … … … … … … … … … … …
Data: …… /…… /………..

SE NÃO FOR O PRÓPRIO A ASSINAR POR IDADE OU INCAPACIDADE
(se o menor tiver discernimento deve também assinar em cima, se consentir)
NOME: …
BI/CD Nº: .... DATA OU VALIDADE …../…../……..
GRAU DE PARENTESCO OU TIPO DE REPRESENTAÇÃO: ....
ASSINATURA …

ESTE DOCUMENTO É COMPOSTO DE 2 PÁGINA/S E FEITO EM DUPLICADO:
UMA VIA PARA O/A INVESTIGADOR/A, OUTRA PARA A PESSOA QUE CONSENTE

Appendix C

Gram staining

Bacterial cells were initially smeared over a microscope slide and heat-fixed. Then, the smear was covered with crystal violet stain for 60 s. The stain was poured off and the excess stain was gently rinsed with running water. Afterwards, the smear was covered with Lugol's solution for 60 s and subsequently rinsed with running water. In the following step, a few drops of 70% ethanol were added and allowed to trickle down the slide. The slide was gently rinsed with running water only when the solvent was no longer coloured. Lastly, the smear was covered with safranin solution for 30 s and gently rinsed with running water. The slide was dried with absorbent paper to remove the excess water. The smears were then examined under a light microscope in order to visualise the morphology (e.g., cocci, bacilli) and the reaction of the bacterial cells to Gram staining (purple, Gram-positive; pink, Gram-negative). *Staphylococcus epidermidis* ATCC 35984 (Gram-positive cocci) and *Escherichia coli* ATCC 25922 (Gram-negative bacilli) were used as controls.

Oxidase testing

Oxidase strips (Oxoid, UK) containing NNN'N'-tetramethyl-p-phenylene-diamine dihydrochloride (TMPD) were used for oxidase testing. On each strip a small amount of the bacteria to be tested was added and observed after 20 s. The strip turned dark blue when in contact with oxidase-positive bacteria (due to the presence cytochrome c oxidase) and remained colourless in the case of oxidase-negative bacteria. *Pseudomonas aeruginosa* ATCC 10145 and *Staphylococcus epidermidis* ATCC 35984 were used as positive and negative controls, respectively.

Catalase testing

A small amount of the bacteria to be tested was placed onto a microscope slide. Then, a few drops of hydrogen peroxide were added over the bacteria. Formation of bubbles was indicative of a positive reaction, whereas no bubble formation indicated a negative reaction. *Staphylococcus epidermidis* ATCC 35984 and *Gardnerella vaginalis* AMD were used as positive and negative controls, respectively.

Appendix D - CFU enumeration

The concentration of bacteria in the growth control wells (medium without antibiotic) of each MIC panel was determined through CFU enumeration. Immediately following inoculation of the MIC panel, 10 µl of the well content were diluted 1:100 in 0.9% NaCl. The obtained suspension was homogenised by vortex and 10 µl were spotted onto a TSA plate and left to dry. Following overnight incubation (18 ± 2 h), the number of colonies present on the plate was counted. CFU enumeration was determined as follows:

CFU/ml = number of colonies x dilution factor (100) x 100