UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA VETERINÁRIA

VIRULENCE CHARACTERIZATION AND ANTIMICROBIAL RESISTANCE OF MAJOR BACTERIAL GENERA FROM DIABETIC FOOT INFECTIONS

CARLA MOTTOLA

Orientadores: Professora Doutora Maria Manuela Castilho Monteiro de Oliveira
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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de Ciências Biológicas e Biomédicas

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“Look up at the stars and not down at your feet.

Try to make sense of what you see, and wonder

about what makes the universe exist. Be curious.”

Stephen Hawking

- *In the memory of my uncle Roberto,*

You left us too early, we miss you everyday

- *In the memory of Professor Cristina Vilela,*

Who taught me to never give up

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Abstract

Virulence characterization and antimicrobial resistance of major bacterial genera from diabetic foot infections

Diabetes *mellitus* is a major chronic disease that continues to increase significantly. One of the most important and costly complications of diabetes is the development of foot ulcers, colonized by pathogenic and antimicrobial resistant bacteria, which may be responsible for impairing its successful treatment. Diabetic foot ulcer (DFU) bacterial communities can be organized in polymicrobial biofilms, which may be responsible for its chronicity. The ability of these communities to produce biofilm was evaluated and was higher when compared to biofilm formation by individual species.

Staphylococcus aureus is one of the most prevalent species in diabetic foot infections (DFI). Staphylococci isolated from DFU in patients from the Lisbon area were identified, genotyped and screened for virulence and antimicrobial resistance traits. The isolates showed high genomic diversity, were resistant to important clinically antibiotics and expressed relevant virulence determinants.

As biofilm formation is one of the most important virulence traits of *S. aureus*, the antimicrobial susceptibility patterns of biofilm-producing *S. aureus* strains were also analysed. The minimum biofilm inhibitory and eradication concentrations were determined for ten antimicrobial compounds. Staphylococci biofilms were resistant to antibiotic concentrations ten to thousand times higher than those effective for planktonic cells. Furthermore, the enterococci frequently isolated from DFI, were also identified and characterized, showing high antimicrobial resistance and important virulence traits.

Since DFI are often caused by resistant bacteria, it is necessary to find alternatives to antibiotic therapy, such as phage therapy. The inhibitory potential of five bacteriophages, previously characterized, was evaluated against established biofilms formed by *S. aureus*, *P. aeruginosa* and *A. baumannii*. A significant cell reduction after phage exposure was observed, mainly after multiple treatments.

DFI are very complex and studies on this topic are scarce. It is necessary to intensify research in order to develop more adequate therapeutic protocols for this type of infection.

Keywords: Diabetic foot infections, biofilm, staphylococci, virulence determinants, antimicrobial resistance.

Resumo

Caracterização da virulência e resistência a antimicrobianos dos principais géneros bacterianos envolvidos em infeções de pé diabético

Diabetes *mellitus* é uma doença crónica com grande impacto em saúde pública e cuja incidência continua a aumentar significativamente em todo o mundo, atingindo atualmente mais de 400 milhões de pessoas. Uma das complicações mais importantes da diabetes e associada a gastos económicos significativos são as úlceras de pé diabético. Uma vez que a camada protetora de pele é danificada, os tecidos profundos ficam expostos à infeção bacteriana, a qual pode evoluir rapidamente. As infeções das úlceras de pé diabético são a causa mais comum de internamento hospitalar de pacientes diabéticos e uma importante causa de morbilidade, levando frequentemente à amputação dos membros inferiores. Estas infeções podem ser promovidas por bactérias potencialmente patogénicas e resistentes aos compostos antimicrobianos, prejudicando assim o sucesso do tratamento. As comunidades bacterianas presentes nas úlceras podem estar organizadas em biofilmes polimicrobianos, que contribuem para que as infeções se tornem crónicas e muito difíceis de resolver.

Foi avaliada a capacidade de produção de biofilme por comunidades polimicrobianas de isolados bacterianos de pé diabético, utilizando um ensaio de microtitulação em placa com "Alamar Blue" (AB) e uma técnica de Hibridação *In Situ* Fluorescente Múltipla (MFISH). Esta avaliação foi realizada em três períodos de incubação distintos (24, 48 e 72 horas), depois da determinação da capacidade de formação de biofilme por 95 isolados de úlceras de pé diabético pertencentes a vários géneros bacterianos (*Staphylococcus*, *Corynebacterium*, *Enterococcus*, *Pseudomonas* e *Acinetobacter*). Todos os isolados apresentaram a capacidade de produzir biofilme às 24 horas, sendo que a quantidade de biofilme produzido aumentou com o tempo de incubação. *Pseudomonas* apresentou a capacidade mais elevada de produção de biofilme, seguida de *Corynebacterium, Acinetobacter*, *Staphylococcus* e por fim, *Enterococcus*. Foram encontradas diferenças estatisticamente significativas na capacidade de formação de biofilme entre os três períodos de incubação. As comunidades polimicrobianas produziram mais biofilme do que as espécies individualmente. As comunidades formadas por *Pseudomonas* + *Enterococcus*, *Staphylococcus* + *Acinetobacter* e *Corynebacterium* + *Staphylococcus* formaram mais biofilme do que as comunidades formadas por *Enterococcus* + *Staphylococcus* e por *Enterococcus* + *Corynebacterium*. O comportamento biológico das diferentes espécies bacterianas nos biofilmes polimicrobianos tem implicações clínicas muito importantes para o sucesso do tratamento deste tipo de infeções. A sinergia entre as bactérias presentes em biofilmes multiespécies foi descrita previamente, sendo que este trabalho representa o primeiro estudo sobre a evolução temporal da formação de biofilme por parte de comunidades polimicrobianas isoladas de úlceras de pé diabético, incluindo várias espécies.

Staphylococcus é um dos géneros bacterianos mais prevalentes nas infeções de pé diabético. Neste estudo, isolados de *Staphylococcus* (n = 53) obtidos a partir de úlceras de pé diabético de doentes da região de Lisboa foram identificados, caracterizados genotipicamente, e rastreados para genes de virulência e de resistência a antimicrobianos. A relação genética entre os isolados foi avaliada através da técnica de eletroforese em gel de campo pulsado (PFGE) e da tipagem de sequências "multilocus" (MLST) de representantes dos pulsotipos identificados. A reação em cadeia de polimerase (PCR) foi aplicada para deteção de doze genes de virulência e a técnica E-teste foi realizada para determinar a concentração mínima inibitória (MIC) em relação a dez antibióticos. Verificou-se que *Staphylococcus* isolados de úlceras de pé diabético são genotipicamente muito variados, apresentam resistência a antibióticos importantes do ponto de vista clínico, nomeadamente ciprofloxacina e eritromicina, e expressam diversos determinantes de virulência. Essas propriedades sugerem que os estafilococos podem contribuir para a persistência e gravidade deste tipo de infeções, levando ao insucesso da terapêutica. Além disso, existe a possibilidade de eles poderem transmitir estas caraterísticas a outros microrganismos que partilham o mesmo nicho ecológico. Neste contexto, os pacientes diabéticos podem tornar-se um veículo de transmissão de clones bacterianos entre o ambiente hospitalar e a comunidade.

S. aureus resistentes à meticilina (MRSA) têm emergido como um dos principais problemas clínicos e epidemiológicos a nível hospitalar. As estirpes MRSA têm a capacidade de resistir à ação da maioria dos antibióticos β-lactâmicos, mas também a uma vasta gama de outros agentes antimicrobianos pertencentes a diferentes classes, tornando o tratamento destas infeções muito difícil e dispendioso. Até à data, existem disponíveis duas cefalosporinas de quinta geração eficazes contra MRSA, a ceftarolina e o ceftobiprole, com espetro de ação semelhante. Tendo em conta que a formação de biofilme é uma das mais importantes caraterísticas de virulência de *S. aureus* e que o seu desenvolvimento desempenha um papel importante na patogénese da infeção, uma vez que representa um mecanismo de defesa bacteriano, os padrões de suscetibilidade antimicrobiana dos isolados de *S. aureus* produtores de biofilme foram analisados, através da determinação da concentração mínima inibitória de biofilme (MBIC) e da concentração mínima de erradicação de biofilme (MBEC), para dez antibióticos incluindo a ceftarolina. Foi igualmente avaliada a presença de genes relacionados com antibiótico-resistência pela técnica de PCR. Foi observado que relativamente aos antibióticos mais utilizados no tratamento de infeções de pé diabético, são necessárias concentrações muito mais elevadas para inibir a formação de biofilme por isolados de *S. aureus in vitro*, o que pode explicar o facto da monoterapia com estes agentes não ser frequentemente eficaz em erradicar a infeção. De facto, os biofilmes analisados foram resistentes a concentrações de antibióticos dez até mil vezes mais elevadas do que as necessárias para matar as células planctónicas correspondentes. Os

únicos antibióticos capazes de erradicar os biofilmes produzidos por 50% dos isolados de *S. aureus* foram a ceftarolina e a gentamicina. Os resultados sugerem que os padrões de sensibilidade aos antibióticos não podem ser extrapolados para infeções com biofilme estabelecido.

Além de estafilococos, existem outras espécies bacterianas frequentemente identificadas em úlceras de pé diabético. Os enterococos são considerados bactérias oportunistas, mas nos últimos anos foram relacionados com infeções clínicas muito graves. *Enterococcus* sp. isolados a partir de infeções de pé diabético foram identificados por multiplex PCR, caracterizados pela análise de macro-restrição através de PFGE, e rastreados para características de virulência e resistência antimicrobiana. A maioria dos *Enterococcus* foi identificada como *E. faecalis*, espécie considerada mais patogénica dentro deste género bacteriano; genotipicamente os isolados mostraram elevada similaridade, revelando uma relação clonal. Todos os isolados foram considerados multirresistentes, produtores de citolisina e gelatinase, e a maioria mostrou ter capacidade de produzir biofilme, demonstrando a importância de *Enterococcus* no desenvolvimento das infeções de pé diabético e na sua persistência, especialmente em relação à sua capacidade de formação de biofilme e resistência a antibióticos clinicamente relevantes.

Uma vez que estas infeções são frequentemente promovidas por bactérias resistentes, torna-se necessário encontrar alternativas terapêuticas, tais como a terapêutica fágica. Para além das bactérias Gram-positivas já mencionadas, estas complexas infeções de pé diabético incluem muitas vezes bactérias Gram-negativas altamente patogénicas, como *Pseudomonas aeruginosa* e *Acinetobacter baumannii*. Um dos objetivos do estudo foi verificar o efeito de cinco bacteriófagos, produzidos pela empresa TecnoPhage, e caracterizados anteriormente, em biofilmes formados pelas espécies bacterianas *S. aureus*, *P. aeruginosa* e *A. baumannii*. A aplicação dos bacteriófagos a células planctónicas revelou-se eficaz às 4 horas após inoculação, mas às 24 horas pós-aplicação observou-se um ressurgimento do crescimento bacteriano. Em relação aos biofilmes e utilizando a atividade metabólica como medida da viabilidade celular determinada através da aplicação de AB, verificou-se uma redução celular significativa após a exposição aos fagos, quer às 4 quer às 24 horas pós-aplicação, mas mais significativa às 4 horas. Um tratamento múltiplo, com aplicação de fagos a cada 4 horas, promoveu uma diminuição ainda mais significativa da atividade celular. Os efeitos inibitórios mais elevados para células planctónicas e biofilme ocorreu num índice de multiplicidade bacteriófago:bactéria de 10. Estes resultados reforçam o potencial clínico da terapêutica fágica para o tratamento de infeções de pé diabético.

As infeções de pé diabético são uma realidade muito complexa e a epidemiologia dos agentes bacterianos envolvidos encontra-se em evolução. Os estudos disponíveis sobre este tema são escassos e é necessário intensificar a investigação quer na vertente

microbiológica quer na vertente clínica no sentido do desenvolvimento de protocolos terapêuticos adequados para este tipo de infeções.

Palavras-Chave: Infeções de pé diabético, biofilme, estafilococos, determinantes de virulência, resistência antimicrobiana.

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LIST OF ABBREVIATIONS

A	absorbance
Aae	*S. epidermidis* autolysin
Aap	accumulation-associated protein
Aas	*S. saprophyticus* autolysin
AB	alamar blue
Abi	abortive infection
agr	acessory gene regulator
AHL	acyl homoserine lactones
AIP-I	autoinducing peptide I
Atl	autolysin
AtlC	*S. caprae* autolysin
AtlE	*S. epidermidis* autolysin
AtlL	*S. lugdunensis* autolysin
AtlWM	*S. warneri* M autolysin
BES	bio-engineered skin
blaZ	penicillin resistance gene
BT	bacteriophage therapy
CA-MRSA	community-acquired MRSA
CC	clonal complex
ccr	cassette chromosome recombinase
CHIPS	chemotaxis inhibitory protein of *S. aureus*
CIP	ciprofloxacin
ClfA	clumping factor A
ClfB	clumping factor B
Cna	colagen binding protein
CoNS	coagulase-negative staphylococci
CoPS	coagulase-positive staphylococci
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CWA	cell wall-anchored
cyl	cytolysin
DFI	diabetic foot infection
DFU	diabetic foot ulcer
DM	diabetes *mellitus*
Eap	Extracellular adherence protein
eDNA	extracellular DNA
Efb	extracellular fibrinogen binding protein
Embp	extracellular matrix-binding protein
EMRSA	epidemic MRSA
EPS	extracellular polymeric substances
erm	erythromycin resistance gene
ES	Electrical Stimulation

Esp	Enterococcal surface protein
ET	exfoliative toxins
Fbe	fibrinogen-binding protein
FDA	U.S. Food and Drug Administration
FEP	functional equivalent pathogroups
FISH	Fluorescent *In Situ* Hybridization
FLIPr	formyl peptide receptor-like-1 inhibitory
FnbpA	fibronectin binding proteins A
FnbpB	fibronectin binding proteins B
fsr	*E. faecalis* regulator
GelE	enterococcal gelatinase
*gyr*A	gyrase subunit A
G-CSF	Granulocyte-Colony Stimulating Factor
HA-MRSA	hospital-associated MRSA
HBOT	hyperbaric oxygen therapy
ica	intercellular adhesion
icaA	intercellular adhesion A
icaADBC	intercellular adhesion operon ABCD
icaD	intercellular adhesion D
icaR	intercellular adhesion regulator
ICTV	International Committee for Taxonomy of Viruses
IDSA	Infectious Disease Society of America
IgG	immunoglobin G
Isd	iron-regulated surface protein
IM	input multiplicity
IWGDF	International Working Group of the Diabetic Foot
LED	light-emitting diodes
LLLT	low-level light therapy
Luk	leukocidin
MBEC	minimum biofilm eradication concentration
MBIC	minimum biofilm inhibitory concentration
MDR	multidrug-resistant
MDT	maggot debridement therapy
MFISH	multiplex fluorescent in situ hybridization
MHC-II	major histocompatibility complex class II
MIC	minimum inhibitory concentration
MLSb	macrolide-lincosamide-streptograminB
MLST	multilocus sequence typing
MRS	methicillin-resistant Staphylococcus
MRSA	methicillin-resistant *S. aureus*
MRSE	methicillin-resistant *S. epidermidis*
MSCRAMM	microbial surface components recognizing adhesive matrix molecules

MSSA	methicillin-susceptible *S. aureus*
NEAT	Near-iron transporter
NICE	National Institute for Health and Care Excellence
norA	ciprofloxacin resistance gene
NPWT	Negative pressure wound therapy
PBP	Penicillin binding protein
PBP2a	penicillin binding protein 2a
PIA	polysaccharide intercellular adhesin
Pls	plasma-sensitive surface protein
PNAG	poly-N-acetylglucosamine
PVL	panton-valentine leucocidin
PSM	phenol-soluble modulin
Rbf	Regulator of biofilm formation
rhPDGF	recombinant human platelet derived growth factor
RM	Restriction-Modification
σA	primary sigma factor
σB	alternative sigma factor
sae	staphylococcal accessory element
sarA	staphylococcal accessory regulator A
sarS	staphylococcal accessory regulator S
sarT	staphylococcal accessory regulator T
sasG	*S. aureus* surface protein G
SCC*mec*	staphylococcal cassette chromosome *mec*
SCIN	staphylococcal complement inhibitor
SCV	small colonial variant
Sdr	serine-aspartate repeat protein
SE	staphylococcal enterotoxins
Sie	superinfection exclusion system
spa	staphylococcal protein A
SSF	staphylococcal scarlet fever
SSSS	staphylococcal scalded skin syndrome
TCRS	Two-Component Regulatory System
tet	tetracycline resistance gene
TSS	toxic shock syndrome
TSST-1	toxic shock syndrome toxin-1
VAN	vancomycin
van	vancomycin resistance gene
VISA	vancomycin-intermediate MRSA
VRE	vancomycin-resistant enterococci
VREF	vancomycin-resistant *E. faecium*
VRSA	vancomycin-resistant MRSA
WHO	World Health Organization

CHAPTER I

Literature Review and Objectives

1.1. Introduction on Diabetic Foot Ulcer

1.1.1. Definitions and epidemiology

Diabetes *mellitus* (DM) is one of the most epidemic chronic diseases worldwide. Its prevalence is increasing mainly due to population growth, but also aging, urbanization and changing lifestyles that lead to reduced physical activity and increased obesity. The last World Health Organization (WHO) report states that in 2014 globally 422 million adults aged over 18 years were living with diabetes (WHO, 2016), an important rise comparing with the 108 million adults affected in 1980. In fact, the global prevalence has increased from 4.7% in 1980 to 8.5% in 2014. Diabetes prevalence is expected to double by the year 2030, also as a result of better health care conditions, which will increase the longevity of people with diabetes (Wild, Roglic, Green, Sicree & King, 2004).

Diabetes distribution varies substantially according to countries' economic status. The majority of diabetic patients in developed countries are aged over 60 years, whereas in developing countries most people with diabetes are of working age, between 40 and 60 years (Shaw, Sicree & Zimmet, 2010). In Portugal, there were over 1 million cases of diabetes registered in 2015. The cost associated to each diabetic patient was estimated to be about 1.880 euros (http://www.idf.org/membership/eur/portugal), proving that diabetes represents a significant health burden in Portugal.

A major concern with diabetes is the high risk of patients in developing one of the many major complications associated with the disease, such as cardiovascular, including myocardial infarction, stroke, angina and heart failure, blindness or nephropathy (Hopkins, Burke, Harlock, Jegathisawaran & Goeree, 2015). A common and most devastating complication of diabetes is the development of diabetic foot ulcers (DFU). Historically, foot ulcers have been estimated to affect 1 to 4% of patients with diabetes annually, but a recent study in the United States indicated that the annual incidence may be as high as 6% (Rice et al., 2014). In Canada and United States, it was observed that diabetic patients have a 25% risk of developing a DFU in their lifetime (Hobizal & Wukich, 2012; Rice et al., 2014).

A DFU can be defined as any full-thickness wound with skin necrosis or gangrene, below the ankle, induced by peripheral neuropathy or peripheral arterial disease in a diabetic patient, independently of its duration (Chuan, Tang, Jiang, Zhou & He, 2015). Most DFU are chronic wounds, defined in standard surgical textbooks as those that have not healed in 3 months. The most common forms are related to diabetes *mellitus*, venous stasis, peripheral vascular diseases and pressure ulcerations (Siddiqui & Bernstein, 2010).

Acute wounds are caused by external damage to intact skin, including more severe traumatic wounds and are expected to heal within a predictable time frame, although the treatment required to facilitate healing will vary according to the type, location and wound depth. In contrast, chronic wounds are most frequently caused by endogenous mechanisms that

compromise the integrity of dermal and epidermal tissues, such as pathophysiological abnormalities including leg ulcers, foot ulcers and pressure sores that include compromised tissue perfusion as a consequence of impaired arterial supply (peripheral vascular disease) or impaired venous drainage (venous hypertension), and metabolic diseases such as diabetes *mellitus* (Eron et al., 2003). Wound healing is characterized by three phases of inflammation, fibroplasia and maturation, resulting in a fine scar with little fibrosis and a return to an almost normal tissue architecture and organ function; if a wound does not heal in an orderly sequence or timely, or if the healing process does not result in structural integrity, then the wound is considered chronic and the healing occurs with the formation of abundant granulation tissue and often with excessive fibrosis leading to scar contraction and loss of function (Stadelmann, Digenis & Tobin, 1998).

DFU require minor or major amputations of lower limbs in 15% to 27% of cases. Infection is the preponderant factor for amputation in 50% of ulcers (Mendes & Neves, 2012), representing a major cause of morbidity and mortality and the most common cause of diabetes-related admission to hospitals, with huge financial, social and psychological consequences (Richard, Sotto & Lavigne, 2011; Mendes & Neves, 2012). A recent report estimated that the risk of hospitalization and lower-extremity amputation was approximately 56 and 155 times greater for diabetic people who had a foot infection than for those without (Richard et al., 2011). In the longer term, DFU have recurrence rates of up to 70%, resulting in repeated interventions and progressive disability that increase sanitary costs (Mendes & Neves, 2012). In fact, nearly one in six patients die within 1 year after their first infection (Hobizal & Wukich, 2012).

1.1.2. Pathophysiology

DFU have a multifactorial nature, but it is well established that absolute or relative insulin deficiency is the primary biochemical abnormality that leads to the organic complications of diabetes *mellitus*. It has also been established that a persistent glycaemic control, with either insulin or oral antidiabetic drugs, is able to stop and probably regress DFU associated microvascular and macrovascular complications (Turner, 1998; Mendes & Neves, 2012). The two major underlying causes of diabetic foot complications are peripheral neuropathy and peripheral vascular disease, by several mechanisms (Figure 1). One of the most frequently described mechanisms is the polyol pathway, in which the hyperglycaemic state leads to an increase in the action of the enzymes aldose reductase and sorbitol dehydrogenase, resulting in the conversion of intracellular glucose to sorbitol and fructose. The accumulation of these sugar products causes a decrease in the synthesis of myoinositol, required by nerve cells for normal progression of the neural impulse (Clayton, 2009). Additionally, the chemical conversion of glucose results in a depletion of nicotinamide adenine dinucleotide phosphate, leading to accumulation of reactive oxygen species and diminished synthesis of the

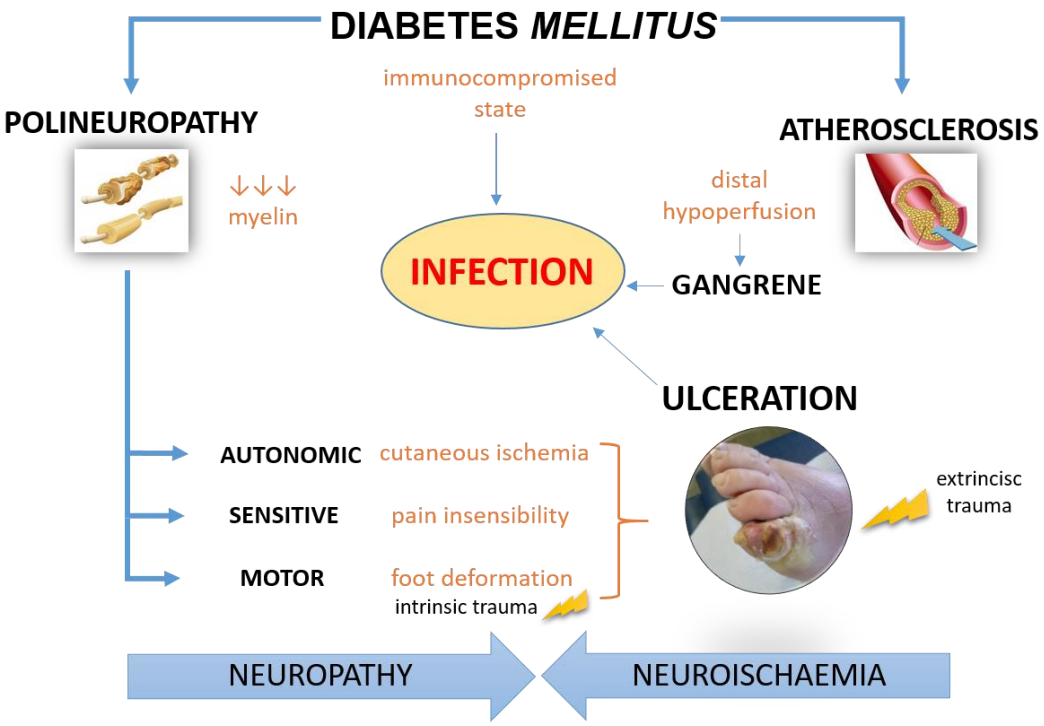
vasodilator nitric oxide (Yagihashi, Mizukami & Sugimoto, 2011). These factors result in oxidative stress in the nerve cell and also in an increased vasoconstriction leading to ischemia, which will promote nerve cell injury and death (Clayton W., 2009).

Several studies have also referred to a potential link between diabetic neuropathy and the mitochondria of sensory neurons located in dorsal root ganglia. These mitochondria are particularly vulnerable, because in the hyperglycaemic state they produce reactive oxygen species, which can damage mitochondrial DNA and membranes, impairing cell functions and leading to degeneration (Leinninger, Edwards, Lipshaw & Feldman, 2006; Said, 2007).

It appears that the size of neurons is also important in diabetes because longer nerve fibres show an earlier decrease in the velocity of nervous impulse transmission. This is why the loss of sensation and reflexes are often observed in the feet first, then progress to other areas, in particular the hands, causing the “glove and stocking” syndrome, which symptoms include numbness, dysesthesia, sensory loss and nocturnal pain (Forbes & Cooper, 2013). The damage to the nerves of the intrinsic foot muscles leads to an imbalance between flexion and extension capacities of the affected foot, producing anatomic deformities that create pressure points, which gradually cause skin breakdown and ulceration (Bowering, 2001) (Figure 1). Moreover, the autonomic neuropathy leads to a decrease in sweat and oil gland function, so the foot becomes dry and keratinized prompting the development of cracks and fissures that constitute a portal for infection development in wounds (Clayton W., 2009). Advanced neuropathy is characterized by altered sensitivities to vibrations and thermal thresholds, which progress to loss of sensory perception. For this reason, many wounds go unnoticed and progressively worsening as the affected area is continuously subjected to repetitive pressure and forces from walk and weight (Forbes & Cooper, 2013). Hyperalgesia, paraesthesia and allodynia can also occur in a proportion of patients, with pain evident in 40 to 50% of those with diabetic neuropathy, decreasing life quality (Obrosova, 2009).

Peripheral vascular disease plays a secondary role in DFU pathophysiology, as a consequence of the persistent hyperglycaemic state. In diabetes there is a decrease in endothelium-derived vasodilators leading to constriction, and an increase in thromboxane A2, a vasoconstrictor and platelet aggregation agonist, which leads to an enhanced risk for plasma hypercoagulability (Clayton W., 2009). Macroangiopathy is also observed, due to atherosclerosis, an obstructive disease of large vessels typically involving the tibial and peroneal arteries, resulting in capillary basement membrane thickening, altered nutrient exchange, tissue hypoxia and microcirculation ischemia (Hobizal & Wukich, 2012) (Figure 1). Cumulatively, these alterations can lead to occlusive arterial disease that result in ischemia in the lower extremity and an increased risk of ulceration in diabetic patients. Moreover, smoking, hypertension and hyperlipidaemia are other factors that contribute to the development of peripheral arterial disease in diabetic patients (Mulder, Tenenhaus & D’Souza, 2014).

Figure 1. Diabetic foot infection pathophysiology. Diabetic foot ulcer results from a complex interaction of risk factors, in which neuropathy plays the central role and causes ulcerations due to trauma or excessive pressure in a deformed foot without protective sensibility. Once the protective layer of skin is broken, deep tissues are exposed to bacterial colonization and an infection can settle (original).



1.1.3. Diagnosis and classification

The establishment of the most adequate therapeutic protocol for DFU must take into account all the risk factors involved and a consistent DFU classification. This evaluation requires the careful assessment of the global medical, foot and wound history, a systemized and detailed physical examination and the execution of complementary diagnostic procedures (Mendes & Neves, 2012).

Many DFU classification systems have been proposed to predict clinical outcome, but many of them have limitations. The International Working Group of the Diabetic Foot (IWGDF) developed the PEDIS classification system to categorize and define DFU objectively and facilitate communication between health-care providers, also allowing the prediction of associated health-care costs. In this system, all DFU are classified according to five categories that consider the most relevant signs and symptoms: perfusion, extent/size, depth/tissue loss, infection and sensation. Moreover, each subcategory is defined according to strict criteria based upon objective techniques, being applicable worldwide (Table 1) (Chuan et al., 2015).

Comparing with previous systems, such as the Wagner and SINBAD systems (Site, Ischemia, Neuropathy, Bacterial, Infection and Depth), PEDIS predicts the clinical outcome more

effectively, as its definition of DFU is based on more strict criteria based upon objective techniques and more comprehensive ulcer healing parameters. PEDIS DFU categorization is based on: 1) perfusion examination, essential for diagnosing peripheral arterial disease, resulting from the physical observation performed by a specialized health-care worker; 2) wound size measurement, which should be determined after debridement, if possible; 3) depth establishment, which is difficult to perform, allowing ulcers to be divided into lesions confined to the skin and those deeper than the skin; 4) infection diagnostic, mainly based on expert opinion; and finally, 5) sensation evaluation, by determining the presence or absence of protective sensation in the affected foot (Schaper, 2004).

Table 1. PEDIS classification system by the International Working Group of the Diabetic Foot (IWGDF) that classifies all ulcers in five main categories. Adapted from Schaper et al. (2004).

Categories	Grades	Description
Perfusion	**grade 1**	No symptoms or signs of peripheral arterial disease in the affected foot
	grade 2	Symptoms or signs of peripheral arterial disease but not of critical limb ischemia
	grade 3	Critical limb ischemia
Extent	Wound size after debridement (measured in square centimeters)	
Depth	**grade 1**	Superficial full-thickness ulcer, non-penetrating deeper than the dermis
	grade 2	Deep ulcer, penetrating below the dermis to subcutaneous structures, involving fascia, muscle or tendon
	grade 3	All subsequent layers, including bone and/or joint
Infection	**grade 1**	No symptoms or signs of infection
	grade 2	Infection involving the skin and the subcutaneous tissue. At least two of the following items are present: - local swelling or duration - erythema >0.5 to 2 cm around the ulcer - local tenderness or pain - local warmth - purulent discharge (thick, opaque to white or sanguineous secretion)
	grade 3	Infection involving structures deeper than skin and subcutaneous tissue *or* erythema >2 cm plus one of the items described above

Table 1. (continuation)

	grade 4	Any foot infection with two or more signs of a systemic inflammatory response syndrome: - temperature >38 or <36 °C - heart rate >90 beats/minute - respiratory rate >20 breaths/minute (or $PaCO_2$ <32 mmHg) - white blood cell count >12.000 or <4.000 cells/mm^3 (or 10% band forms)
Sensation	**grade 1**	No loss of protective sensation
	grade 2	Loss of protective sensation

$PaCO_2$: partial pressure of carbon dioxide in the arterial blood.

1.2. Diabetic Foot Infections

1.2.1. Definition and pathophysiology

Infection is a frequent (40-80%) and costly complication of DFU and represents a major cause of morbidity and mortality (Boyanova & Mitov, 2013). The healing impairment of DFU is caused by several factors that favour the overgrowth of bacteria, and has an essential role in the rapid spread of infection in diabetic ulcers (Jeffcoate & Harding, 2003) (Figure 2). These include intrinsic factors, such as neuropathy and vascular problems, and extrinsic factors, including callus formation and excessive local pressure. Traditionally, this set of predisposing abnormalities in diabetes has been referred to as “the pathogenic triad of neuropathy, ischaemia, and trauma” (Falanga, 2005).

The significance of bacteria in wounds presents a continuum, from contamination through colonization, to critical colonization and, finally, to infection (Siddiqui & Bernstein, 2010). Some wound specialists believe that the presence of a high concentration of microorganisms, usually superior to $>10^5$ colony-forming units [CFU] per gram of host tissue, represents ‘increased bioburden’ or ‘critical colonization’ even in the absence of clinical evidence of infection. There is, however, no universal techniques to define critical colonization, no routine laboratory tests available for quantitative bacteriology and no convincing evidence of its association with adverse clinical outcomes, for example, failure of healing or development of overt infection (Spichler, Hurwitz, Armstrong & Lipsky, 2015). This threshold may be altered by the status of the immune system and number and type of bacteria involved. Signs of critical colonization include atrophy or deterioration of granulation tissue, discoloration of granulation tissue to deep red or grey, increased wound friability and increased drainage (Siddiqui & Bernstein, 2010).

A diabetic foot infection (DFI) is defined as any infra-malleolar infection in a person with diabetes *mellitus*. These infections may include paronychia, cellulitis, myositis, abscesses, necrotizing fasciitis, septic arthritis, tendonitis and osteomyelitis. However, the most common and classical lesion is the infected diabetic "*mal perforans*" foot ulcer, in which, after the breach of the protective layer of skin, underlying tissues are exposed to bacterial colonization; this wound may progress to become actively infected, involving deeper tissues. This sequence of events can be rapid, occurring over days or even hours especially in ischemic limbs (Lipsky, 2004a). Although most infections, such as cellulitis, remain superficial, in around 25% of the cases will spread from the epidermal layer to deeper regions, reaching subcutaneous tissues and bones, as observed in necrotic fasciitis, septic arthritis and osteomyelitis (Noor, Zubair & Ahmad, 2015).

Wound contaminants are likely to originate from three main sources: the environment, including exogenous microorganisms in the air or those introduced by traumatic injury; the surrounding skin; and endogenous sources, involving mucous membranes, such as the gastrointestinal, oropharyngeal and genitourinary mucosae (Bowler, Duerden & Armstrong, 2001). After colonization, transition to infection occurs when bacterial proliferation overcomes the host's immune response (Siddiqui & Bernstein, 2010). This transition involves a multitude of microbial and host factors, including type, location, size and depth of the wound, the level of blood perfusion of the wound, the health and immune status of the host, the microbial load and the virulence potential expressed by the microorganisms involved. The immunocompromised state of diabetic patients, namely the neutrophil dysfunction, constitutes a relevant aspect because facilitates infections. The hyperglycaemia state seems to be the main factor altering the neutrophil chemotaxis, phagocytosis and intracellular killing of bacteria (Boyanova & Mitov, 2013).

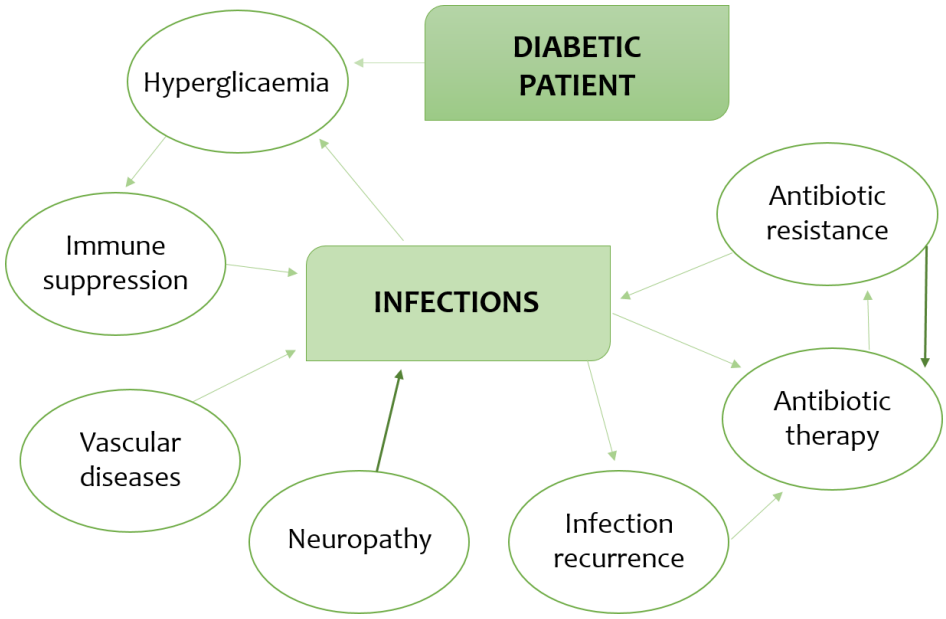
Due to frequent infections, diabetic patients are more exposed to antibacterial agents, which can lead to increased antibiotic resistance rates; also, the presence of peripheral vascular disease can leads to poor antibiotic penetration in the infected tissue, favouring the development of antibiotic resistance (Lipsky et al., 2012a). There is a vicious cycle in which the infections can worsen the glycaemic control of the diabetic patient and, vice-versa, the poor glycaemic control and other factors associated with DM can facilitate or aggravate the development of infections (Figure 2).

1.2.2. DFI diagnosis

DFI diagnosis begins with a clinical suspicion accompanied by a comprehensive history and physical exam, validated with a complete laboratory evaluation, microbiology assessment and diagnosis imaging. Since all chronic wounds are colonized by microorganisms, the DFI diagnosis is based on clinical findings. According to the Infectious Disease Society of America (IDSA) guidelines, infection is present if there is obvious purulent drainage and/or the

presence of two or more signs of inflammation (erythema, pain, tenderness, warmth or induration), being divided in four grades of severity according to the extension of tissue involved and the presence of systemic toxicity or metabolic derangement (Hobizal & Wukich, 2012). In some patients, especially those with peripheral neuropathy or vasculopathy, symptoms and signs may be diminished, leading some clinicians to evaluate the presence of "secondary" findings before diagnosing infection, such as foul odour and the presence of friable or discoloured granulation tissue (Spichler et al., 2015).

Figure 2. Interplay among factors associated with diabetes, infections and antibiotic resistance in diabetic patients (original).



1.3. Microbiology of DFI

1.3.1. Bacteria involved in DFI

To properly identify the microorganisms involved in DFI it is important to choose the correct sampling procedure. Tissue biopsy following initial debridement and cleansing of superficial debris is known as the most advantageous and standard sampling method. However, due to fear of infections spreading and loss of adjacent structure in limb and ischemia tissue, sometimes biopsy is not advisable, being replaced by swab sampling. This method renders sample collection easier and can be performed in any kind of wound. This method is widely used for the identification of causative microorganisms and antimicrobial susceptibility testing, despite the fact that isolated bacteria also include colonizers (Noor et al., 2015).

In the presence of a large volume of wound fluid, sampling by needle aspiration can be performed, being well established as the most useful procedure for sampling purulent fluid from intact cutaneous abscesses (Bowler et al., 2001). Cultures of specimens obtained from patients with mixed infections generally yield 3 to 5 isolates, including Gram-positive and Gram-negative aerobes and anaerobes (Lipsky, 2004a). Many studies from western countries report that prevailing microorganisms cultured from DFI are Gram-positive aerobes (Dang, Prasad, Boulton & Jude, 2003; Citron, Goldstein, Merriam, Lipsky & Abramson, 2007; Mendes et al., 2012), whereas studies from eastern countries have described the presence of Gram-negative aerobes as predominant organisms (Gadepalli et al., 2006; Zubair, Malik, Ahmad & Rizvi, 2011; Noor et al., 2015).

Generally, aerobic Gram-positive cocci are the predominant microorganisms that colonize and cause acute DFI, with *Staphylococcus aureus* being the most common isolated pathogen, followed by *Staphylococcus epidermidis* and *Streptococcus* spp., especially the ones belonging to β-haemolytic groups (Shankar, Mohan, Premalatha, Srinivasan & Usha, 2005; James et al., 2007; Galkowska et al., 2009; Tascini et al., 2011). Staphylococci are perhaps the most virulent pathogens in DFI, showing a correlation between specific virulence genotypic markers from DFU isolates and ulcer outcome (Sotto, Lina & Richard, 2008). Chronic wounds develop a more complex colonizing microbiota and infections are more frequently polymicrobial, often including aerobic or facultative Gram-positive bacteria such as *Enterococcus* and *Corynebacterium*, Gram-negative bacilli such as *Pseudomonas aeruginosa* and *Acinetobacter*, and obligate anaerobic bacteria (Citron et al., 2007; Dowd et al., 2008a; Zubair et al., 2011; Małecki, Rosiński & Adamiec, 2013; Sekhar, Vyas, Unnikrishnan, Rodrigues & Mukhopadhyay, 2014). In fact, foot ulcers with ischemia and deep tissue necrosis have been reported to present anaerobic growth (Hobizal & Wukich, 2012; Lipsky, Richard & Lavigne, 2013; Noor et al., 2015). It is also important to remember that fungi have also been reported in DFI and can be a major contributor to the bioburden or biofilm formation in wounds (Dowd et al., 2011).

Acute infections in patients who have not recently received antimicrobials are often monomicrobial, being the majority promoted by an aerobic Gram-positive coccus, whereas chronic infections are often polymicrobial (Noor et al., 2015). The pathogenic role of each isolate in a polymicrobial infection is often unclear, but some studies suggest that the interactions of organisms within these polymicrobial communities lead to the production of virulence factors, such as collagenases, proteases and haemolysins, and short-chain fatty acids that cause inflammation, obstructed wound healing, increasing the chronicity of infection (Noor et al., 2015). The impaired host defences around necrotic soft tissue or bone may allow low-virulence colonizers, such as coagulase-negative staphylococci and *Corynebacterium* spp. (“diphtheroids”), to assume a pathogenic role (Lipsky, 2004a).

Generally, the presence of one microorganism generates a niche for the colonisation by other pathogenic microorganisms; on the other hand, two or more non-pathogenic microorganisms together can cause disease. The major polymicrobial interactions that have been studied are synergism and antagonism. In synergy there is a cooperative interaction between two or more species of microbes, allowing increased growth, virulence, antimicrobial tolerance and enhanced production of exopolysaccharide (EPS) (Brogden, Guthmiller & Taylor, 2005). Antagonism, also called antibiosis, is characterized by the suppression of one microbial species by another; it can include production of chemical signs and factors inhibiting the growth of neighbours and/or storage of nutrients that promote their starvation (Gabrilska & Rumbaugh, 2015).

Hospitalization, surgical procedures and, especially, prolonged or broad-spectrum antibiotic therapeutics may predispose patients to colonization and/or infection with antibiotic-resistant microorganisms. Among hospitalized patients, the prevalence of methicillin-resistant *Staphylococcus aureus* (MRSA) in DFI can range from 15 to 30%, depending on the geographical location of affected patients (Hobizal & Wukich, 2012). Although MRSA strains have been previously isolated mainly from hospitalized patients, community-associated strains are now becoming common, being already associated with severe outcomes in patients with DFI (Dang et al., 2003). For example, the first reported cases of vancomycin-resistant *S. aureus* in the United States and in Europe have been isolated from diabetic patients with foot infections (Lipsky, 2004a; Melo-Cristino, Resina, Manuel, Lito & Ramirez, 2013).

In summary, bacterial genera frequently associated with DFI include *Staphylococcus*, *Enterococcus*, *Corynebacterium*, *Pseudomonas* and *Acinetobacter*.

1.3.2. Staphylococci

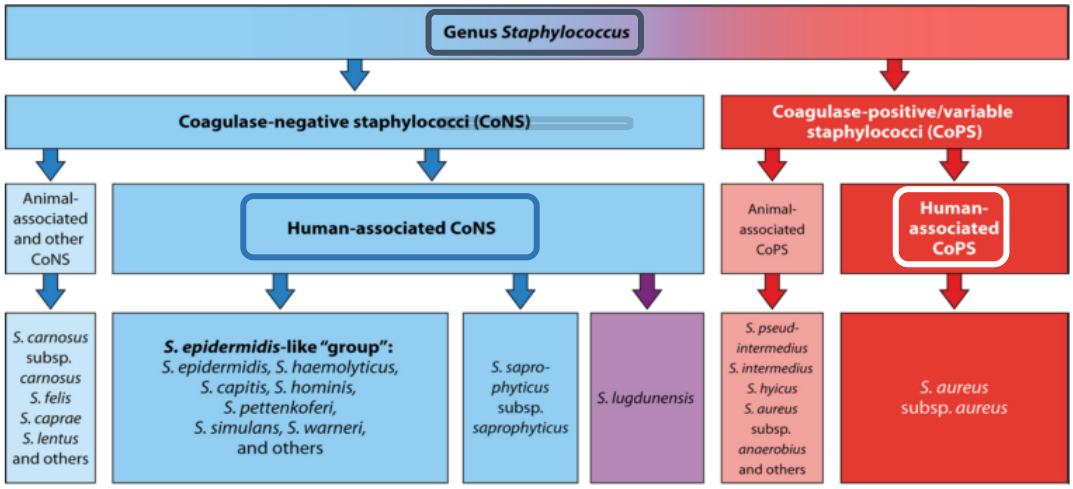
1.3.2.1. Generalities and classifications

Staphylococcus, named after the Greek words *staphyle* (bunch of grapes) and *kokkos* (berry), is a genus of Gram-positive bacteria frequently associated with surgical and skin infections, respiratory disease and food poisoning (Licitra, 2013). They were observed for the first time in 1880, by the Scottish surgeon Sir Alexander Ogston that observed staphylococci in pus samples from a surgical abscess in a knee joint, describing them as “the masses looked like bunches of grapes.” In 1884, German physician Friedrich Julius Rosenbach differentiated the bacteria species by the colour of their colonies growing in agar medium: *S. aureus* (from the Latin *aurum*, gold) due to the presence of carotenoids, and *S. albus* (Latin for white); *S. albus* was later renamed as *S. epidermidis* due to its ubiquitous presence on human skin (Orenstein, 2008). Staphylococci are characterized by being non-motile, non-spore-forming, spherical cells with 0.5 to 1.5 µm in diameter, usually forming clusters; they are facultative anaerobes with

complex nutritional requirements including several amino acids and vitamins B; they tolerate high concentrations of sodium chloride and are resistant to heat (Becker & Eiff, 2011).

Staphylococci are catalase-positive and their cell wall peptidoglycan structure contains multiple glycine residues in the cross-bridge, responsible for their susceptibility to lysostaphin action (Plata, Rosato & Wegrzyn, 2009). The *Staphylococcus* genus is traditionally divided into two groups based on the bacteria's ability to produce coagulase, an enzyme that causes blood clotting by converting fibrinogen to fibrin: coagulase-positive staphylococci (CoPS), the group that includes *S. aureus*, a frequent ethological agent of diseases that exhibits resistance to a growing number of therapeutic agents; and the coagulase-negative staphylococci (CoNS), a group composed by common skin commensals (Figure 3). CoNS include *S. epidermidis* and *S. haemolyticus* as the most prevalent species, along with *S. capitis*, *S. hominis*, *S. simulans*, *S. warneri* and *S. saprophyticus* (Becker, Heilmann & Peters, 2014) (Figure 3).

Figure 3. Clinical and epidemiological classification of staphylococcal species. Adapted from Becker et al. (2014).



S. aureus is the most pathogenic species of the genus *Staphylococcus*, implicated in both community-acquired and nosocomial infections and associated with considerable morbidity and mortality rates (Carbon, 2000). It has been estimated that about 20 to 30% of the population are permanently colonized by this bacterium, constituting healthy carriers, while other 30% are transient carriers. The anterior nose nares are the most frequent location for *S. aureus* colonization, followed by skin, perineum, pharynx, and less frequently, the gastrointestinal tract, axillae and vagina (Wertheim et al., 2005). Colonization represents an increased risk of infection particularly for the immunocompromised host. In such cases, *S. aureus* can be responsible for the development of bacteraemia, infective endocarditis as well as osteoarticular, skin and soft tissue, pleuropulmonary and device-related infections (Tong, Davis, Eichenberger, Holland & Fowler, 2015).

Staphylococcus, especially *S. aureus*, is by far the most common and virulent pathogen in DFI (Jones, Edwards, Finch & Jeffcoate, 1985; Sotto et al., 2008; Lipsky et al., 2013). Many studies have described a high *S. aureus* prevalence in these infections, ranging from about 50% (Dang et al., 2003; Citron et al., 2007; Galkowska et al., 2009; Mendes et al., 2012) to 30% (Richard et al., 2011; Tascini et al., 2011; De Vries, Ekkelenkamp & Peters, 2014; Zenelaj, Bouvet, Lipsky & Uckay, 2014). These studies also describe CoNS as the second most frequently encountered aerobic Gram-positive isolates. MRSA have emerged as a serious and common problem in patients with diabetic foot ulcers, and infections promoted by such strains may result in prolonged stay hospital and increased economic costs (Tentolouris et al., 2006). High rates of MRSA were already reported in several studies on DFI, normally representing 20 to 40% of all *S. aureus* isolates (Bowling, Salgami & Boulton, 2007; Tascini et al., 2011; Boyanova & Mitov, 2013); also one study described a 68% MRSA rate in DFI (Malik, Mohammad & Ahmad, 2013). In case of diabetic foot osteomyelitis, the majority of infections are polymicrobial, with *S. aureus* being the most common etiologic agent, isolated in about 40% of cases, followed by *S. epidermidis*, isolated in about 25% (van Asten et al., 2015).

1.3.2.2. Staphylococci virulence traits

S. aureus virulence determinants can be divided into cell wall-anchored (CWA) surface proteins (adhesins) and secreted factors (exotoxins). These factors allow this bacterial species to adhere to surfaces/tissues and avoid or invade the host immune system causing toxic effects.

1.3.2.2.1. Cell wall-anchored (CWA) surface proteins

The first step of staphylococcal invasion is bacterial adherence that can occur via direct interaction with host cells or via interaction with host-factor binding proteins (Schroeder et al., 2009). These include autolysins, adhesins, binding proteins, clumping factors, iron transporters and surface proteins.

Direct *S. aureus* adherence to a surface may be mediated by the major autolysin Atl that has both adhesive and enzymatic functions. It is involved in the initial attachment of the cells to a polymeric surface and in biofilm formation. It also binds to vitronectin, suggesting a role in not only colonizing polymer surfaces, coated materials and host tissue (Heilmann, Hartleib, Hussain & Peters, 2005). This enzyme also participates in the hydrolysis of cell wall peptidoglycan, leading to autolysis and release of extracellular DNA (eDNA), which has been shown to be an important component of staphylococcal biofilms (Becker et al., 2014). *S. aureus* Atl is highly homologous to the *S. epidermidis* autolysin/adhesin AtlE, which is involved in the attachment to polymer surfaces. Recently, homologous Atl proteins with similar functions were also reported for other CoNS, such as *S. caprae* (AtlC), *S. saprophyticus* (Aas),

S. lugdunensis (AtlL) and *S. warneri* M (AtlWM), as well as a novel autolysin/adhesin Aae in *S. epidermidis* (Becker et al., 2014).

S. aureus has numerous CWA surface proteins that promote bacterial adherence to host glycoproteins, including the "microbial surface components recognizing adhesive matrix molecules" (MSCRAMM) (Extremina, Costa, Aguiar, Peixe & Fonseca, 2011) (Figure 4). MSCRAMM bind to host molecules such as collagen, fibronectin and fibrinogen, and appear to play a key role in the onset of endovascular, bone, joint and prosthetic-device infections. Different MSCRAMM may adhere to the same host-tissue component and different *S. aureus* strains may have different MSCRAMM groups, being related with particular infections (Gordon & Lowy, 2008).

Fibronectin binding proteins A and B (FnbpA and FnbpB) are also MSCRAMM that participate in the attachment of bacterial cells to biofilm extra-cellular matrix components, to fibronectin and to plasma clots, also having an invasive function. A fibrinogen-binding protein, Fbe, has also been identified in *S. epidermidis* (Hartford, O'Brien, Schofield, Wells & Foster, 2001; Hussain et al., 2009). The collagen binding protein, Cna, is necessary for *S. aureus* adherence to collagenous tissues and cartilage (Elasri et al., 2002).

Clumping factors A and B (ClfA and ClfB) mediate clumping and adherence of *S. aureus* cells to fibrinogen in the presence of fibronectin. These proteins are also antiphagocytic, protecting bacteria from opsonophagocytosis by degradation of complement element, C3b. *S. lugdunensis* expresses a fibrinogen-binding surface protein (Fbl) with considerable similarity to *S. aureus* ClfA (Geoghegan et al., 2010; Becker et al., 2014). Finally, serine-aspartate repeat proteins C, D and E (SdrC, SdrD, SdrE) are fundamental to the adhesion prior to nasal colonization (Geoghegan et al., 2010).

Besides MSCRAMM, CWA surface proteins include many more molecules. Belonging to the Near-iron transporter (NEAT) Motif Family, the domain proteins capture heme group from hemoglobin and help bacteria to survive in the host, where accessibility to iron is restricted. Heme group is transported via several CWA proteins called iron-regulated surface (Isd) proteins, to a membrane transporter and then to the cytoplasm, where haemoxygenases promote the release of free iron trough the lysis of the heme group (Geoghegan et al., 2010).

Protein A is a cell wall-associated protein that binds to the Fc domain of immunoglobin G (IgG), produced by over 95% of *S. aureus* strains, being encoded by the staphylococcal protein A (spa) gene (Adesida et al., 2006). The N-terminal region of protein A comprises a tandem array of five separately folded three-helical bundles that can bind to several different ligands. Protein A induces a defective binding of IgG to the surface of *S. aureus* cells, disrupting opsonization and phagocytosis; it also exhibits the ability to bind to the von Willebrand factor, a large glycoprotein that mediates platelet adhesion at sites of endothelial damage, playing a role in adherence and induction of endovascular diseases (Adesida et al., 2006; Plata et al., 2009).

The G5-E Domain Family includes *S. aureus* surface protein G (SasG) and the plasma-sensitive surface protein (Pls), which are very similar in structure and organization to the accumulation-associated protein Aap of *S. epidermidis*. Both proteins promote cell aggregation and biofilm formation. The Pls protein, once processed by plasmin, participates in both fibrinogen and fibronectin binding (Hussain et al., 2009).

Non-proteinaceous adhesins are also observed, including the polysaccharide intercellular adhesin PIA, also known as poly-N-acetylglucosamine [PNAG], and cell wall teichoic acids. PIA is encoded by the intercellular adhesion operon, *icaADBC* that was first identified in *S. epidermidis*, being also present in *S. aureus*, in which it is involved in biofilm formation (Schroeder et al., 2009). In *S. epidermidis*, biofilm production is also mediated by proteins, namely the accumulation-associated protein Aap and extracellular matrix-binding protein (Embp) (Schommer et al., 2011).

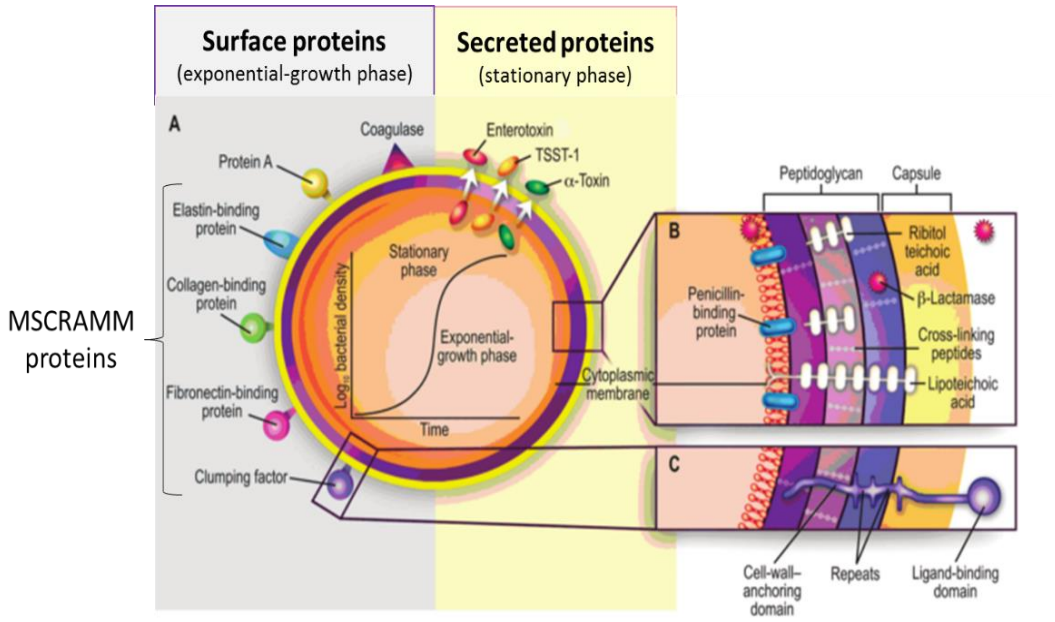
1.3.2.2.2. Secreted factors (exotoxins)

Another important virulent feature of *S. aureus* is the ability to secrete toxins (Figure 4), playing an active role in impairing host immunity action. These secreted factors can be classified into four categories: superantigens, cytolytic toxins, exoenzymes and miscellaneous proteins.

The superantigens are a group of powerful immune-stimulatory proteins that induce different human diseases, and include staphylococcal enterotoxins (SE) A-E and G-J, toxic shock syndrome toxin-1 (TSST-1) and exfoliative toxins (ET) A, B and D. These toxins were initially described as being responsible for specific acute staphylococcal toxaemia syndromes, such as: toxic shock syndrome (TSS) and staphylococcal scarlet fever (SSF), both promoted by TSST-1, SE type B and SE type C; scalded skin syndrome (SSSS), related to ET; and staphylococcal food poisoning due to SE (Podbielska, Galkowska & Olszewski, 2011). TSST-1 cross-links the T-cell receptor with major histocompatibility complex class II (MHC-II) on antigen-presenting cells, triggering large-scale T-cell activation and massive cytokine release, leading to an overwhelming systemic inflammatory response syndrome, resulting in septic shock with organ failure (Tong et al., 2015). SE have the same action mechanism of TSST-1, causing diarrhoea and vomiting when ingested, and are responsible for staphylococcal food poisoning (Podbielska et al., 2011). ET toxins, associated with SSS, cause separation within the epidermis, by promoting intra-epidermal cleavages between the living layers and the superficial dead layers (Jarraud, Mougel & Thioulouse, 2002).

Figure 4. Structural and secreted pathogenic factors of *S. aureus.*

Adapted from Gordon et al. (2008).



A, surface and secreted proteins; B and C, cross-sections of cell-envelope; TSST-1, toxic shock syndrome toxin 1.

The cytolytic toxins, α-hemolysin, β-hemolysin, γ-hemolysin, leukocidin and Panton-Valentine leucocidin (PVL), have different structures and targets but share a similar function on host cells. They form β-barrel pores in the cytoplasmic membranes of target cells, namely leucocytes, erythrocytes, lymphocytes and platelets, causing leakage of their content or cell lysis (Costa et al., 2013). PVL targets mononuclear and polymorphonuclear cells and causes cell death by necrosis or apoptosis, being strongly associated with community-associated MRSA (CA-MRSA). PVL toxin consists of two synergistic proteins, LukF-PVL and LukS-PVL, encoded by *lukF* and *lukS*, present on a temperate bacteriophage (Rossney et al., 2007). Haemolysins cause pore formation on susceptible host cell membranes, triggering alterations in ion gradients, loss of membrane integrity, activation of stress-signalling pathways and cell death (Bien, Sokolova & Bozko, 2011). Leukocidin is a multicomponent protein toxin forming an octameric pore, composed of four LukF and four LukS subunits, and therefore haemolytic. It is only present in 2% of all *S. aureus* isolates collected from severe dermonecrotic lesions (Podbielska et al., 2011).

Nearly all strains of *S. aureus* secrete several other extracellular enzymes whose functions include the disruption of host tissues, inactivation of host antimicrobial mechanisms, acquisition of nutrients for bacterial growth and facilitation of bacterial dissemination. These exoenzymes include lipases, nucleases, proteases, hyaluronidase and staphylokinase (Bien et al., 2011). Lipases inactivate fat acids; nucleases cleave nucleic acids; proteases, that include serine, cysteine and aureolysin, inactivate the host defense mechanisms, including antibodies,

and destruct the tissue proteins, promoting invasiveness; and hyaluronidases degrade hyaluronic acid (Costa et al., 2013). Staphylokinase contributes to bacteria spread by activating plasminogen, promoting clot disruption and destruction of fibrin fibres that hold cells together (Bokarewa, Jin & Tarkowski, 2006).

Other specific proteins produced by staphylococci have a profound impact on the innate and adaptive immune system and include: staphylococcal complement inhibitor (SCIN), a C3 convertase inhibitor which blocks the formation of C3b on the surface of the bacterium, impairing phagocytosis by human neutrophils; *S. aureus* extracellular fibrinogen binding protein (Efb) that inhibits both classical and alternative pathways of complement activation; chemotaxis inhibitory protein of *S. aureus* (CHIPS) and formyl peptide receptor-like-1 inhibitory protein (FLIPr) that block neutrophil receptors for chemo-attractants; and the extracellular adherence protein (Eap) that blocks migration of neutrophils from blood vessels into the tissue (Bien et al., 2011; Costa et al., 2013).

Another *S. aureus* strategy that might play a role in bacterial survival in biofilms is phage induction. Stress factors, like nutrient limitation, unfavourable oxygen concentrations and high cell densities in biofilms, lead to phage release resulting in cells lysis (Gilbert, Collier & Brown, 1990). This lysis may can promote biofilm persistence because the remaining cells obtain nutrients from the lysed ones (Resch, Fehrenbacher, Eisele, Schaller & Götz, 2005).

1.3.2.2.3. Virulence regulatory systems

To control the production of virulence determinants during infection, *S. aureus* expresses several regulatory systems that “cross-talk” to ensure that specific genes are expressed only in favourable conditions. These systems can be divided in two main categories: two-component signal transduction systems and global transcriptional regulators. The two-component regulatory systems include the accessory regulator gene (*agr*) and the staphylococcal accessory element gene (*sae*) (Cheung, Bayer, Zhang, Gresham & Xiong, 2004).

The *agr* locus regulates more than 70 genes, 23 of which are related to virulence. This system is responsible for upregulating the expression of many exoproteins and downregulating the synthesis of cell wall-associated proteins, such as Spa, through the negative regulation of the *spa* gene expression (Bien et al., 2011). The *sae* locus codes for another Two-Component Regulatory System (TCRS) that regulates the expression of many virulence factors involved in bacterial adhesion, toxicity and immune evasion. This includes the upregulation of α-, β- and γ- haemolysins and the downregulation of *spa* (Goerke et al., 2005).

Several global regulatory systems have been identified in *S. aureus*, including the staphylococcal accessory regulator A gene (*sarA*), and its several homologues, such as staphylococcal accessory regulator S (*sarS*) and T (*sarT*) (Bronner, Monteil & Prévost, 2004); *sarA* upregulates the expression of some virulence factors (e.g. α- and β-haemolysins) and downregulates others. Opposite to the *agr* system, *sarS* binds to the *spa* promoter and

activates its expression; in turn, *agr* down-regulates *sarS* and maybe also *spa* expression, by suppressing its activator *sarS* (Tegmark, Karlsson & Arvidson, 2000).

The regulation of virulence determinants may also involve sigma factors (σ), which are proteins that bind to the RNA polymerase core to form an holoenzyme linking to specific promoters (Schmidt, Manna, Gill & Cheung, 2001). *S. aureus* has two sigma factors: the primary sigma factor, σA, responsible for the expression of housekeeping genes essential for growth, and the alternative sigma factor σB, which regulates the expression of different genes involved in cellular functions (e.g. stress response) and of at least 30 virulence genes (Deora & Misra, 1996; Deora, Tseng & Misra, 1997). σB upregulates capsule, FnbpA and coagulase production, and downregulates haemolysins and serine protease A expression. All the mentioned regulators constitute an interactive network, influencing each other, to ensure that specific virulence genes are expressed only when required (Costa et al., 2013).

As described above, the genes and products of the *ica* locus [*icaR* (regulatory) and *icaADBC* (biosynthetic) genes] are necessary for biofilm formation and virulence in *Staphylococcus* and are upregulated in response to anaerobic growth, in conditions similar to biofilm environment. A strong negative regulation is conferred by *icaR*, whereas the protein regulator of biofilm formation, Rbf, represses transcription of *icaR* leading to increased *ica* gene expression (Archer et al., 2011). However, biofilms can occur in an *ica*-independent manner through the action of the accumulation-associated protein (Aap) (Hennig, Nyunt Wai & Ziebuhr, 2007).

Another important component of the staphylococcal biofilm matrix is eDNA, first documented in *Pseudomonas aeruginosa* (Steinberger & Holden, 2005). The opposing activities of the *cid* and *irg* operon, regulators of murein hydrolase and cell death, control the cell lysis and genomic DNA release during biofilm development in *S. aureus*. eDNA has the ability to increase biofilm adherence (Mann et al., 2009). The regulatory factors involved in *S. aureus* biofilm formation are summarized in the Figure 5.

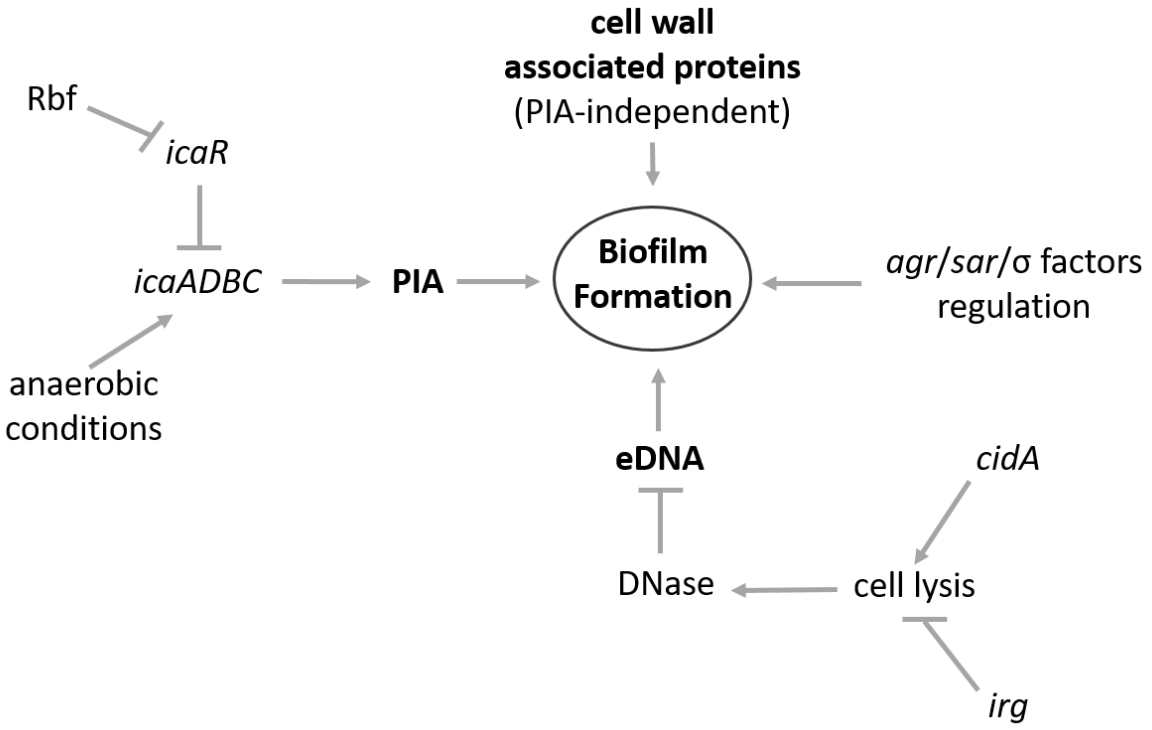
1.3.2.2.4. Phenotypic switching

Regarding staphylococci virulence traits, it is also important to refer that chronic and exacerbated staphylococcal infections have been associated with altered phenotypes, resulting from mutations. These bacteria evolved fitness-compensatory mechanisms and reversible stress-resistance mechanisms such as phenotypic switching. This ability consists in a reversible conversion of phenotypic states due to environmental changes, based on an on/off mechanism that can greatly accelerate the adaptive evolution of bacteria, having an impact on antibiotic susceptibility and virulence factors expression (Sousa, Machado & Pereira, 2011).

Figure 5. Regulatory factors involved in *S. aureus* biofilm formation.
Adapted from Archer et al. (2011).

- PIA-dependent biofilm formation: expression of the *icaADBC* gene cluster results in PIA expression and biofilm formation; *icaADBC* can be suppressed by *icaR*, resulting in downregulation of PIA and thus biofilm formation; the *icaR* expression can be downregulated by the protein Rbf, leading to upregulation of *icaADBC*, PIA production and biofilm formation. Additionally, anaerobic conditions induce expression of the *icaADBC* gene cluster, PIA production and biofilm formation.
- PIA-independent biofilm formation: in *ica*-deleted mutants, PIA-independent biofilm formation can be mediated through cell wall-associated proteins.
- eDNA and biofilm formation: eDNA leads to enhanced biofilm formation. DNase treatment degrades eDNA and inhibits eDNA-mediated biofilm formation. DNA release is arbitrated through cell lysis and controlled by *irg* and *cidA* gene expression. Upregulation of *irg* gene results in inhibition of cellular lysis, DNA release and biofilm formation, although *cidA* expression enhances cellular lysis, DNA release and biofilm formation.

The biofilm formation is regulated by a complex interaction of *agr*, *sar* and σ systems.



icaADBC, intercellular adhesion biosynthetic genes; PIA, polysaccharide intercellular antigen; icaR, intercellular adhesion regulatory gene; Rbf, protein regulator of biofilm formation; eDNA, extracellular DNA; lrg, regulator of murein hydrolase and cell death; cidA, regulator of murein hydrolase and cell death; *agr*, accessory gene regulator; *sar*, staphylococcal accessory regulator; σ, sigma factors.

This morphologic heterogeneity of staphylococci is particularly associated to the “small colonial variant” (SCV), a specific phenotype resulting from a switch of the wild-type phenotype, which can also be assumed for CoNS SCV (Becker et al., 2014). The SCV phenotype is characterized by a reduced growth rate and drastic changes in cellular metabolism: the colonies are tiny, typically 10 times smaller, with reduced or lost pigmentation

and haemolysis when compared to their wild-type counterparts. They are located intracellularly, which provides a survival niche in the host environment, shielding SCV from host defences and antimicrobial agents, representing a major challenge concerning disease management (Proctor et al., 2006).

1.3.2.3. Staphylococci antimicrobial resistance ability

The WHO published in the past February its first ever list of antibiotic-resistant "priority pathogens" in which *Staphylococcus aureus* is referred to as belonging to the high priority category (http://www.who.int//mediacentre/news/releases/2017/bacteria-antibiotics-needed/).

Before the introduction of antibiotics in the 1940s, invasive staphylococcal infections were often fatal and penicillin greatly reduced the corresponding mortality; however, just few years after its introduction, the first clinical cases of MRSA were identified (Costa et al., 2013). In 1960, around 80% of all *S. aureus* strains were resistant to penicillin. Methicillin, a semi-synthetic antibiotic derived from penicillin but resistant to β-lactamase inactivation was introduced in 1959 (Deurenberg et al., 2007b). However, in 1961 there were reports from the United Kingdom that *S. aureus* isolates had acquired resistance to methicillin and, rapidly MRSA were reported in several countries, until becoming endemic in many hospitals worldwide (Chambers & Deleo, 2009; Carvalho, Mamizuka & Filho, 2010). Methicillin resistance is associated with acquisition of a large transmissible genetic element known as staphylococcal cassette chromosome *mec* (SCC*mec*). SCC*mec* elements are currently classified into types based on the nature of the *mec* and *ccr* gene complexes, and further classified into subtypes according to their joining ("junkyard") regions DNA segments, which constitute non-essential components that may carry additional antimicrobial resistance determinants (Hiramatsu, Katayama, Yuzawa & Ito, 2002).

The *ccr* gene complex consists of cassette chromosome recombinase (*ccr*) genes (*ccr*C or *ccr*A and *ccr*B pair) encoding recombinases which mediate the integration and excision of SCC*mec* and surrounding genes (Carbon, 2000). The *ccr* complex is currently classified into eight types, while to date, 11 types and several subtypes have been reported for the SCC*mec* element (http://www.sccmec.org/Pages/SCC_TypesEN.html).

The *mec* gene complex consists of *mec*A, encoding for the penicillin binding protein (PBP) PBP2a, regulatory genes and associated insertion sequences, and is classified into six different classes: A, B, C1, C2, D and E (http://www.sccmec.org/Pages/SCC_TypesEN.html). PBP2a consists of a transpeptidase with low affinity for β-lactams that replaces the wild type PBP and is directly responsible for resistance to methicillin and all other β-lactam antibiotics (Liu, 2009).

The first MRSA isolate, the archaic clone, harboured the SCC*mecI* and circulated in hospitals throughout Europe, disappearing from European hospitals in the 1980s for unknown reasons. In the mid to late 1970s, new MRSA strains containing SCC*mecII* and SCC*mecIII* emerged,

leading to a worldwide pandemic of MRSA (Chambers & Deleo, 2009). For a long time MRSA infections were limited to hospitalized patients, but during the 1990s CA-MRSA infections among healthy individuals without associated risk factors were reported.

These types have genetic differences: SCC*mec* types I, II and III are characteristic of hospital-acquired MRSA (HA-MRSA) strains and show resistance to multiple classes of antibiotics; while SCC*mec* types IV, V and VI are generally associated with CA-MRSA strains, which in most cases do not contain additional antimicrobial resistance genes, and therefore, only show resistance to β-lactam antibiotics (Enright et al., 2002; Robinson & Enright, 2003; Lindsay, 2013). Moreover, these types affect distinct populations and cause different clinical syndromes: CA-MRSA infections tend to occur in previously healthy children and young adults and have been linked to soft-tissue and severe infections; HA-MRSA strains are usually isolated from older adults and immunocompromised people, being a common cause of invasive infections, pneumonia and bacteraemia (David & Daum, 2010). The evolution of sequence-based molecular methods for bacterial genotyping, particularly the multilocus sequence typing (MLST) technique, has made possible to unravel the molecular epidemiology of *S. aureus* and to distinguish between HA-MRSA and CA-MRSA strains. MLST is based on the sequencing conserved genes, known as housekeeping genes, allowing to group strains into clonal complexes (CC). The vast majority of nosocomial MRSA strains detected worldwide belong to five CC: 5, 8, 22, 30 and 45 (Enright et al., 2002).

The global distribution and impact of HA-MRSA infections led to an increase in the administration of vancomycin, the main antimicrobial agent used for treatment of serious infections by methicillin-resistant staphylococci and biofilm producing strains. Fortunately, resistance to vancomycin among such isolates remains rare, with less than 15 cases reported worldwide (Archer et al., 2011). The glycopeptides vancomycin and teicoplanin exercise their effect by binding irreversibly to the terminal D-alanyl-D-alanine (D-Ala-D-Ala) of the bacterial cell wall precursors, inhibiting the synthesis of the *S. aureus* cell wall (Sieradzki, Pinho & Tomasz, 1999). The reduced susceptibility to vancomycin in vancomycin-intermediate MRSA (VISA) strains is due to the synthesis of an unusually thickened cell wall containing D-alanyl-D-alanine dipeptides capable of binding to vancomycin, effectively sequestering this compound, thereby reducing the availability of the drug that cannot reach its intracellular target molecules (Appelbaum, 2006). The genetic basis responsible for these cell wall alterations has not yet been determined. On the other hand, vancomycin resistance in vancomycin-resistant MRSA (VRSA) occurs due to the plasmid-mediated transfer of the *van*A gene cluster, which includes *van*R, *van*S, *van*H, *van*A and *van*X genes, usually carried by the mobile genetic element Tn1546 from vancomycin-resistant enterococci (VRE) (Weigel, 2003). If vancomycin resistance emerges, linezolid or daptomycin may be alternatives, which may be responsible for the development of reduced susceptibility to these antibiotics (Fraimow, 2009).

Recently significant increases in the percentage of resistant staphylococcal isolates were also described regarding oxacillin, ciprofloxacin, clindamycin, erythromycin and gentamicin (Becker et al., 2014). Staphylococcal antibiotic resistance represents a serious clinical problem worldwide and the establishment of adequate therapeutic protocols for treating life-threatening infections continues to be a very difficult challenge.

1.3.3. Enterococci

The term "*enterococcus*" has its origin at the end of the 19th century when Thiercelin described a saprophytic *coccus*, of intestinal origin, capable of causing infection (Lewis & Zervos, 1990). In same year, MacCallum and Hastings characterized a similar organism from a lethal case of endocarditis, which called *Mycrococcus zymogenes.* The name *Streptococcus faecalis* (faecalis, relating to feces) was first used in 1906 by Andrewes and Horder, who isolated this organism from a patient with endocarditis and considered that this *streptococcus* was "so characteristic of the human intestine that the term '*Streptococcus faecalis*' justly be applied to it" (Murray, 1990). At last, the same term was used for organisms that could be grew at 10 and 45°C, in 6.5% NaCl, and at pH 9.6 and which survived 60°C for 30 minutes. These and many others characteristics became widely used to distinguish between enterococci and non-enterococcal streptococci. These descriptions constitute the basis for the paradigm of enterococci classification as pathogenic bacteria or as a commensal opportunist (Lebreton, Willems & Gilmore, 2014).

Enterococci are non-sporing facultative anaerobes and obligatory fermentative chemoorganotrophs, with an optimal growth temperature of 35°C, catalase negative (with the exception for some species), usually homofermentative, producing lactic acids through glucose fermentation, without gas production (Klein, 2003). They are primarily located in the human and mammals small and large intestines, particularly in the jejunum, ileum, cecum and recto-sigmoid. Therefore, they are frequently found in human feces, especially *E. faecalis* and *E. faecium*, and are common in the oral cavity, being rarely found in the stomach (Bik et al., 2006).

Enterococci taxonomy has considerably changed and at the present the genera includes 35 recognized species (http://www.antimicrobe.org/new/b03.asp). Two major bacterial species account for the vast majority of enterococcal infections: *E. faecalis* is the most common one, causing 80 to 90% of such infections, while *Enterococcus faecium* about 10 to 15%. The primary sites of infection are the urinary tract and the soft tissues adjacent to the intestines where enterococci are resident. *E. faecalis* is generally non-pathogenic to healthy humans, being an extremely adaptable organism capable of surviving in hard environmental conditions (Ballering et al., 2009).

Little is known about the main mechanisms used by enterococci to colonize the gastro-intestinal (GI) tract of either healthy individuals or hospitalized patients. The exposure of

hospitalized patients to antibiotics results in major modifications of the gut microbiota, which facilitates colonization of the GI tract by drug-resistant enterococci (Donskey et al., 2000). In such conditions, indigenous commensal enterococci can act as opportunistic pathogens and translocate across the mucosal barrier to cause systemic infections in immune-compromised hosts. However, infection more commonly results from the colonization, overgrowth and translocation of hospital-adapted antibiotic-resistant strains with enhanced pathogenicity (Donskey, 2004). In fact, *Enterococcus* have emerged in recent years as pathogenic microorganisms associated with serious nosocomial infections, despite their usually low level of virulence. They cause infection almost exclusively in hospitalized patients who have significantly compromised immune defences. Such hospital-related strains are frequent, especially in intensive care units, where enterococci can be transmitted via the hands of clinical staff (Dufour, Leung & Lévesque, 2012).

Enterococcus is one of the most frequently isolated genera from DFU (James et al., 2007; Bowling, Jude & Boulton, 2009) and several studies reported a frequency of approximately 20% (Gadepalli et al., 2006; Mendes et al., 2012; Swarna, Madhavan, Gomathi & Thamaraiselvi, 2012; Małecki et al., 2013) or even as high as 35% (Citron et al., 2007). In compromised patients, such as diabetics, enterococci can act as opportunistic pathogens (Tascini et al., 2011). Currently, there is increasing evidence that diabetes is a risk factor for vancomycin resistant enterococci (VRE) (Boyanova & Mitov, 2013).

1.3.3.1. Enterococci virulence traits

The pathogenicity islands of *Enterococcus* were first identified in an *E. faecalis* multi-drug resistant (MDR) strain in the 1980's and included virulence traits associated with surface proteins, namely the enterococcal surface protein Esp, the secreted cytolysin toxin and aggregation substances. Other important virulence factors produced by enterococci include haemolysin, gelatinase and surface adhesins (Giridhara Upadhyaya, Ravikumar & Umapathy, 2009).

In order to cause disease, enterococci must first adhere to host tissues. They express surface adhesion proteins, such as the aggregation substance previously mentioned, that allow them to bind to human intestinal cells (John & Carvalho, 2011). Escape to the immune system is facilitated by cytolysin that can lyse macrophages and neutrophils, representing an advantage for growth and survival (Clewell, 2007). Enterococcal cytolysin consists of two structural subunits, coded in an operon of five genes, in which the extracellular activator serine protease gene, *cylA*, activates the cytolysin precursor components (Semedo et al., 2003).

Biofilm formation may be an important factor in the pathogenesis of enterococcal infections (Figure 6) and previous results suggest that *E. faecalis* produce biofilm more frequently than *E. faecium.* Biofilm formation seems to be dependent on environmental conditions, since, for example, high osmolality has a negative effect, but the exact mechanisms involved in biofilm

formation by enterococci are still unknown (Mohamed & Huang, 2007). *E. faecalis* gelatinase (GelE) is an extracellular zinc metalloprotease that can hydrolyse gelatin, collagen and casein, and seems to be essential for biofilm formation (Mohamed & Murray, 2005). The *fsr* locus (*E. faecalis* regulator) in *E. faecalis*, which includes the *fsr*A, *fsr*B and *fsr*C genes, being homologue of the staphylococcal *agr*BCA loci, has been characterized and seems to have a similar effect (Qin, Singh, Weinstock & Murray, 2000).

Enterococcal surface protein (Esp) is associated with increased virulence, colonization and persistence in the urinary tract and also with biofilm formation; in fact, the *esp* gene has been identified as a marker with high prevalence in vancomycin-resistant *E. faecium* (VREF) clones among hospitalized patients (Willems et al., 2001).

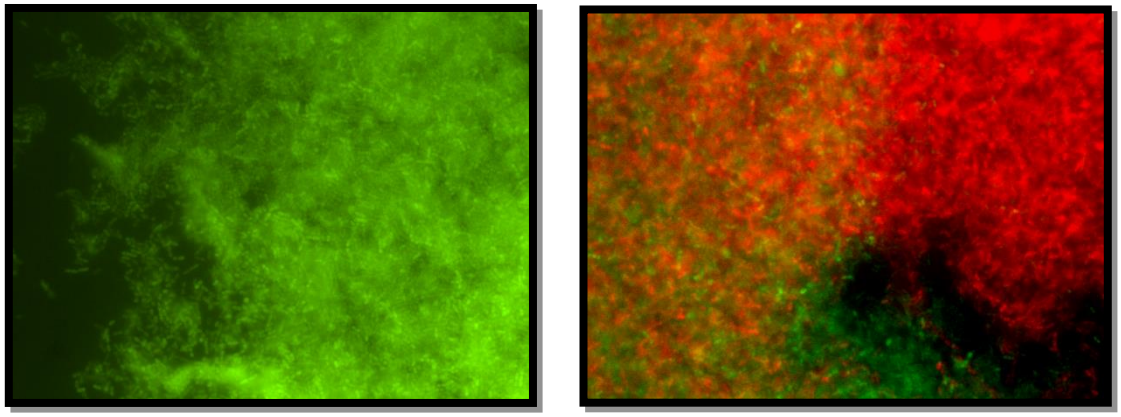
1.3.3.2. Enterococci antimicrobial resistance ability

Traditionally, treatment for serious enterococcal infections was based on the synergistic effects of an aminoglycoside and a cell wall-active antibiotic such as ampicillin or vancomycin (John & Carvalho, 2011). However, these organisms have been exposed to multiple antibiotics in hospital settings, thus providing them an evolutionary pressure for the selection of mechanisms involved in resistance genes transfer, including conjugation via plasmid, as well as transfer of resistance genes via transposons or bacteriophages (Moellering, 1992). Considering that enterococci reside in the GI tract, they can exchange resistance genes with other bacterial genera present in the same ecological niche (Sung & Lindsay, 2007). Enterococci are resistant to many antibiotics: some have acquired resistance to aminoglycosides via aminoglycoside-modifying enzymes, to tetracyclines via acquisition of resistance genes such as *tet*M and *tet*N, to chloramphenicol via chloramphenicol acetyltransferase and to glycopeptides via enzymes that modify or remove the vancomycin target (Courvalin, 2006). There are also strains of enterococci that produce β-lactamases capable of inactivating penicillin, ampicillin and related drugs, acquired via transferable plasmids (Herman & Gerding, 1991).

In terms of vancomycin resistance, six gene clusters have been discovered that appear to be associated with this phenotype (VanA to VanG) (Courvalin, 2006). Some *Enterococcus* strains have the potential to be reservoirs of glycopeptide resistance genes and transfer them to more virulent pathogens such as MRSA (Sung & Lindsay, 2007): in fact, it has been demonstrated that the genes conferring the VanA phenotype could be transferred from *E. faecium* to MRSA (Noble, Virani, & Cree, 1992). The phenotype VanA, with high-level resistance to vancomycin and teicoplanin, and VanB, with variable resistance to vancomycin, are the most common forms of acquired glycopeptide resistance, being transferable via plasmids or transposons (Woodford, Johnson, Morrison & Speller, 1995). The VanA phenotype has been found in approximately 60% of VRE and VanB found in approximately 40% of such isolates (Clark, Cooksey, Hill, Swenson & Tenover, 1993)

The emergence of MDR enterococci over the past 50 years has substantially increased and antibiotic resistant enterococci are now leading causes of nosocomial infection worldwide (Lebreton et al., 2014).

Figure 6. Biofilm-producer *E. faecalis* DFU strain (left) after FISH (×1000; original); polymicrobial biofilm formed by *Corynebacterium* (green) and *E. faecalis* (red) DFU strains after FISH (right) (×1000; original).



DFU, diabetic foot ulcer; FISH, Fluorescent *In Situ* Hybridization.

1.3.4. Other bacteria frequently involved in DFI

1.3.4.1. *Corynebacterium*

The genus *Corynebacterium*, a member of the class Actinobacteria, includes aerobic, asporogenous, irregular shaped Gram-positive ubiquitous rods that belong to the human skin microbiota. Nearly all strains are catalase and oxidase positive and express a range of pigments (Bernard, 2012). Until now, there are 88 established species: 53 are occasional or rare causes of infection in humans, being accidentally transmitted by zoonotic contact, with the remaining 35 species originating solely from animals, including birds, the environment, water, foodstuffs or synthetic materials (Bernard & Funke, 2012). *Corynebacterium* is increasingly being recognized as a relevant opportunistic pathogen under specific circumstances, especially for immunocompromised patients, with prosthetic devices, or committed to hospitals/nursing homes for long-term periods (Funke, von Graevenitz, Clarridge & Bernard, 1997).

The species *C. urealyticum*, *C. amycolatum*, *C. jeikeium* and *C. striatum* have been reported as the most important pathogens of this genus. However, the most significant pathogen of this group is *Corynebacterium diphtheriae*, the primary cause of diphtheria (Riebel, Frantz, Adelstein & Spagnuolo, 1986; Renom et al., 2007).

Different studies have described high resistance rates in *Corynebacterium* regarding several antimicrobials, such as β-lactams, aminoglycosides, quinolones, macrolides, lincosamides and

tetracyclines (Goldstein et al., 2003; Gómez-Garcés, Alos & Tamayo, 2007). The high level of macrolide resistance is of particular concern due to its wide use in infections caused by these microorganisms, being an alternative to β-lactams and tetracyclines (Ortiz-Pérez et al., 2010). Macrolide resistance is mainly due to the presence of methylase enzymes that are codified by *erm*, with the presence of the *erm*X methylase gene being definitively linked to the resistant macrolide-lincosamide-streptograminB (MLSb) phenotype, expressed as resistance to erythromycin and clindamycin and also associated with resistance to other antimicrobial agents, including chloramphenicol and trimethoprim-sulfamethoxazole (Roberts et al., 1999). Mutations in *gyr*A have also been linked to quinolone and vancomycin resistance in coryneform bacteria, being inappropriate to recommend glycopeptides as first-line drugs for the treatment of these infections, since some corynebacteria are intrinsically vancomycin resistant (Bernard, 2012).

Bessman, Geiger & Canawati in 1992 referred the importance of this organism in DFI; afterword several studies have reported a high prevalence of *Corynebacterium* in foot ulcers (Citron et al., 2007; Gontcharova, Youn, Sun, Wolcott & Dowd, 2010; van Asten et al., 2015), being in some cases the most frequently isolated species (Dowd et al., 2008a). Although often considered non-pathogenic since it belongs to the human skin and mucous membranes microbiota, this genus appears to be a common and prevalent microorganism in polymicrobial DFI.

1.3.4.2. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa has emerged worldwide as one of the most relevant pathogen in health care institutions, contributing significantly to high morbidity and mortality in hospitalized patients (Peleg et al., 2009). After *S. aureus*, *P. aeruginosa* is the bacterial species most frequently isolated from diabetic foot ulcers, as reported by several studies (Sekhar et al., 2014), representing about 7 to 23% of total DFI isolates. In a study conducted by Swarna et al. in 2012, it was the most frequently isolated bacteria from DFI (Swarna et al., 2012). *P. aeruginosa* may cause severe tissue damage in diabetics and should never be ignored as insignificant in DFU because it may result in sepsis and in the need for amputation (Sivanmaliappan & Sevanan, 2011).

P. aeruginosa is a non-fermentative Gram-negative, rod-shaped (Figure 7), asporogenous and monoflagellated bacterium that has an incredible nutritional versatility (http://www.antimicrobe.org/new/b112.asp). It is an opportunistic pathogen causing severe infections, including ventilator-associated pneumonia, urinary and peritoneal dialysis catheter infections, bacterial keratitis, otitis externa and burn wound infections. *P. aeruginosa* plays a particularly important role in patients with cystic fibrosis, in whom chronic and recurrent infections of the sinopulmonary tract by *P. aeruginosa* are common (Driscoll, Brody & Kollef, 2007).

The mechanisms involved in *P. aeruginosa* adhesion have been increasingly investigated over the last decade. Flagella and type IV pili, *cup* fimbria and *pel* genes are involved in bacterial adhesion, being also observed that *P. aeruginosa* is able to produce biofilm through a complex and highly regulated mechanism, in which each stage has a unique phenotype (Bonfiglio et al., 1998; Sauer et al., 2002; Macé et al., 2008). The pathogenicity of these organisms is based on their ability to produce a variety of toxins and proteases and also to resist to phagocytosis (Sivanmaliappan & Sevanan, 2011).

Besides being noted for its metabolic versatility and its exceptional ability to colonize a wide variety of environments, *P. aeruginosa* is also relevant due to its intrinsic resistance to a wide diversity of antimicrobial agents (Carmeli, Troillet, Eliopoulos & Samore, 1999). The antibiotic agents to which these strains are not regularly resistant include fourth-generation and some third-generation cephalosporins (cefepime, ceftazidime and cefoperazone), β-lactam/β-lactamase inhibitor combinations (piperacillin/tazobactam and ticarcillin/clavulanic acid), ureidopenicillin (piperacillin), carboxypenicillins (ticarcilin), aminoglycosides (gentamicin, tobramycin and amikacin), monobactams (aztreonam), polymyxins (colistin), some quinolones (levofloxacin and ciprofloxacin) and carbapenems (imipenem, meropenem and ertapenem) (Driscoll et al., 2007; Sivanmaliappan & Sevanan, 2011); however, cross-resistance between these agents has been reported. *P. aeruginosa* exhibits resistance to a variety of antimicrobials including β-lactams, with carbapenems being often used for treating infections caused by β-lactam resistant *P. aeruginosa*, and also by strains resistant to aminoglycosides and fluoroquinolones, the traditional antipseudomonal antimicrobials (Murugan & Lakshmi, 2010).

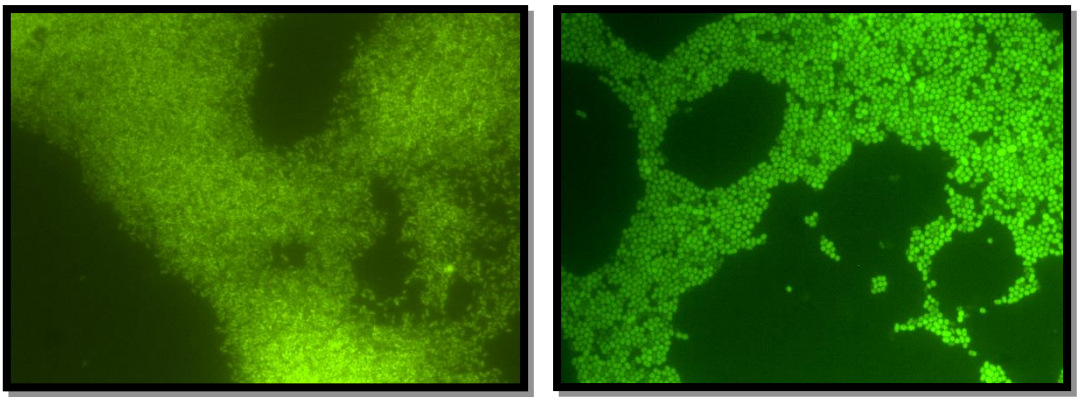
1.3.4.3. *Acinetobacter baumannii*

Acinetobacter baumannii is another common Gram-negative DFI isolates, besides Enterobacteriaceae as *E. coli*, *Proteus* spp., *Klebsiella* spp. and *Enterobacter* spp. (Ramakant et al., 2011). *A. baumannii* represents approximately 2 to 5% of total DFI isolates as described in several studies (Ge et al., 2002; Gadepalli et al., 2006; Dowd, Sun et al., 2008; Mendes et al., 2012; Swarna et al., 2012; Malik et al., 2013). The same reports referred that *Acinetobacter* DFI isolates showed significant antimicrobial resistant profiles associated to biofilm production.

The genus *Acinetobacter* is formed by a genetically diverse group of aerobic Gram-negative non-fermenting bacteria (Rao, Karthika & Singh, 2008). The most relevant species is *A. baumannii,* an important short rod (*coccobacillus*), considered to be an opportunistic pathogen (Figure 7) with the ability to colonize and persist in the hospital setting and on medical devices (Lee et al., 2008). It was previously reported as an important pathogen in wounded soldiers returning from Iraq and Afghanistan, in patients affected by tsunami in Southeast Asia in December 2004 and survivors from the earthquake that occurred in Turkey in 1999. Since

these patients are often transferred to tertiary care centers, sometimes in distant geographic areas, they may become a source of transmission in previously non-endemic hospitals (Abbo, Carmeli, Navon-Venezia, Siegman-Igra & Schwaber, 2007).

Figure 8. *P. aeruginosa* (left picture) and *A. baumannii* (right picture) DFU strains after FISH (×1000; original).



DFU, diabetic foot ulcer; FISH, Fluorescent *In Situ* Hybridization.

A. baumannii nowadays represents a significant problem especially in intensive care units, causing pneumonia, wound and urinary tract infections, bacteraemia and meningitis (Espinal, Martí & Vila, 2012). The mortality rate associated to *Acinetobacter* nosocomial infections is high, ranging from 26 to 68% (Ibrahim & Somily, 2012). It ranks second after *P. aeruginosa* among the nosocomial, aerobic, non-fermentative, Gram-negative bacilli pathogens (Rao et al., 2008). *A. baumannii* can survive for extended periods on fingertips and on inanimate objects such as glass, plastic and other environmental surfaces, even in dry conditions (Jawad, Heritage & Snelling, 1996). The survival of *A. baumannii* has been attributed to its ability to form biofilms that, added to desiccation resistance, may enhance colonization and persistence in the hospital environment and also increase the probability of acquiring antimicrobial resistance genes, that confer ability to cause nosocomial infections and outbreaks (Espinal et al., 2012). Biofilm formation in *A. baumannii* is multifactorial and diverse, being dependent on the adhesion surface; however, many of the molecular mechanisms responsible for bacterial attachment remain obscure (Gaddy & Actis, 2009). Several reports have shown that *csu*C and *csu*E, which belong to a gene cluster encoding pili assembly proteins, are required for the early steps of biofilm formation (Tomaras, Dorsey, Edelmann & Actis, 2003).

A significant association between biofilm production and resistance to a variety of antimicrobial agents has been reported in *A. baumannii* (Peleg et al., 2009; Swarna et al., 2012). Whereas multi-drug resistance is rarely found in community isolates of *A. baumannii*, the prevalence of the MDR phenotype among hospital isolates has increased during the last decade, and MDR

A. baumannii has become a leading pathogen in many hospitals worldwide (Falagas, Bliziotis & Siempos, 2006). Therefore, *A. baumannii* infections represent a global medical challenge.

1.4. Biofilms in DFI

1.4.1. Introduction

Bacteria exist as suspended growth forms known as “planktons” that multiply rapidly, are highly motile and are more susceptible to the effects of antibiotics, environment and host factors. Under nutrient limitations bacteria grow more slowly, have restricted mobility and are termed “sessile”, ultimately forming large aggregates known as biofilms.

A biofilm can be defined as “a community of bacteria and their extracellular polymers that is attached to surfaces” (Sekhar, Ohri & Chakraborti, 2010). The concept of biofilm was first addressed by the Dutch scientist Antonie van Leeuwenhoek, in the seventeenth century, who described “animalcules” as the accumulation of tiny living animals in the plaque of his own teeth (Dufour et al., 2012); however the general theory of biofilm occurrence was only established in 1978 by Costerton, Geesey & Cheng (1978). This theory stated that the majority of bacteria grow in matrix-enclosed biofilms adherent to surfaces in all nutrient-sufficient ecosystems; he stated that these bacteria persisted in coordinated, spatially organized, and metabolically integrated biofilm communities.

The definition of biofilm has evolved over the last 25 years, becoming a more dynamic concept in which single-cell organisms assume a temporary complex process that is multifaceted and dynamic in nature. Costerton et al. in 1995 stated that the cellular adhesion triggered the expression of genes controlling the production of bacterial components required for adhesion and biofilm formation, emphasizing that the process of biofilm production was regulated by specific genes transcribed during initial cell attachment.

1.4.2. Biofilm formation

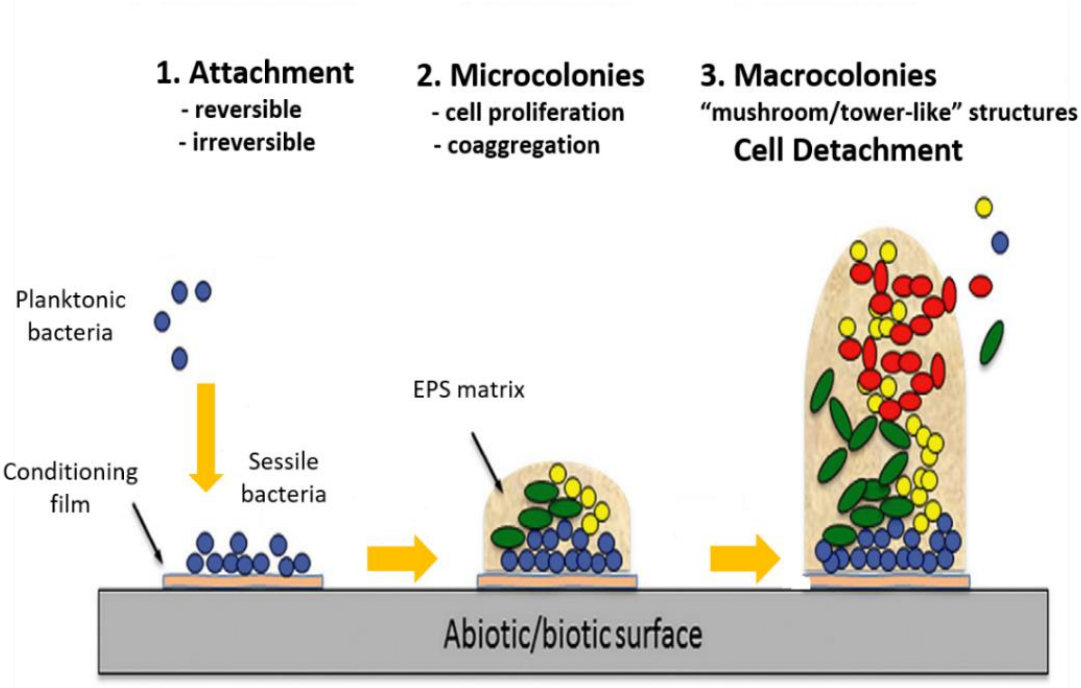
Formation of biofilms involves several stages (Figure 8). The first step is a reversible attachment of the bacterial cells to a biotic or abiotic surface, mediated by weak Van der Waals, Lewis acid-base and electrostatic forces. This transient attachment is reinforced by host and tissue-specific adhesins located on the bacterial surface, such as LPS and exopolysaccharides, or by appendages such as pili and fimbriae that allow the establishment of irreversible interactions (Kaplan, 2010).

The second stage of biofilm development involves the multiplication of bacteria on the attached surface forming micro-colonies, and the synthesis of an extracellular polymeric matrix that strongly attaches the bacterial mass to the underlying surface. This matrix also contributes to biofilm-mediated antimicrobial resistance, either by acting as a diffusion barrier, or by

binding directly to antimicrobial agents and preventing their access to the biofilm cells (Mah & O'Toole, 2001). Microorganisms account for less than 10% of the biofilm dry mass, with the self-produced polymeric matrix representing 90% of it. This matrix is mainly composed by EPS that can vary greatly between biofilms, depending on the microorganisms present, the shear forces experienced, the temperature and the availability of nutrients. The EPS is commonly called "the dark matter of biofilms" due to the large range of matrix biopolymers that include polysaccharides, proteins, pili, flagella, nucleic acids, lipids and eDNA (Flemming & Wingender, 2010).

In the third step occurs the development of mature biofilms, composed by macro-colonies containing multiple layers of packed cells gathered into "mushroom" and "tower-like" structures. Macro-colonies are surrounded by water channels that allow the exchange of nutrients and cell signalling (Hall-Stoodley, Costerton & Stoodley, 2004).

Figure 8. Schematic representation of the biofilm formation steps: initial attachment to surface, cell proliferation with formation of a monolayer and biofilm maturation and cell dispersal. Adapted from Dufour et al. (2012).



EPS: extracellular polymeric substances.

The final stage of biofilm development comprises the individual or group detachment of cells from mature biofilms and their dispersal into the environment. This is an essential stage of the biofilm life cycle that allows the translocation of cells to new locations where they can attach, allowing biological dispersal, bacterial survival and disease transmission (Kaplan, 2010). There are diverse mechanisms of biofilm dispersal used by different bacterial species, including the production of extracellular enzymes that degrade adhesive components in the

biofilm matrix, such as glycosidases, proteases and deoxyribonucleases (Boles & Horswill, 2008). The deoxyribonuclease known as thermonuclease has been implicated in cell detachment from *S. aureus* biofilms, and may function as an endogenous mediator of biofilm dispersal in this species (Mann et al., 2009).

Alternative mechanisms of biofilm dispersal include the production of biofilm colonies containing internal cavities that become filled with planktonic cells, which can be released into the surrounding medium. These cavities have been observed in biofilms produced by the human pathogens such as *Aggregatibacter actinomycetemcomitans*, *P. aeruginosa*, *Serratia marcescens* and *S. aureus* (Boles, Thoendel & Singh, 2005; Ma et al., 2009).

1.4.3. Biofilm and Quorum Sensing

In 2000, Watnick & Kolter (Watnick & Kolter, 2000) compared biofilm to a “bustling city”, in which microbes are distributed geographically based on the environment and neighbourhood, being considered “social” organisms able to communicate with each other. Cell-to-cell communication is generally carried out by secrete signalling molecules, produced and released by bacteria in response to population density variations, through a process called quorum sensing. Low molecular weight molecules called “autoinducers” are synthesised intracellularly and released in response to population increases, reaching a quorum sensing threshold that triggers signal transduction cascades resulting in changes of cellular gene expression with a consequent increased transcription of biofilm-specific genes (Ng & Bassler, 2009). Autoinducers have been shown to control several stages of biofilm formation, including surface attachment, matrix synthesis, formation of fluid channels and pillar-like architecture and dispersal (Hall-Stoodley et al., 2004; Stanley & Lazazzera, 2004).

Acyl homoserine lactones (AHL) are the major and best-studied class of autoinducers used by Gram-negative proteobacteria for interspecies quorum sensing. The first AHL autoinducer was discovered in the bioluminescent marine bacterium *Vibrio fischeri*, in which two proteins LuxI and LuxR are essential for control of bioluminescence (Sensing, Fuqua, Parsek & Greenberg, 2001).

One of the best-studied AHL quorum sensing systems include the LasI/LasR-RhlI/RhlR of *P. aeruginosa* that controls virulence factor gene expression and biofilm formation (Winson et al., 1995). Another autoinducer produced by *P. aeruginosa* is the 2-heptyl-3-hydroxy-4-quinolone, also known as *Pseudomonas* quinolone signal or PQS, that mediates cell death, DNA release and induces dispersal of wild-type *P. aeruginosa* biofilms cultured in microplate wells (Dong, Zhang, An, Xu & Zhang, 2008).

Gram-positive bacteria primarily use modified oligopeptides as autoinducers in quorum sensing systems. Staphylococci produce and secrete a number of peptide signals called phenol-soluble modulins (PSM) that accumulate in the extracellular environment. Evidences suggest that two of these peptides may play a role in biofilm dispersal, namely the δ-toxin

produced by *S. epidermidis* and *S. aureus* and the autoinducing peptide I (AIP-I) produced by *S. aureus* (Novick & Geisinger, 2008). When the density of AIP throughout the bacterial community reaches the quorum sensing threshold, the *agr* gene expression is induced. This result in the upregulation of the expression of detergent-like peptide, a protease and a thermostable nuclease, leading to release of bacterial cells from the mature biofilm (Archer et al., 2011). Autoinduction is not only responsible for biofilm formation, but also for many other processes, such as expression of virulence factors and biosynthesis of antibiotics (Dickschat, 2010).

1.4.4. Multi-drug resistance in biofilm

Inadequate exposure to antimicrobial agents is one of the principal factors underlying chemotherapeutic failure and presumably limits the efficacy of some agents regarding biofilm-associated infections (Lynch & Robertson, 2008).

Biofilm bacteria are particularly recalcitrant to antibiotic treatments due to many mechanisms that makes them up to 1000-fold more resistant to antibiotics than their planktonic counterparts (Høiby, Bjarnsholt, Givskov, Molin & Ciofu, 2010). The first experiment showing that biofilm cells were more tolerant to drugs than planktonic cells was probably performed by Leeuwenhoek, when he failed to kill *in situ* plaque bacteria present on his teeth using prolonged rinsing with vinegar, while the treatment was effective if they were first removed from the teeth and mixed with vinegar in the laboratory (Dufour et al., 2012).

It is important to distinguish between tolerance and resistance, although their distinction remains ambiguous. “Resistance” is used to describe the inherited ability of microorganisms to grow at high concentrations of an antibiotic, independently of treatment duration, and is quantified by the MIC of a particular antibiotic, whereas “tolerance” is more generally used to describe the ability, inherited or not, of microorganisms to survive transient exposure to high concentrations of an antibiotic without a change in the MIC, which is often achieved by slowing down an essential bacterial process (Brauner, Fridman, Gefen & Balaban, 2016).

Regardless the resistance mechanisms, bacterial biofilms use several strategies to prevent a drug to hit its target and can include one or more of the following: destruction of the antibiotic, for example by β-lactamases; restricted penetration of the antimicrobial agent through the biofilm matrix; direct binding of the antibiotics to the EPS matrix; altered growth rate of biofilm bacteria present in the inner layers; target modifications (Dufour et al., 2012). In fact, biofilm structure and the extracellular polymeric substances represent an impermeable barrier to drugs, limiting antimicrobial penetration inside the biofilm and shielding its cells. Already in 1994, Suci et al. demonstrated the occurrence of delayed penetration of ciprofloxacin into *P. aeruginosa* biofilms, requiring 21 minutes in contrast with the usual 40 seconds (Donlan & Costerton, 2002). Upon antibiotic exposure, cells at the top of the biofilm interface die due to their closer contact with the antimicrobial compound, while bacteria embedded deep inside the

biofilm are able to survive. This cannot be applicable to all antibiotics because the EPS matrix provides little or no barrier to some of them (Donlan, 2002).

The development of dormant persister cells is another mechanism of resistance phenotype in biofilms (Dufour et al., 2012). This phenotype was first described in *S. aureus* in 1942 by Hobby et al., who found that 1% of cells were not killed by penicillin and became persister cells (Wood, Knabel, & Kwan, 2013). Persisters are not mutants, as they reach this state without undergoing any genetic change. They are phenotypic variants of actively dividing cells produced stochastically in the population and arising due to a state of dormancy, in which cells are metabolically inactive. This allows them to survive to stressful conditions and prevents death because some antibiotics only target dividing cells; for this reason persister cells are extremely tolerant to high concentrations of antibiotics (Lewis, 2008).

Another mechanism related to biofilm resistance to antimicrobial agents is that biofilm-associated cells grow significantly more slowly than planktonic cells and, as a result, take up antimicrobial agents more slowly. This occurs especially in the microbial cells present in the biofilm deeper layers where nutrients and oxygen are limited, leading to a lower growth rate that may account for the enhanced tolerance toward antibiotics and persistent infections (Sekhar et al., 2010). As some antibiotics only target dividing cells, persister cells are extremely tolerant to high concentrations of antibiotics (Lewis, 2008).

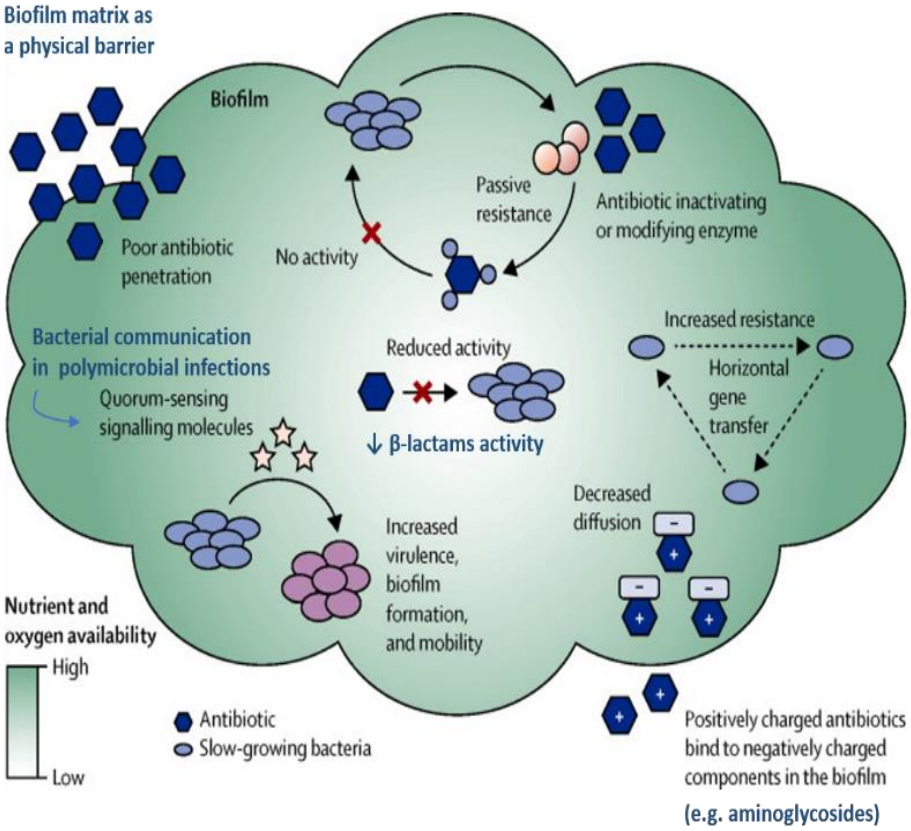
One of the mechanisms more explored to explain the recurrence of biofilm infections, is the drug efflux systems that represent a key mechanism of resistance in Gram-negative bacteria, being also found in Gram-positives. These systems pump solutes out of the cell, allowing the microorganisms to regulate their internal environment by removing toxic substances, including antimicrobial agents, metabolites and quorum sensing signal molecules (Soto, 2013).

Moreover, bacteria in biofilms are protected against stress factors, and can switch to more tolerant phenotypes when they are under environmental stressors, such as starvation, heat or cold shock. These general stress responses in bacteria are regulated by sigma factors (Dufour et al., 2012).

Furthermore, mobile antibiotic resistance genes, associated with plasmids and transposons can be shared among bacteria occupying the same environment by horizontal gene transfer, especially in bacterial communities (Sherrard, Tunney & Elborn, 2014).

Finally, the role of quorum sensing and intracellular signalling molecules in antimicrobial resistance of biofilms has also been investigated, and studies have shown that the use of quorum sensing inhibitors were able to enhance susceptibility of *P. aeruginosa* biofilms to antimicrobial treatments (Bjarnsholt et al., 2005). However, these cell-cell communication mechanisms are not yet fully understood and require further investigation. Figure 9 summarises some of the bacterial mechanisms of antimicrobial resistance in biofilms.

Figure 9. Antibiotic resistance mechanisms in bacterial biofilms.
Adapted from Sherrard et al. (2014).



1.4.5. Biofilm in infectious diseases

Several pathogens associated with chronic infections are linked to biofilm infections. Persistent bacterial infections are typically biofilm-related diseases, developing slowly, rarely resolved by immune defences, and with the ability to persist for a long time in the human body (James et al., 2007). In fact, 80% of bacterial chronic inflammatory and infectious diseases involve biofilms (Wood, Hong & Ma, 2011).

About 60 to 70% of hospital-acquired infections are due to biofilms as they have the ability to colonize biomedical devices, including prosthetic heart valves, orthopaedic devices, vascular prostheses and urinary catheters. The most frequent organisms that colonize implanted medical devices are staphylococci and enterococci, two commensal inhabitants of the human skin microbiota, upper respiratory tract, lower gastro-intestinal tract and urogenital tract (Bryers, 2009).

A vast majority of chronic wounds involve polymicrobial interactions that usually occur within biofilms (Sauer et al., 2002). Biofilms are responsible for the non-healing nature of chronic wounds, where their presence not only delays the wound healing process, but also

complicates treatment outcomes. In fact, polymicrobial wound infections that involve biofilms exhibit increased tolerance to antibiotic treatment as compared to monomicrobial infections (Wei, Chong & Kline, 2016). Infected DFU share these characteristics and it has been hypothesized that biofilm may play a role in these infections (Neut, Tijdens-Creusen, Bulstra, van der Mei & Busscher, 2011). In view of the diverse population observed in DFU, Dowd et al. in 2008 introduced the concept of functional equivalent pathogroups (FEP) (Dowd et al., 2008a). Their hypothesis suggested that single non-pathogenic species might not cause disease, but when in mixed populations, the synergistic effect obtained contribute to the chronicity of diabetic foot wounds. The presence of FEP could explain the failure of antibiotic monotherapies, because diverse pathogenic biofilms are more stable than less diverse ones.

1.5. DFU Treatment

1.5.1. Therapeutic protocols

1.5.1.1. Debridement, wound healing agents and surgery

Based on National Institute for Health and Clinical Excellence strategies, diabetic DFU healing must be managed by a multidisciplinary team, that consists of a general practitioner, a nurse, an educator, an orthotic specialist and a podiatrist, being sometimes necessary to consult other specialists such as vascular surgeons, infectious disease specialists, dermatologists, endocrinologists, dieticians and orthopaedic specialists (National Institute for Health and Care Excellence [NICE], 2015). A holistic approach with a multi-disciplinary team can reduce amputation rates, lowering the associated costs and leading to better quality of life for patients with DFU (Yazdanpanah, Nasiri & Adarvishi, 2015).

First of all, foot self-management is considered the cornerstone to prevent DFU, and 50% of DFU cases can be prevented by effective education (Mensing et al., 2004). Patients with DFU should be educated about risk factors and the importance of foot care including the need of self-inspection, foot temperature monitoring, appropriate daily foot hygiene, use of proper footwear and regular blood sugar control. In fact, glucose control is the most important metabolic factor, being reported that inadequate blood sugar control is the primary cause of DFU (McMurry, 1984). A greater elevation in blood glucose level is associated with a higher potential for suppressing inflammatory response and decreasing the host's response to infection (Alavi et al., 2014).

Debridement, consisting in the removal of necrotic and senescent tissues as well as foreign and infected materials from the wound, is considered as the first and most important therapeutic step allowing the wound closure and decreasing the possibility of limb amputation (Davis, Martinez & Kirsner, 2006). Debridement also seems to decrease bacterial counts and

stimulates production of local growth factors. Moreover, it also reduces pressure, allows for the evaluation of the wound bed and facilitates wound drainage (DiPreta, 2014). There are different kinds of debridement techniques including surgical, enzymatic, autolytic, mechanical and biological. The debridement method of choice may depend on the patient's level of comfort and the practitioner's level of expertise (Lebrun, Tomic-Canic & Kirsner, 2010).

A long time used debridement technique that is categorized as biological debridement, is maggot debridement therapy (MDT), indicated for open wounds and ulcers that contain gangrenous or necrotic tissues, with or without infection (Mumcuoglu, 2001). In this method, sterile and live forms of *Lucilia sericata* larvae are applied to the wound to achieve debridement, disinfection and ultimately wound healing, due to a powerful autolytic enzyme secreted by the larvae that liquefies necrotic tissues, stimulating the healing process and destroying bacterial biofilms (Sherman, 2009). MDT was routinely used until the mid-1940s, becoming rare with the advent of antibiotics; however, it is now considered by many therapists as a practical solution for many non-healing wounds. Many studies have reported that MDT can significantly diminish wound odour and bacterial count, including MRSA, prevent hospital admission and decrease the number of outpatient visits of individuals with DFU (Bowling et al., 2007).

Despite its advantages, adequate debridement must always be accompanied by the application of topical wound healing agents, dressings or wound closure procedures, which may be expensive (Yazdanpanah et al., 2015). The use of offloading techniques, commonly known as pressure modulation, is considered the most important component for the management of neuropathic ulcers in patients with diabetes, promoting DFU healing (Mendes & Neves, 2012). Many types of devices can offload the infected wound, but it is important to choose one that permits easy inspection.

Over the last decades, novel dressings have appeared for DFU management, aiming to enhance the life quality of patients, alleviate pain, deliver drugs and reduce odours. Ideally, dressings should confer moisture balance, protease sequestration, growth factor stimulation, antimicrobial activity, oxygen permeability and the capacity to promote autolytic debridement, facilitating the production of granulation tissues and the re-epithelialization process (Moura, Dias, Carvalho & De Sousa, 2013). Dressings are used based on DFU characteristics and can include natural or synthetic polymers that have been combined or cross-linked for this purpose. Hydrogels have been found to be the most popular choice of dressing for all DFU types but do not yet represent a practical option since application of these compounds tends be very expensive and difficult to regulate/control (Cos, Toté, Horemans & Maes, 2010).

Surgery procedures for DFU healing include non-vascular and vascular foot surgery, and in some cases amputation. While the primary goal of DFU management focuses on limb salvage, in some cases amputation may offer a better functional outcome. Surgical procedures range from drainage and excision of infected and necrotic tissues, to revascularization of the lower

extremity and reconstruction of soft-tissue defects or mechanical misalignments (Lipsky, 2004a). Unfortunately, surgical treatment of diabetic foot infections is based on even less-structured evidence than that for antibiotic therapy (Citron et al., 2007).

1.5.1.2. Antibiotic therapy

Antibiotic therapy is always relevant in the treatment of chronic wounds when clinically infected, but the role of systemic antibiotics is limited by the lack of blood supply to the wound surface. Systemic antibiotics have a clear role in infected wounds with surrounding cellulitis, but their efficacy in the routine treatment is limited (Hernandez, 2006). Antibiotic therapy is associated with frequent adverse effects, high financial costs and increasing risk of antibiotic resistance dissemination; therefore, the reasonable use of antibiotics for DFI treatment is a very crucial concern for clinicians (Chu et al., 2015). According to the 2012 clinical practice guidelines for the diagnosis and treatment of DFI established by the Infectious Diseases Society of America (IDSA) (Lipsky et al., 2012b), it is recommended that, in the case of infected wounds, the clinicians send appropriately obtained specimens for culture prior to starting empiric antibiotic therapy. The empiric antibiotic regimen is based on the severity of the infection and the most probable etiologic agent(s), and the definitive protocol is based on the results of appropriately culture and sensitivity testing from a wound specimen as well as the patient's clinical response to the empiric regimen. The duration of antibiotherapy for DFI should be based on the severity of infection, presence or absence of bone infection and clinical response to therapeutics. In fact, it can generally be discontinued when signs and symptoms of infection are resolved (Lipsky et al., 2012a). In a recent study, Chu et al. (Chu et al., 2015) demonstrated that continuing antibiotic regimens could improve clinical outcomes for patients with moderate/severe infections when signs and symptoms have resolved, but more studies are required.

Topical antibiotics have been accepted as useful in the presence of high bacterial counts that translate into a highly exudative wound, but their role in colonized wounds is less clear (Mustoe, 2004). Their use is controversial because the lack of proved efficacy, the reports of cytotoxicity and the risk of antibiotic-resistance induction. Regardless of the decision to initiate topical antimicrobial therapeutics, general consensus exist about the fact that a protracted courses of antibiotics may inhibit wound healing and promote the development of resistant organisms (O'Meara, Cullum, Majid & Sheldon, 2001; Mendes, 2014). Antiseptics may be preferable to topical antibiotics because of decreased rates of bacterial resistance and contact susceptibility (Richmond, Vivas & Kirsner, 2013).

Sometimes, if all these treatment protocols fail or cannot be considered, amputation is the only possible option rendering novel approaches a major necessity.

1.5.1.3 Advanced therapeutics

Advanced therapeutics for DFU treatment are also available. Hyperbaric oxygen therapy (HBOT) has shown promise in the treatment of serious cases of non-healing DFU, which are resistant to other therapeutical methods, but does not substitute antibiotic therapy, local humid therapy or surgical wound debridement (Barnes, 2006). It involves intermittent administration of 100% oxygen, usually in daily sessions, but has a limited availability and is expensive. This treatment aims to increase oxygen delivery to ischemic tissues, which may help fight infection and improve wound healing in the high-risk foot (Lipsky, 2004b).

Another technique reported to stimulate DFU healing is Electrical Stimulation (ES). It can improve common deficiencies that have been associated with faulty wound healing in DFU, such as poor blood flow, infection and deficient cellular response (Thakral et al., 2013). ES also has an antibacterial effect by stimulating growth factors and collagen synthesis (Santamato et al., 2012). This therapy is safe, inexpensive and easy to perform.

Negative pressure wound therapy (NPWT) is a non-invasive wound closure system that uses controlled and localized negative pressure to help heal chronic and acute wounds. While available evidences for the efficacy of NPWT in DFU patient treatment are promising, this method does not replace but is used in association with surgical wound debridement to improve blood circulation in all DFU patients (Vikatmaa, Juutilainen, Kuukasjärvi & Malmivaara, 2008).

Bio-engineered skin (BES) has been used during the last decades as a new therapeutic method to treat DFU. This method replaces the degraded extra cellular matrix, introducing a new one with cellular components that allow for a new healing trajectory. Currently, many kinds of BES products are approved in the United States and are available for DFU treatment, such as Dermagraft, Apligraf and TheraSkin (Yazdanpanah et al., 2015). This method cannot be used in infected diabetic foot and requires surgical revascularization and decompression (Marston, Hanft, Norwood & Pollak, 2003). BES with stem cells has been applied to diabetic animals allowing earlier abundant neo-vessel formation and better tissue remodelling; however, the clinician must be aware that the majority of biological products present high protein levels that may be very susceptible to the inflammatory enzymatic activity in a wound. Therefore, controlling high levels of inflammation is as critical as debridement in promoting wound healing (Mulder et al., 2014).

Low-level light therapy (LLLT) uses either low-level, low-power lasers or light-emitting diodes (LED) to alter cellular function and molecular pathways. The biologic mechanism of LLLT resides in the absorption of light by photo-acceptors or chromophores at the molecular, cellular, and tissue levels, resulting in cellular changes such as synthesis of collagen and extracellular matrix, recruitment of cytokines and growth factors, migration, proliferation and differentiation of cells (Tchanque-Fossuo et al., 2016). The evidence is limited but LLLT may be a promising treatment for DFU.

Since diabetes is associated with immunological deficiencies that help to explain the clinical failure of treatments, several investigators have sought adjunctive therapies for treating DFI. Two types of growth factors have been used in clinical practice of diabetic patients: the platelet-derived growth factors and the Granulocyte-Colony Stimulating Factor (G-CSF) (Frykberg & Banks, 2015). The recombinant human platelet derived growth factor (rhPDGF), becaplermin (brand name Regranex), produced by the yeast *Saccharomyces cerevisiae*, remains the only growth factor approved by the US Food and Drug Administration (FDA) for application in chronic diabetic wounds (Barrientos, Stojadinovic, Golinko, Brem & Tomic-Canic, 2008). Available as a topical gel, the clinical use of rhPDGF remains limited because of its high cost and uncertain patient-specific clinical benefits. Some studies have even indicated that it would be biologically possible that topical administration of rhPDGF could promote cancer (Richmond et al., 2013; Mulder et al., 2014).

G-CSF is an endogenous haematopoietic growth factor that improves the function of both normal and defective neutrophils in people with diabetes (Nelson et al., 2000). Its purified cloned recombinant form, usually injected, is commercially approved and has been used to treat various difficult infectious problems. The available evidence regarding adjunctive G-CSF treatment in people with a DFI is limited; it does not appear to influence infection resolution and healing but it seems to reduce the need for surgical interventions, especially amputations, and the duration of hospitalisation. Therefore, it should be considered as a complement to appropriate care for DFI (Cruciani, Lipsky, Mengoli & de Lalla, 2013).

In summary, HBOT, NPWT and G-CSF can be included in the management of infected DFU, although the other advanced therapies available can only be applied in non-infected DFU.

1.5.2. Bacteriophage therapy

1.5.2.1. Bacteriophages: general features and life cycle

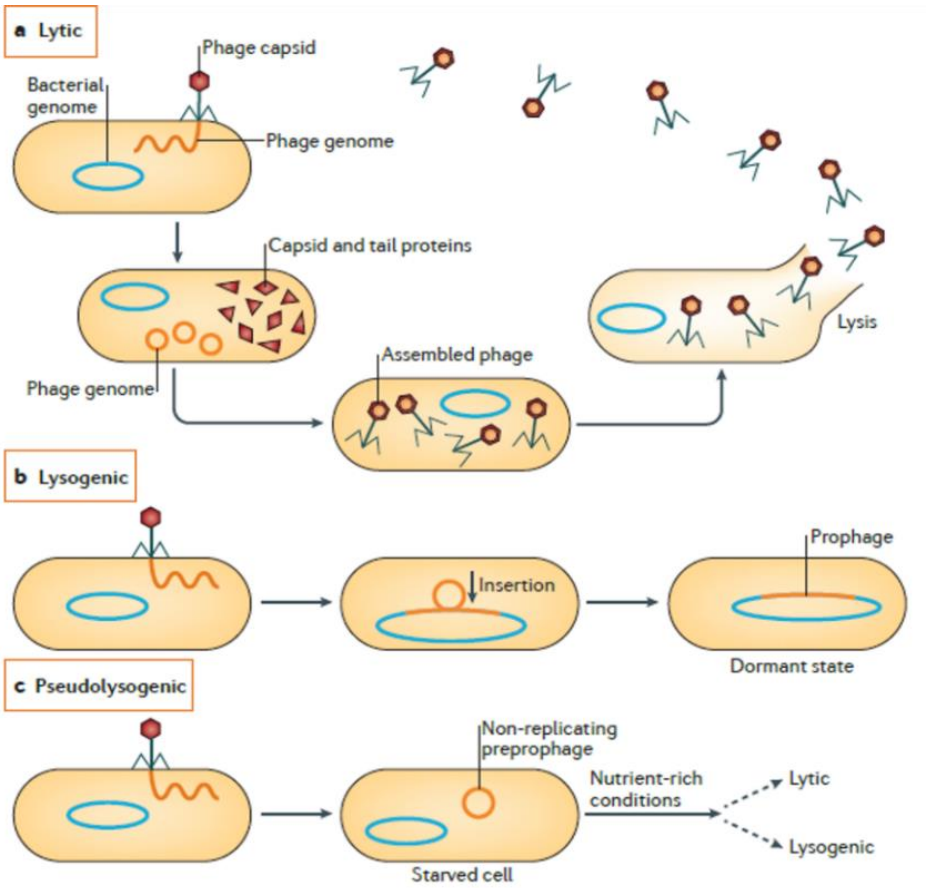
Bacteriophages are viruses that specifically infect bacteria, consisting of a DNA or RNA genome contained within a protein coat. In 2011, Alexander Sulakvelidze defined bacteriophages as “the most ubiquitous organisms on Earth, playing a significant role in maintaining microbial balance on this planet” (Sulakvelidze, 2011). Indeed, bacteriophages, or phages, are present everywhere together with their bacterial host, and play key roles in regulating the microbial balance in every ecosystem. The discovery of phages was achieved by two microbiologists, independently: in 1915 by the British Felix Twort and in 1917 by the French-Canadian Felix d’Hérelle; both investigated the nature of bacteriophages and explored their ability to function as therapeutic agents (Wittebole, De Roock & Opal, 2013).

The International Committee for Taxonomy of Viruses (ICTV), responsible for phage classification, actually considers 14 phage families, 11 of which are not grouped in a superior taxonomical category, while the other three are included in the order *Caudovirales.* This order

includes the most known bacteriophages, with DNA genomes, a regular symmetric capsid head and a symmetric helical DNA injection tail. The tail morphology allows the definition of the three *Caudovirales* families: *Myoviridae* (long contractile tail), *Syphoviridae* (long non-contractile tail) and *Podoviridae* (short tail) (Ackermann, 2009).

Phages are obligate parasites that can sustain two types of replication cycle, lytic and lysogenic, defined by their genetic interaction with the bacterial host. Upon infection, lytic phages immediately enter a productive cycle, in which the phage genome is replicated and phage capsid and tail proteins are synthesized using bacterial cell machineries; the phage genome is then packaged into progeny phage particles, which are liberated via bacterial lysis (Figure 10). By contrast, temperate phages can enter a lysogenic cycle, during which the phage genome is integrated into bacterial chromosome becoming a prophage, persisting in a dormant state until, at some point, phage genes are activated and phage virions are produced, eventually lysing the host cell and releasing progeny phages (Figure 10).

Figure 10. The phage replication cycles. Schematic of lytic, lysogenic and pseudolysogenic cycles. Adapted from Feiner et al. (2015).



On the other hand, virulent or lytic phages do not enter into a prophage state and immediately begin viral reproduction upon infection, resulting in rapid destruction of their host cell within minutes to hours (Burrowes, Harper, Anderson, McConville & Enright, 2011). Another less common phage replication cycle is pseudolysogeny, an unstable situation in which the phage genome fails to replicate as lytic or to become a prophage. This occurs most frequently under nutrient-deprived conditions, when bacterial cells cannot support DNA replication or protein synthesis, and persists until nutrition is restored (Feiner et al., 2015) (Figure 10).

The lysogenic process can promote gene transfer, which disseminates toxin proteins or pathogen factors among bacterial species, including antibiotic resistance genes, representing a selective advantage for bacterial host; however, the same process could be exploited therapeutically by using phages to transfer genes aiming at rendering bacteria more susceptible to some antibiotics (Wittebole et al., 2013).

1.5.2.2. Pros and cons of bacteriophage therapy

Phages effectively constitute a powerful tool to fight resistant infections and they can be used in combination with antibiotics for improved performance (Pires, Vilas Boas, Sillankorva & Azeredo, 2015).

Bacteriophage therapy (BT) is the use of lytic bacteriophages to reduce or eliminate pathogenic bacteria (Mendes et al., 2014). Bacteriophages are natural antibacterial organisms able to regulate bacterial populations by the induction of bacterial lysis; they are highly numerous, usually readily isolated and active against Gram-positive as well as Gram-negative bacteria, including MDR pathogens (Matsuzaki et al., 2003; Wang et al., 2006). Due to its specificity, phage therapy action is directed to one single species or even strain, narrowing the collateral damages of antibiotic therapy and allowing the preservation of the existing microbiome. Some phages, however, exhibit broad host ranges and are able to infect a large subset of a given species or even multiple species (Chen & Novick, 2009). Another advantage of BT is that phages replicate at the site of infection, ensuring their safety and absence of side effects, even after their wide distribution upon systemic administration (Haq, Chaudhry, Akhtar, Andleeb & Qadri, 2012). Unlike antibiotics, phages do not necessarily need to be delivered to the site of infection in high concentrations; *in situ* replication leads to far higher numbers of phages exactly where they are needed (Burrowes et al., 2011). One of the major benefits of phage therapy is that antibiotic resistance and phage resistance are largely unrelated phenomena, so bacterial strains that are resistant to antibiotics can nevertheless be susceptible to phage infection. Another pro of BT include the easy manipulation of phages by several techniques. Finally, the economic aspects of phage therapy look promising because phage production is simple and relatively inexpensive (Haq et al., 2012; Wittebole et al., 2013).

The most serious concern regarding BT is the fact that phages are not always lytic, being dependent on host growth and infection temperatures, which make phage therapy use limited to optimal conditions (Haq et al., 2012). There is no guarantee that lytic phages under laboratory conditions remain lytic *in vivo*, and if the conversion occurs, bacterium containing prophage can be immunized against the corresponding lytic phage and can encode or mobilize bacterial virulence factors (Ghannad & Mohammadi, 2012). For this reason, only exclusively virulent lytic phages are generally considered clinically useful and the presence of temperate bacteriophages must be strictly excluded.

Another con to be considered is the large-scale lysis of bacteria, especially Gram-negative species, may lead to the release of toxic cellular components, namely endotoxins, producing an autoimmune response (Herxheimer effect). This phenomenon was observed regarding some bactericidal antibiotics when used to treat severe bacterial infections but has not yet been reported with bacteriophages. It seems that the sequential multiplication of bacteriophages produces a longer duration of bacteriolytic effect, reducing the effects of such release (Rac, Greer & Wendel, 2010). However, this is an area to monitor in phage therapy clinical trials and more detailed studies are necessary.

Another problem to be considered in phage therapy *in vivo* is the promotion of strong antibody response, which could clear phages more quickly, impairing their use for extended periods of time (Clark & March, 2006). Since bacteriophages are viruses, they can be rapidly eliminated from the systemic circulation by both humoral and innate immunity, leading to the production of anti-phage antibodies and a decreased efficacy in the case of prolonged or repeated applications (Dabrowska, Switała-Jelen, Opolski, Weber-Dabrowska & Gorski, 2005).

Bacteria have evolved adaptive mechanisms protecting them from phages and they can inhibit the phage cycle by several resistance mechanisms (Drulis-kawa, Majkowska-skrobek, Maciejewska, Delattre & Lavigne, 2012). The most common resistance mechanism to phage infection is the prevention of phage adsorption through the lack of a bacterial receptor, which blocks phage adsorption on the bacterial surface, resulting in a complete loss of ability to generate virus progeny; the phage adsorption can also be avoided by the production of extracellular matrix or of competitive inhibitors, rendering the receptors unavailable to phages (Labrie, Samson & Moineau, 2010).

Another well-studied phage defence mechanisms include the Restriction-Modification (RM) defence system and the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) that cause degradation of phage DNA (Makarova, Brouns, Horvath, Sas & Wolf, 2012). If a phage enters the host cell and avoids restriction by the host RM and CRISPR systems, it proceeds to develop, replicate and release its progeny; the interruption of phage development in any of these stages is called abortive infection (Abi), promoting host mechanisms that lead to cell death and prevent the spread of infectious particles (Stern & Sorek, 2012). Finally, Superinfection exclusion (Sie) systems are proteins that block the entry

of phage DNA into host cells, thereby conferring immunity against specific phages (Labrie et al., 2010).

Thus far, the frequency of *in vivo* resistance to phage therapy is reportedly low as opposed to the observed in *in vitro* resistance testing; furthermore, unlike antibiotics, the isolation of novel active phages from environmental sources or progressive adaptation of viral parasite to resistant host population are possible, so the resolution of the anti-phage resistance problem is relatively fast and easy to achieve (Wittebole et al., 2013).

1.5.2.3. Bacteriophage therapy over time

The use of bacteriophages as antibacterial agents for suppurated infections treatment began shortly after the discovery of bacteriophages. Bruynoghe and Maisin first applied BT to treat *S. aureus* skin infections in 1921 (Wittebole et al., 2013), but in fact phage therapy started back in 1896, when Ernest Hankin demonstrated that the waters from the Indian rivers Ganga and Yamuna contained a biological principle that destroyed cultures of cholera-inducing bacteria (Haq et al., 2012). In the 1930s, after the introduction of antibiotics, the enthusiasm for phage therapy declined and BT was abandoned in western countries, but continued to develop within the Soviet Union and eastern Europe, where it is still practised today and where a large number of reports are available, mainly in Poland and Georgia (Lu & Collins, 2007). Phage therapy was “rediscovered” by 1980s, starting with the work of Smith and Huggins. However, the western phage therapy “renaissance” only gained strength in the 1990s. By the year 2000 started a progression in genomics and broad ecology-based phage research that continues to this day (Smith, Huggins & Shaw, 1987; Abedon, Kuhl, Blasdel & Kutter, 2011).

Despite bacteriophages being one of the best-studied microbes known to science, there is a lack of formal and large-scale clinical studies on their safety and efficacy as therapeutic agents. The FDA and the European Medicines Agency (EMA) are actively working towards this goal, using the Biological Medicinal Products guidelines for European trials, and the guidelines of the Division of Vaccines and Related Product Applications in the USA trials (Wittebole et al., 2013). Specific phage guidelines and standardised protocols to evaluate safety and efficacy of bacteriophage products are necessary because individual countries have their own regulations, which levels can be highly variable. Detailed molecular characterization of the bacteriophage genome is also mandatory to exclude the presence of any toxin or antibiotic-resistance genes.

The problem is that phage therapeutics do not fit into a single category and they are actually classified as biological human medicinal products (Parracho et al., 2012). The current regulatory regime for human biological medicinal products implies the conduct of clinical trials and the submission of a full product dossier compliant with directive 2001/83/EG (Huys et al., 2013). To address these issues, a European organization, the Phages for Human Applications

Group Europe (P.H.A.G.E.), was created for the promotion of research and clinical trials using BT.

As mentioned, in the last few years the emergence of antibiotic resistant bacteria and lack of effective novel antibiotics have revived interest in bacteriophage therapeutics as an approach to controlling bacterial infections (Knezevic & Petrovic, 2008). The first bacteriophage-based product formally approved by the United States government regulatory agency was AgriPhageTM, in 2005, to treat crop diseases. A few years later was approved the first food safety-related bacteriophage product, ListShield™, a phage cocktail that targets *Listeria monocytogenes* contaminants on ready-to-eat foods containing meat and poultry products (Parracho et al., 2012).

Although bacteriophages represent a serious threat to the dairy industry, causing losses in the production of cheese and fermented products, they are also seen as positive agents, replacing antibiotics in the control of pathogens with two main applications: preventing food contamination and/or treating bacterial infections in animals or plants. There has been an increase in the number of patents regarding the application of phages in this field, with a number of products becoming available (Maura & Debarbieux, 2011). Strategies targeting single pathogens, such as *Listeria* in cheese production and *E. coli* in the meat industry are currently being developed, and bacteriophages active against three major human pathogens *E. coli* O157:H7, *Salmonella* and *Campylobacter*, have been identified and are currently being used in experimental treatments (Goodridge & Bisha, 2011; Hungaro, Mendonça, Gouvêa, Vanetti & Pinto, 2013).

Regarding the Polish and Georgian historical experiences related to the topical and systemic application of BT in wounds, the set of studies by Slopek et al., involving 550 patients, constitutes to date the most detailed documentation of phage application for the treatment of human infections. The authors used bacteriophage-soaked compresses applied in localized infections, in addition to oral BT, with a treatment success of about 92%. It is important to point out that BT is actually a primary tool integrated in the successful treatment of multi-resistant infections in major tertiary care centres of Georgia (Mendes, 2014).

Despite the hundreds of phage therapy-related publications in the last decade, no phage preparation has been approved for market authorization. A very complete microbiological and molecular characterization of a phage for *S. aureus* control, *Staphylococcus aureus* ISP, has been published, contributing to the further development of phage therapeutic applications (Vandersteegen et al., 2011). A first phase I randomized controlled trial conducted in the United States was published in 2009, which evaluated the safety of a phage cocktail directed against *E. coli*, *S. aureus* and *P. aeruginosa* in 42 patients with chronic venous leg ulcers. The study demonstrated BT to be safe and effective in managing wounds but it has to be complemented by a phase 2 efficacy study because the posology used (allowed by the FDA) did not agree with existing data on bacteriophage pharmacokinetics (Rhoads et al., 2009). A

project funded by the European Commission under the 7th Framework Programme for Research and Development, the Phagoburn, was launched on June 1st 2013 and will last 45 months. The project aims is to evaluate the ability of phage therapy for the treatment of burn wounds infected with *Escherichia coli* and *Pseudomonas aeruginosa,* and is currently running the implementation of a phase I-II clinical trial (http://www.phagoburn.eu/).

1.5.2.4. Bacteriophage therapy and biofilm

Although the application of phages against antibiotic resistant bacteria have been largely described in veterinary and medical applications, there are few studies on the interaction of phages with biofilm (Sillankorva, Oliveira, Vieira, Sutherland & Azeredo, 2004). Many studies describe phage infection of cells, but most of them only consider planktonic bacteria, and bacteria attached to surfaces present very different characteristics. Considering that phage infection and replication cycle are generally strongly dependent on the growth stage of the host bacterium, the treatment of slowly growing cells in biofilms is a challenge. Some studies have already studied the application of phages to eradicate biofilm-forming bacteria (Doolittle, Cooney & Caldwell, 1996; Hibma, Jassim & Griffiths, 1997; Hughes, Sutherland, Jones & Rutherford, 1998; Merril, Scholl & Adhya, 2003), but more understanding of phage action in biofilm is still required (Tait, Skillman & Sutherland, 2002; Curtin & Donlan, 2006; Cerca, Oliveira & Azeredo, 2007).

Infected chronic wounds constitute one of the best models for the application of BT, and DFI represents a good target for BT due to some intrinsic adverse characteristics that include poor vascularization and the presence of biofilm-associated infections. BT application for DFI treatment has been used in both Europe and the United States (Mendes, 2014), but it is not an established treatment protocol in the western world, rendering studies proving its efficacy urgent to revert this trend.

1.6. Thesis objectives and scope

The objectives proposed in this thesis aimed to complement the objectives established for the research project “Biofilms in diabetic foot: microbial virulence characterization and cross-talk of major isolates” (PTDC/SAL-MIC/122816/2010), focusing the study on virulence characterization of the main bacterial genera involved in diabetic foot infections. The isolates under study were obtained from diabetic patients with clinically infected foot ulcers.

The main objectives of this research project were:

- To evaluate the influence of polymicrobial communities in the ability of DFU isolates to produce biofilm after evaluating their individual biofilm-forming ability. The isolates belonged to several bacterial genera, including *Staphylococcus*, *Corynebacterium*, *Enterococcus*, *Pseudomonas* and *Acinetobacter* **(Chapter II)**.
- To characterize the staphylococci isolated from diabetic foot ulcers, regarding their genotype, virulence and antimicrobial resistance profiles **(Chapter III)**.
- To analyse the antimicrobial susceptibility patterns of biofilm-producing *S. aureus* strains isolated from diabetic foot infections, by determination of MIC, MBIC and MBEC, followed by identification of genetic determinants of biofilm production and antimicrobial resistance **(Chapter IV)**.
- To perform a characterization of enterococci isolated from diabetic foot ulcers by evaluation using macrorestriction analysis, and screening for virulence traits and antimicrobial resistance **(Chapter V)**.
- To evaluate the antimicrobial activity and wound-healing potential, of five previously characterized bacteriophages, against biofilms formed by *S. aureus*, *P. aeruginosa* and *A. baumannii* isolated from chronic DFU infections **(Chapter VI)**.

This thesis provides relevant information regarding DFU bacterial strains in Portugal, contributing to the important and innovative characterization of the microbiological isolates, but also to the investigation of a possible new strategy for the treatment of clinically relevant resistant diabetic foot infections. These data intend to be a strong contribution to the development of new clinical approaches for the treatment of DFU infections.

CHAPTER II

Polymicrobial biofilms by diabetic foot clinical isolates

2.1. Polymicrobial biofilms by diabetic foot clinical isolates

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* The author participated in the conception and design of the study, conducted the experiments, performed the data analysis process and drafted the manuscript.

Abstract

Background: Diabetes *mellitus* is a major chronic disease that continues to increase significantly. One of the most important and costly complications of diabetes is foot ulceration that may be colonized by pathogenic and antimicrobial resistant bacteria, which may express several virulence factors that could impair treatment success. These bacterial communities can be organized in polymicrobial biofilms, which may be responsible for diabetic foot ulcer (DFU) chronicity.

Methods: We evaluated the influence of polymicrobial communities in the ability of DFU isolates to produce biofilm, using a microtiter plate assay and a multiplex fluorescent *in situ* hybridization, at three time points (24, 48, 72h), after evaluating biofilm formation by 95 DFU isolates belonging to several bacterial genera (*Staphylococcus*, *Corynebacterium*, *Enterococcus*, *Pseudomonas* and *Acinetobacter*).

Results: All isolates were biofilm positive at 24 h, and the amount of biofilm produced increased with incubation time. *Pseudomonas* presented the higher biofilm production, followed by *Corynebacterium*, *Acinetobacter*, *Staphylococcus* and *Enterococcus*. Significant differences were found in biofilm formation between the three time points. Polymicrobial communities produced higher biofilm values than individual species. *Pseudomonas+Enterococcus*, *Acinetobacter+Staphylococcus* and *Corynebacterium+Staphylococcus* produced higher biofilm than the ones formed by *E. faecalis+Staphylococcus* and *E. faecalis+Corynebacterium*.

Conclusions: Synergy between bacteria present in dual or multispecies biofilms has been described, and this work represents the first report on time course of biofilm formation by polymicrobial communities from DFU including several species. The biological behavior of different bacterial species in polymicrobial biofilms has important clinical implications for the successful treatment of these infections.

Keywords: Diabetic foot infection, Biofilm, Polymycrobial communities, Microtiter plate assay, Fluorescent *in situ* hybridization.

Background

Diabetes *mellitus*, one of the most prevalent chronic diseases worldwide, continues to increase significantly. Recent estimates indicate that in 2030, there will be 347 million diabetic patients throughout the world and that diabetes will be the seventh leading cause of death (WHO Geneva, 2011). Foot ulceration is one of the major complications of diabetes, which occurs in approximately 25% of the diabetic patients. In fact, infected diabetic foot ulcers (DFU) are responsible for 60% of non-traumatic lower limb amputations (Bowling et al. 2009).

There is no consensus about the causes of DFU, but most studies state that they are usually related with impairments in innate immunity and with the microbiocidal activity of polymorphonuclear cells, which are more evident in the presence of hyperglycemia (Richard, Lavigne & Sotto, 2012). DFU may be colonized by pathogenic and antimicrobial resistant bacteria, which could influence treatment outcome. In fact, Xu et al. (2007) have shown that a high bacterial load was associated with an inferior rate of wound healing. These bacteria may express several virulence factors, such as biofilm formation, characterized by organized polymicrobial bacterial populations which may be responsible for DFU chronicity (Dowd et al. 2008; Donlan and Costerton 2002). In fact, biofilms are highly resistant to many traditional therapeutic protocols that generally target the individual causative agents without considering the synergies occurring in polymicrobial communities (Dowd et al. 2008; Brogden et al. 2005). More recently, James et al. (2008) reported that 60% of chronic wounds exhibited biofilms as opposed to 6% of acute wounds.

Since DFUs are colonized by several bacterial species, the antimicrobial therapy protocols selected should cover a variety of synergistic microorganisms and not simply target specific pathogens that are frequently perceived to be the causative agents. A previous study conducted by our research team showed that 83.7% of patients with DFU presented polymicrobial infections, with a rate of isolation of 3.0±1.4 bacteria per patient (Mendes et al. 2012). Most isolates from acute infections were aerobic Gram-positive cocci, while more complex bacterial communities were isolated from chronic wounds, including Gram-negative and anaerobic bacteria (Mendes et al. 2012).

In spite of the increasing research efforts on DFU bacteria, only a few studies on biofilm formation by DFU isolates are available, which include a small number of different bacterial species.We aimed to evaluate if the presence of polymicrobial communities in the same DFU affected isolates' ability to produce biofilm, after screening biofilm production by 95 DFU isolates belonging to several bacterial genera: *Staphylococcus*, *Corynebacterium*, *Enterococcus*, *Pseudomonas* and *Acinetobacter*.

Methods

Bacterial isolates

Bacterial isolates tested belong to a larger bacterial collection obtained from 55 DFU clinical samples (including aspirates, n=6; biopsies, n=14; swabs, n=35) using conventional microbiological procedures as previously described by Mendes et al. (2012). Isolates were identified through their biochemical profile (API System, BioMérieux) and kept frozen at -80 °C until further processing.

From the 95 isolates tested, 57 were identified as *Staphylococcus*: *S. aureus* (n=31); *S. intermedius* (n=1); *S. epidermidis* (n=9); *S. haemolyticus* (n=3); *S. capitis* (n=2); *S. lugdunensis* (n=2); *S. caprae* (n=1); *S. chromogenes* (n=1); *S. saprophyticus* (n=1); *S. schleiferi* (n=1); *S. simulans* (n=1); S. hominis (n=1); *S.* sp. (n=3); 15 as *Corynebacterium*: *C. striatum* (n=8); *C. striatum/amycolatum* (n=4); *C. accolens* (n=1); *C. auris* (n=1); *C. macginleyi* (n=1); *C.* sp. (n=1); 12 as *Enterococcus* (*E. faecalis* n=10; *E. faecium* n=2); seven as *Pseudomonas* and four as *Acinetobacter baumannii/calcoaceticus*.

Isolates biofilm-forming ability was evaluated in pure cultures and in cocultures formed by clinical isolates obtained from the same DFU sample. Therefore, polymicrobial communities tested (n=34) included the following combinations: *Corynebacterium*+*Staphylococcus* (n=14); *Enterococcus faecalis*+*Staphylococcus* (n=7); *Pseudomonas aeruginosa*+*Enterococcus* (n=5); *Acinetobacter*+*Staphylococcus* (n=4); *Enterococcus faecalis*+*Corynebacterium* (n=4) (Table 1).

Evaluation of biofilm formation by a microtiter biofilm assay

Assays were performed using flat-bottom, polystyrene, microtiter plates (Orange Scientific, Belgium) containing 100 µL of bacterial suspensions in Mueller Hinton Broth (MHB) (Liofilchem, Italy), with a concentration of 5 x 10^5 CFU/mL for each bacteria. According to the protocol described by Pettit et al. (2005), plates were incubated in a humid chamber at 37°C for 24, 48, and 72h; after each time point, 5 µL of resazurin was added to each well (Alamar Blue, AB, ThermoScientific, Spain), and the plates were incubated for 1h at 37°C. Their absorbance (A) values at 570 and 600 nm were registered. Absorbance cutoff (Ac) was defined as three standard deviations above the mean A of the negative control, and isolates were classified as nonbiofilm producers if $A \leq Ac$; as weak biofilm producers if $Ac < A \leq 2 \times Ac$; as moderate biofilm producers if $2 \times Ac < A \leq 4 \times Ac$; and as strong biofilm producers if $A > 4 \times Ac$ (Stepanović, Cirković, Ranin & Svabić-Vlahović, 2004). Assays were performed in triplicate, repeated in different occasions, and results were averaged.

Table 1. Composition of polymicrobial communities tested by a microtiter biofilm assay and by MFISH.

Polymicrobial communities composition	Nº
Corynebacterium + *Staphylococcus*	14
C. striatum + *S. aureus*	7
C. spp. + *S. aureus*	2
C. spp. + *Staphylococcus* spp.	1
C. striatum/amycolatum + *S. caprae*	1
C. striatum/amycolatum + *S. aureus*	1
C. striatum/amycolatum + *S. haemolyticus*	1
C. auris + *S. haemolyticus*	1
Enterococcus + *Staphylococcus*	7
E. faecalis + *S. aureus*	3
E. faecalis + *S. epidermidis*	1
E. faecalis + *S. hominis*	1
E. faecalis + *S. capitis*	1
E. faecalis + *Staphylococcus* sp.	1
Pseudomonas + *Enterococcus*	5
P. aeruginosa + *E. faecalis*	3
P. aeruginosa + *E. faecium*	2
Acinetobacter + *Staphylococcus*	4
A. baumannii/calcoaceticus + *S. aureus*	3
A. baumannii/calcoaceticus + *S. epidermidis*	1
Enterococcus + *Corynebacterium*	4
E. faecalis + *C. striatum*	3
E. faecalis + *C. macginleyi*	1
Total	34

Evaluation of biofilm formation by a microtiter biofilm assay

Assays were performed using flat-bottom, polystyrene, microtiter plates (Orange Scientific, Belgium) containing 100 µL of bacterial suspensions in Mueller Hinton Broth (MHB) (Liofilchem, Italy), with a concentration of $5x10^5$ CFU/mL for each bacteria. According to the protocol described by Pettit et al. (2005), plates were incubated in a humid chamber at 37°C for 24, 48 and 72 h; after each time point, 5 µl of resazurin was added to each well (Alamar Blue, AB, ThermoScientific, Spain) and the plates were incubated for 1h at 37°C. Their

Absorbance (A) values at 570 nm and 600 nm were registered. Absorbance cut-off (Ac) was defined as three standard deviations above the mean A of the negative control and isolates were classified as non-biofilm producers if $A \leq Ac$; as weak biofilm producers if $Ac < A \leq 2 \times Ac$; as moderate biofilm producers if $2 \times Ac < A \leq 4 \times Ac$; and as strong biofilm producers if $A > 4 \times Ac$ (Stepanović et al. 2004). Assays were performed in triplicate, repeated in different occasions and results were averaged.

Biofilm detection by Multiplex Fluorescent *In Situ* Hybridization (MFISH)

Biofilm production by dual-species communities was confirmed using a Multiplex Fluorescent *in situ* Hybridization (MFISH) protocol adapted from Oliveira et al. (2007).

Overnight bacterial suspensions from each isolate were obtained and polymicrobial suspensions (Table 1) with a 1:1 ratio were made and diluted at 1:40 in Tryptone Soya Broth (TSB, Liofilchem, Italy). Then, 10 µl of each polymicrobial suspension were placed in 10-well teflon slides (Heinz Herenz, Germany), used as hybridization supports, and incubated for 24, 48 and 72h, at 37°C, to allow biofilm formation. After incubation and air-drying, bacteria were fixed with a 4% paraformaldehyde (Sigma-Aldrich, USA) (w/v) solution in PBS (Sigma-Aldrich, USA) for 2h at room temperature. After fixation, bacteria were permeabilized with ethanol (Merck, Portugal) at 50, 80 and 96%, during 3 min at each concentration (Merck, Portugal); for the staphylococci isolates, permeabilization was achieved using lysostaphine 0.01 mg/mL (Sigma-Aldrich, USA) during 4 min at room temperature after ethanol serial incubations. Afterwards, 10 µl of hybridization buffer (0.9 mol/L NaCl, 20 mmol/L Tris–HCl, pH 7.2, 0.01% SDS), containing 5 ng/µl of a specific probe for each bacterium (Table 2), was added to each well.

Slides were incubated in a humid chamber (Omnislide Thermal Cycling Block, USA) at 45°C for 3h and then washed using a buffer solution (0.9 mol/L NaCl, 20 mmol/L Tris-HCl, pH 7.2, 0.1% SDS) at 45°C during 15 min. Afterwards, slides were mounted in Vectashield Mounting Medium (Vector Laboratories, United Kingdom) and immediately visualized by fluorescent microscopy in a Leica DMRA2 fluorescence microscope, equipped with a mercury lamp of 100W, an I3 filter for excitation between 450 and 490 nm and a N2.1 filter for excitation between 515 and 560 nm (Leica Microsystems Lda., Portugal).

All assays were performed in triplicate and repeated on different occasions.

Statistical analysis

Statistical analysis of the microtiter plate assay results was performed using the SPSS 19.0 software for Windows. To evaluate the significance of the increase in biofilm production with time by individual isolates and by the bacterial communities, Friedman test was applied. Post hoc analysis with Wilcoxon signed-rank test was conducted with a Bonferroni correction. The corrected P-value ≤ 0.017 was considered statistically significant for this test.

Results from different species and results from the dual-species communities, at each time point, were compared using Kruskal-Wallis test followed by Bonferroni correction. A corrected P-value ≤ 0.0125 was considered significant for this test.

Finally, to compare biofilm production by individual isolates and dual-species communities and to evaluate the significance of the increase in biofilm production with time by the dual-species communities, Friedman and Wilcoxon Signed Rank Test post-hoc with a Bonferroni correction were applied. The corrected P-value ≤ 0.025 was considered statistically significant for this test.

Table 2. Probes used in the Multiplex Fluorescent *In Situ* Hybridization (MFISH) protocol.

Probe	Target	Sequence	Fluorescently label
Sta	*Staphylococcus* spp.	5'-TCCTCCATATCTCTGCGC-3'; *E. coli* 697	5'-rhodamine
Sau	*S. aureus*	5'-GAAGCAAGCTTCTCGTCCG-3'; *E. coli* 69	5'-rhodamine
Eub 338	*Eubacteria*	5'-GCTGCCTCCCGTAGGAGT-3'; *E. coli* 338	5'-fluorescein
EFAECI	*E. faecium*	5'- AGCTCCCGGTGGAAAAAGAAG-3'; *E. coli* 1204	5'-rhodamine
EFAEC	*E. faecalis*	5'- TTATCCCCCTCTGATGGG-3'; *E. coli* 135	5'-rhodamine or 5'-fluorescein*

*The choice of the fluorescent label depends on the bacterial composition of the polymicrobial community tested

Results

Microtiter biofilm assay

All DFU isolates tested produced biofilm at 24h. *Pseudomonas* isolates were the higher biofilm producers, followed in descending order by *Corynebacterium*, *Acinetobacter*, *Staphylococcus* and *Enterococcus.* Significant differences were found in biofilm formation between different bacterial species (Kruskal-Wallis test, P ≤ 0.0125) (Figure 1), namely between the *Enterococcus* in comparison with *Staphylococcus* and *Pseudomonas* at 24 and 48h. At 72h, significant differences were found between *Enterococcus* when compared with *Staphylococcus* and *Acinetobacter*.

In staphylococci, biofilm production increased with incubation time, and differences between A values at three time points were statistically significant (Friedman test, P=0.000) (Figure 1). According to the classification of biofilm production introduced by Stepanović et al. (2004), at

24h all staphylococci isolates were weak biofilm producers and only at 72h a large percentage (84.2%) revealed a moderate ability to produce biofilm; no strong producers were observed at the three time points tested (Table 3).

Regarding *Corynebacterium*, biofilm production significantly increased from 24 to 48h (Friedman test P=0.014), decreasing afterwards (Figure 1). Moderate (66.7%) and strong (26.7%) biofilm producers were observed already at 24h. The percentage of moderate biofilm producers increased after a 48h incubation (86.7%), while the percentage of strong biofilm producers decreased (6.7%) (Table 3). At 72h, the percentage of moderate producer isolates decreased to 73.3%, while 13.3% of the isolates were able to produce strong biofilms (Table 3).

Biofilm formation by enterococci also increased with time (Figure 1). A significant difference was found between biofilm production at 24 and 72h (Friedman test P=0.017) (Figure 1). All the enterococci isolates were weak biofilm producers at the three time points (Table 3).

Figure 1. Time course of one-species biofilm formation (24, 48 and 72h). In staphylococci, differences between biofilm production at three time points were statistically significant (ANOVA repeated measures P<0.001); in *Corynebacterium*, biofilm production significantly increased from 24 to 48h (Friedman test P=0.014); in enterococci and *Acinetobacter*, a significant difference was found between biofilm production at 24 and 72h (Friedman test P=0.017 and P=0.034, respectively); in *Pseudomonas* no significant differences were found between the three time points (Friedman test P=0.565)

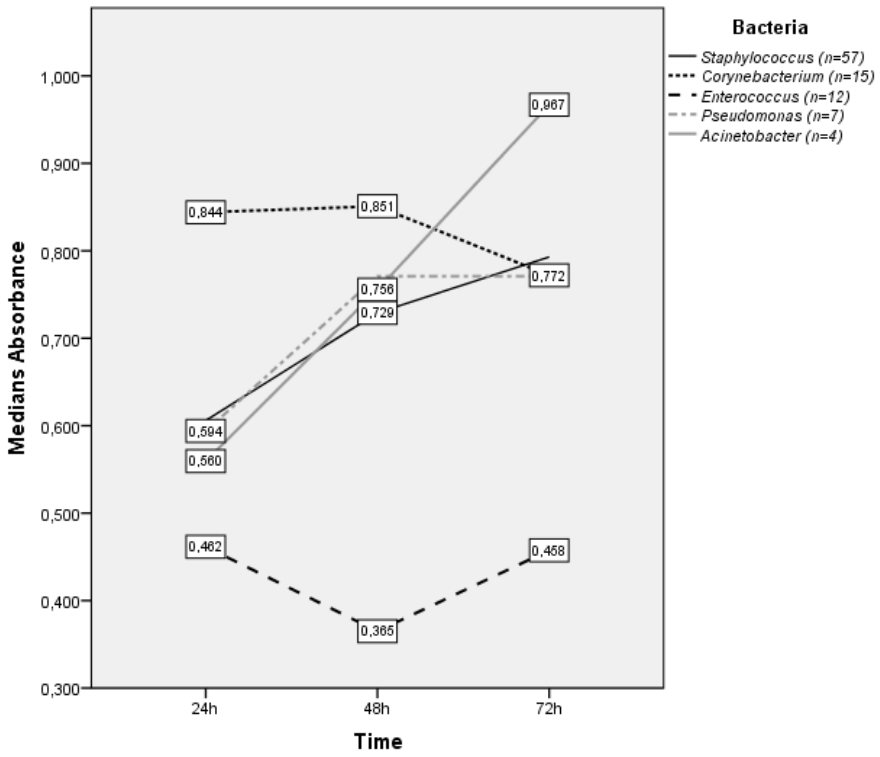
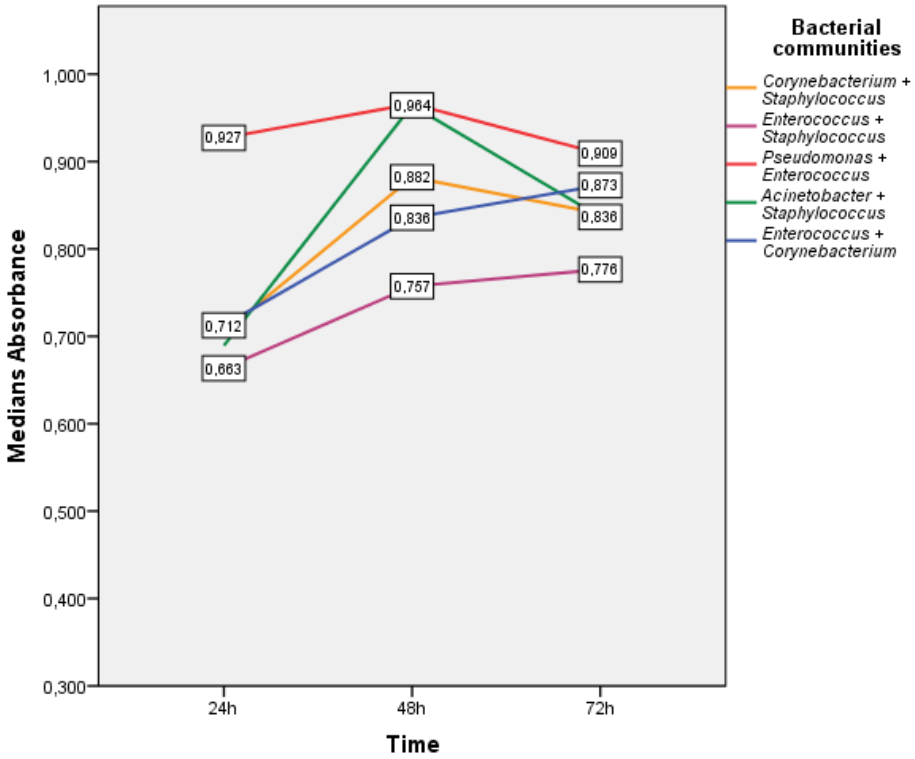


Table 3. Classification of biofilm production by the DFU clinical isolates, as determined by a microtiter biofilm assay.

Bacterial genera		Biofilm 24h			Biofilm 48h			Biofilm 72h			Total
		weak	moderate	strong	weak	moderate	strong	weak	moderate	strong	
Staphylococcus	n=	57	0	0	56	1	0	9	48	0	57
	%	100%			98.2%	1.8%		15.8%	84.2%		
Corynebacterium	n=	1	10	4	1	13	1	2	11	2	15
	%	6.7%	66.6%	26.7%	6.7%	86.7%	6.6%	13.3%	73.3%	13.3%	
Enterococcus	n=	12	0	0	12	0	0	12	0	0	12
	%	100%			100%			100%			
Pseudomonas	n=	0	5	2	0	6	1	0	6	1	7
	%		71.4%	28.6%		85.7%	14.3%		85.7%	14.3%	
Acinetobacter	n=	4	0	0	3	1	0	3	1	0	4
	%	100%			75%	25%		75%	25%		
Total	n=	74	15	6	72	21	2	26	66	3	95
	%	77.9%	15.8%	6.3%	75.8%	22.1%	2.1%	27.3%	69.5%	3.2%	

Figure 2. Time course of polymicrobial communities' biofilm formation (24, 48 and 72h).



Significant differences were found between biofilm production by the different communities from 24 to 48h (Friedman test P<0.05) but not from 48 to 72h (P>0.05)

For *Corynebacterium*+staphylococci (n=14), the increase in biofilm production observed in the polymicrobial community when compared with the production by the individual species was not significant at 24 and 72h, but significant at 48h for staphylococci (Friedman test, P < 0.001). No significant differences were found between *Acinetobacter*+staphylococci (n=4) and between the isolates individually (Table 4).

For *E. faecalis*+staphylococci (n=7), the increase in biofilm production observed in the dual-species community was significant when compared with the enterococci results at all three time points tested (P=0.005), but for staphylococci was only significant at 24h (P=0,023). Similar results were observed for *P. aeruginosa*+*Enterococcus* (n=5), in which significant differences were found only for enterococci at three time points (P < 0,025) (Table 4).

For *E. faecalis*+*Corynebacterium* (n=4), no significant differences were found between the increase in biofilm production observed in the polymicrobial community when compared with the production by the individual species (Table 4).

Regarding the evaluation of time course biofilm production by dual-species communities, significant differences were only found in *Corynebacterium*+staphylococci communities at three time points (Friedman test, P < 0.017).

Multiplex Fluorescent *In Situ* Hybridization (MFISH)

Heterogeneous multi-species biofilms were successfully detected with the MFISH protocol. For *Corynebacterium*+staphylococci, biofilm was composed mainly by staphylococci at 24 and 48h (Figure 3), while at 72h species distribution was homogeneous. Similar results were observed for *E. faecalis*+staphylococci, in which enterococci are more prevalent in the mixed biofilms at 72h, except when the dual-species community included *S. aureus*. In these cases, *S. aureus* was more prevalent at 72h. Regarding enterococci+*Pseudomonas*, *Pseudomonas* was predominant in biofilms produced with *E. faecium*, while in mixed biofilms with *E. faecalis* distribution was homogeneous. In *Acinetobacter*+staphylococci communities, *Staphylococcus* was predominant at 24 and 48h, but at 72h the dual-species biofilms presented a homogeneous composition (Figure 4). The same was observed for *Corynebacterium*+*E. faecalis* biofilms, where *Corynebacterium* predominated over *E. faecalis* at 24 and 48h.

Table 4. Comparison of biofilm production between the polymicrobial community and the individual species, using Friedman's test and Wilcoxon post-hoc with Bonferroni correction.

	Biofilm formation		
	24h	48h	72h
Combination 1 (n=14)			
Corynebacterium	NA	P=0.032	NA
Staphylococcus	NA	P=0.000*	NA
Corynebacterium + *Staphylococcus*	P=0.199	P=0.000*	P=0.058
Combination 2 (n=7)			
Enterococcus faecalis	P=0.023*	P=0.010*	P=0.023*
Staphylococcus	P=0.010*	P=1.000	P=1.000
E. faecalis + *Staphylococcus*	P=0.005*	P=0.005*	P=0.005*
Combination 3 (n=5)			
Pseudomonas aeruginosa	P=1.000	P=1.000	P=0.342
Enterococcus	P=0.034	P=0.034	P=0.005*
P. aeruginosa + *Enterococcus*	P=0.022*	P=0.022*	P=0.007*
Combination 4 (n=4)			
Acinetobacter	NA	P=0.040	NA
Staphylococcus	NA	P=0.231	NA
Acinetobacter + *Staphylococcus*	P=0.368	P=0.039	P=0.420
Combination 5 (n=4)			
Enterococcus faecalis	P=0.102	NA	NA
Corynebacterium	P=1.000	NA	NA
E. faecalis + *Corynebacterium*	P=0.050	P=0.105	P=0.223

* values with P-value <0.025 were considered statistical significant; NA=not applicable

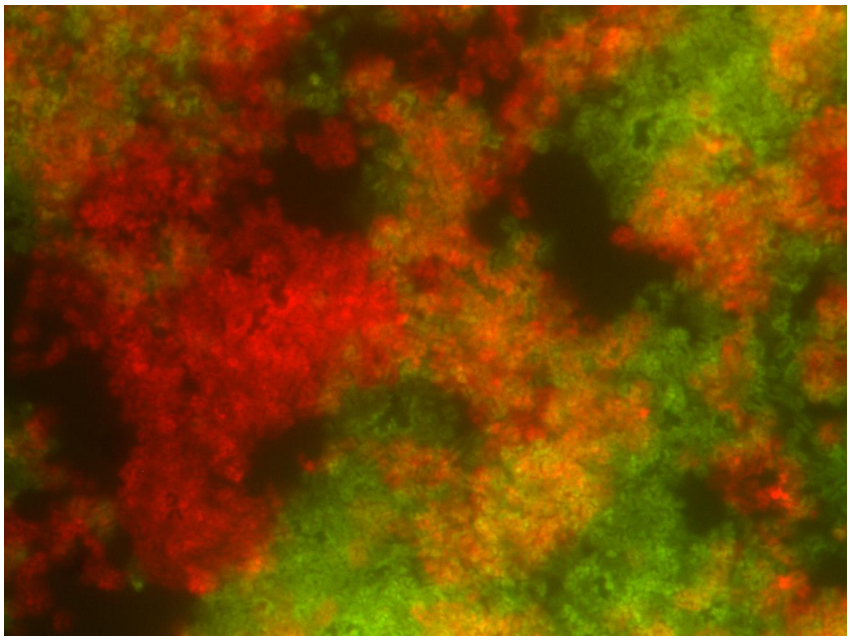
Discussion

The biological behavior of different species in polymicrobial communities has important clinical implications for the control and eradication of infections promoted by biofilm producing bacteria, and different approaches must be applied for better understanding these complex communities (Yang et al. 2011). Studies regarding the diversity of DFU's bacterial populations are scarce. However, clinicians and researchers are beginning to acknowledge the importance of multi-species biofilms as major contributors to the development of chronic infections such as these. To our knowledge, this work represents the first report on time course biofilm formation by polymicrobial communities from diabetic foot ulcers that include several species. Co-cultures tested represent the most frequent communities obtained from the same DFU sample in a previous study by our research team (Mendes et al. 2012).

Staphylococci are recognized as the most frequent cause of biofilm-associated infections; amongst these, *S. aureus* is described as the more persistent species, due to its virulence profile (Otto et al. 2008). A high percentage of biofilm producing DFU staphylococci was

detected at 24h, as already described by other authors (Malik et al. 2013; Swarna et al. 2012). In our study, biofilm formation by this bacterial species significantly increased with time, which points out for the importance of time course studies, as evaluation only after a 24h incubation may originate false-negative results.

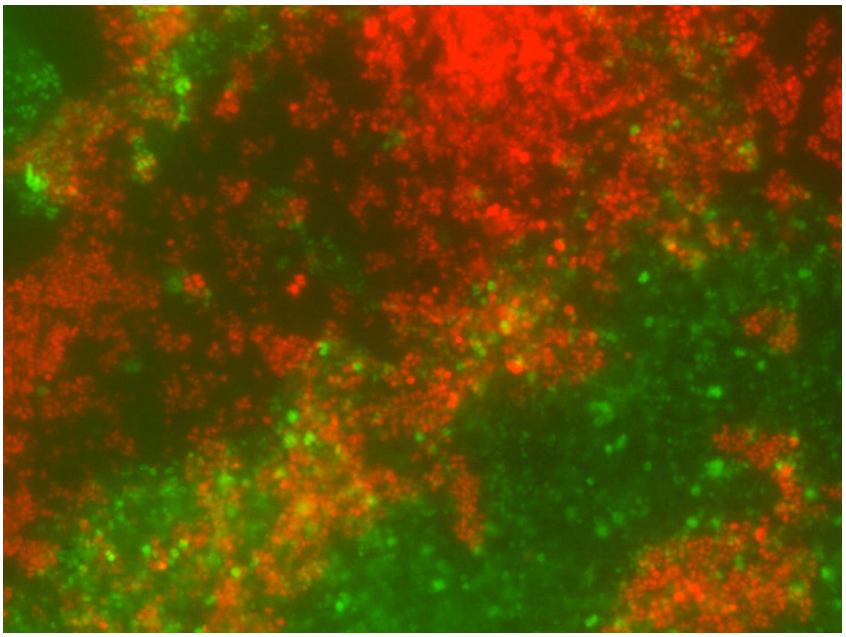
Figure 3. Polymicrobial biofilm formed by *Corynebacterium* (green) and *Staphylococcus* (red) after a 24h incubation (x1000; original).



Corynebacterium was the second major biofilm producer, with an increase from 24 to 48h. This bacterial genus is commonly considered as a contaminant, so studies concerning *Corynebacterium* are scarce. However, they appear to be frequent players in DFU as demonstrated by Bessman et al. (1992) and Dowd et al. (2008). This last author showed that, in DFU, *Corynebacterium* probably interacts with anaerobes to create a pathogenic group with a virulent potential equivalent to commonly noted pathogens such as *P. aeruginosa* or *S. aureus*.

Pseudomonas produced the higher biofilm values at 24h, which remained constant; in contrast, enterococci isolates produced the lower biofilm values, and were considered weak producers at all time points tested. *Pseudomonas* has been related with severe tissue damage in diabetic patients, being able to produce a variety of toxins and to resist to phagocytosis (Sivanmaliappan and Sevanan 2011). Enterococci were also weak biofilm producers. Biofilm formation by these isolates is dependent on multiple genetic factors and influenced by many environmental factors and signals (Mohamed and Huang 2007), and this multifactorial dependence may be responsible for the low values in biofilm production by *Enterococcus* observed in this study.

Figure 4. Polymicrobial biofilm formed by *Acinetobacter* (green) and *Staphylococcus* (red) after a 72h incubation (x1000; original).



Acinetobacter showed high biofilm forming ability at 24h. Although biofilm production by these bacteria increased with time, with a maximum at 72h, this increase was not significant, as observed for *Pseudomonas.* The presence of *A. baumannii* biofilm-producing isolates in DFU is a major cause for concern, since this species is able to survive under a wide range of environmental conditions and to persist for extended periods, being also extremely resistant to antimicrobial therapy (Espinal et al. 2012; Ibrahim et al. 2012). It would be important to evaluate more *Acinetobacter* DFU isolates since the number of tested was low.

Species synergy in polymicrobial biofilms has been previously reported but mainly focused on bacterial isolates from the oral cavity (Thornton et al. 2011). In this study all dual-species communities tested showed higher biofilm formation ability when compared with single species. The enhanced biofilm biomass formed by these communities may result from the earlier nutrient depletion that occurs in one-species biofilms (Burmølle et al. 2006). Differences between dual and single species biofilms were not significant, with the exception for *Enterococcus* and *Staphylococcus*, which may be due to the fact that these two bacterial genera showed the lowest biofilm production when analysed individually.

Interestingly, our results show that biofilm production within communities may be influenced by the specie or genera that are able to produce higher biofilm values. The significance in biofilm production by polymicrobial communities can be merely attributed to differences in biofilm formation, and the increase in absorbance to a cumulative effect rather than synergistic.

However, other interactions between bacteria may occur in polymicrobial communities that may lead to inhibition of growth and biofilm production, such as nutrient competition and toxic substances secretion. In fact, *P. aeruginosa* was reported to kill *Candida* in multi-species biofilms, due to virulence factors production (Yang et al. 2011). These phenomena may be responsible for the fact that the increase in biofilm production by the different communities analyzed in this study was not significant.

There were also differences between species belonging to the same genera when present in polymicrobial communities. For example, in enterococci the species with higher biofilm producing values was *E. faecalis.* These results were confirmed by direct observation using MFISH, and are in accordance with other studies, which already described that *E. faecalis* produces more biofilm than *E. faecium* (Mohamed et al. 2007).

Conclusions

Diabetic foot infections represent a significant burden for patients and the health care system. New research approaches are required to characterize these infections, which are mostly promoted by polymicrobial communities, frequently organized in biofilms, which may be responsible for resistance to therapeutics and for infection chronicity. The complexity of polymicrobial communities remains an inherent characteristic of these biofilms, and our results confirm that data regarding single-species biofilms cannot be extrapolated directly to multi-species communities.

CHAPTER III

Molecular typing, virulence traits and antimicrobial resistance of diabetic foot staphylococci

3.1. Molecular typing, virulence traits and antimicrobial resistance of diabetic foot staphylococci

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* The author contributed to the conception and design of the study, conducted the experiments, performed the data analysis process and drafted the manuscript.

Abstract

Background: Diabetes *mellitus* is a major chronic disease that continues to increase significantly. One of the most important and costly complications of diabetes are foot infections that may be colonized by pathogenic and antimicrobial resistant bacteria, harboring several virulence factors, that could impair its successful treatment. *Staphylococcus aureus* is one of the most prevalent isolate in diabetic foot infections, together with aerobes and anaerobes.

Methods: In this study, conducted in the Lisbon area, staphylococci isolated (n=53) from diabetic foot ulcers were identified, genotyped and screened for virulence and antimicrobial resistance traits. Genetic relationship amongst isolates was evaluated by pulsed-field-gel-electrophoresis with further multilocus sequence typing of the identified pulsotypes. PCR was applied for detection of 12 virulence genes and e-test technique was performed to determine minimal inhibitory concentration of ten antibiotics.

Results: among the 53 isolates included in this study, 41 *Staphylococcus aureus* were identified. Staphylococcal isolates were positive for intercellular adhesins *icaA* and *icaD*, negative for biofilm associated protein *bap* and pantone-valentine leucocidin *pvl*. *S. aureus* quorum sensing genes *agrI* and *agrII* were identified and only one isolate was positive for toxic shock syndrome toxin *tst*.

36% of staphylococci tested were multiresistant and higher rates of resistance were obtained for ciprofloxacin and erythromycin. Clonality analysis revealed high genomic diversity and numerous *S. aureus* sequence types, both community- and hospital-acquired, belonging mostly to clonal complexes CC5 and C22, widely diffused in Portugal nowadays.

Conclusions: this study shows that diabetic foot ulcer staphylococci are genomically diverse, present resistance to medically important antibiotics and harbour virulence determinants. These properties suggest staphylococci can contribute to persistence and severity of these infections, leading to treatment failure and to the possibility of transmitting these features to other microorganisms sharing the same niche. In this context, diabetic patients may become a transmission vehicle for microorganisms' clones between community and clinical environments.

Keywords: Diabetic foot staphylococci, PFGE, HA-MRSA, CA-MRSA, MLST, Virulence factors.

Background

Foot ulcers are an increasing problem in patients with Diabetes *mellitus* and infection is a frequent complication that actually constitutes the most common cause of hospitalization in diabetic patients, often related to lower-extremity amputation (Spichler et al., 2015). Several studies have demonstrated that they represent an economic burden worldwide, comparable with the costs associated with cancer, depression, lung and musculoskeletal diseases (Rice et al., 2014; Van Acker, Léger, Hartemann, Chawla & Kashif Siddiqui, 2014). Diabetic foot infections (DFI) are often polymicrobial and can be caused by several pathogens, mainly Gram positive bacteria, being *Staphylococcus* the most predominant bacterial genus, as already described (Mendes et al., 2012; Zenelaj et al. 2014).

Staphylococcus is a frequent commensal bacteria of human skin and mucosa, being one of the major cause of infections in humans, ranging from minor skin infections to severe infections such as septicaemia, endocarditis and osteomyelitis (Harastani, Araj & Tokajian, 2014). These bacteria may produce several virulence factors, one of the most important being biofilm formation, which consists in adherent bacterial populations growing inside their polymeric structures that confer the ability of evasion to immune system and to multiple antibiotic treatments (Sekhar et al., 2010). Several virulence genes are implicated in biofilm formation, like *icaA* and *icaD*, responsible for the biosynthesis of polysaccharide intercellular adhesion (PIA) molecules, containing N-acetylglucosamine, the main constituent of the biofilm matrix in the accumulation phase (Cos & Tote, 2010). A biofilm associated protein, coded by the gene *bap*, was also described as essential in biofilm production of some *Staphylococcus* spp. isolated from nosocomial infections (Potter et al., 2009).

One of the bacterial properties that allow the development and growth of multicellular biofilm is cell communication and signalling, in which the bacterial signals reach a specific density or “quorum” activating regulatory genes that control some cellular processes (Sifri, 2008); the *S. aureus* accessory gene regulator (*agr*) was the first peptide signal discovered (Novick & Geisinger, 2008).

Many virulence determinants including toxins, tissue degrading enzymes and immune evasion factors, are secreted by staphylococci, particularly by *S. aureus* (Gordon & Lowy, 2008). *ClfA* is a gene responsible for causing platelet activation through binding to fibrinogen and fibrin and for inhibiting phagocytosis in *S. aureus* (Chambers & Deleo, 2010). One of the major threats in severe tissue necrosis is the presence of the cytotoxin panton-valentine leukocidin (*pvl*), whose locus is carried on a bacteriophage, manifesting commonly in strains isolated from community-acquired skin and soft tissue infections and especially from pneumonia (Holmes et al. 2005). Some *S. aureus* isolates also secrete the toxic shock syndrome toxin 1 (TSST-1), a superantigenic toxin responsible for staphylococcal scarlet fever and toxic shock syndrome, encoded by the *tst* gene (Durand, Bes & Meugnier, 2006). *S. aureus* and coagulase-negative staphylococci (CoNS) infections occur in the community or in healthcare

settings and an extremely high percentage of these isolates are resistant to methicillin. In Europe, methicillin-resistant *S. aureus* (MRSA) are predominantly acquired in healthcare settings representing a major challenge to the control of antibiotic resistance in hospitals (Grundmann et al., 2014). Portugal is one of the European countries presenting higher rates of MRSA in hospitals, reaching 53.8% according to last report data (European Centre for Disease Prevention and Control [ECDC], 2012) and hospital-associated MRSA (HA-MRSA) have been extensively characterized (Aires de Sousa, Conceicão, Simas & Lencastre, 2005; Amorim et al., 2009; Tavares, Faria, De Lencastre & Miragaia, 2014). However, less is known about the epidemiology of MRSA in the community (CA-MRSA), which remains poorly understood (Almeida et al., 2014). Epidemic MRSA (EMRSA)-15 clone (ST22-IV), is currently the most predominant clone in Portuguese hospitals, accounting for 72 % of all MRSA isolates, followed by the NY/Japan clone (NY/JP) (ST5-II). More recently a variant of this clone (ST105) appeared as the second most predominant clone in Portuguese hospitals (Faria, Miragaia & De Lencastre, 2013; Tavares et al., 2014).

In the last years the complications of DFI have raised due to the increased rate of multidrug-resistant (MDR) isolates, so a better knowledge of these bacteria is necessary in order to institute an effective antibiotic therapy (Zenelaj et al., 2014; Spichler at al., 2015). This study aimed to investigate the molecular types, virulence traits and antimicrobial susceptibility pattern of *Staphylococcus* spp. isolated from diabetic foot ulcers in Portugal.

Methods

Bacterial isolates

A total of 53 staphylococci clinical isolates from diabetic foot ulcers, belonging to 49 samples collected in a transversal observational study conducted at four clinical centers in Lisbon, from January 2010 to July 2010 (Mendes et al., 2012), were used in this study. Only eight patients were hospitalized during the collection of samples. All isolates were processed, isolated and identified by standard methods (Mendes et al., 2012). Each isolate corresponds to a different patient, with the exception of following pairs, which belonged to the same patient: *S. aureus* A2-1a and A2-1b, *S. aureus* B3-2 and B3-3, *S. aureus* Z1-1 and Z1-2, *S. aureus* Z3-1 and Z3-2, *S. aureus* Z21-1 and Z-21-3, *S. aureus* Z27-2 and Z27-3 and *S. aureus* Z33-1 and Z33-2. Although being recovered from the same patient, such staphylococci were included in further analysis due to the distinct colony morphologies observed during isolation and purification procedures.

Identification at species level

After inoculation in Columbia Agar + 5% sheep blood (Biomerieux), plates were incubated at 37°C for 24 hours. Rapid DNA extraction was performed by suspending four to five bacterial colonies in 100 µL of TE (10 mM Tris, 1 mM EDTA, pH 7.8) buffer and heating to 97°C for

seven min. After centrifugation at 15 000 g for five min, supernatant was collected and stored at -20°C for subsequent PCR screening.

Staphylococcus *aureus* and *Staphylococcus epidermidis* identification was confirmed using a multiplex PCR protocol described elsewhere (Pereira et al., 2010). Amplified products were analysed by electrophoresis using 0.5X Tris-Borate-EDTA (TBE) buffer in a 2% agarose gel (Bioline) stained with GreenSafe (NZYTech) and visualized by transillumination under UV (Pharmacia Biotech, Thermal Imaging System FTI-500). NZYDNA ladder VI (NZYTech) was used as a molecular weight marker. *S. aureus* ATCC 29213 and *S. epidermidis* ATCC 35984 were used as PCR amplification controls.

For the remaining staphylococcal isolates, Biomerieux API Staph galleries were used for species identification.

Screening for virulence factors

The presence of virulence determinants was evaluated by PCR amplification using primers and protocols previously described. Genes tested included coagulase gene *coa* (Akineden et al., 2001), protein A gene *spa* (Akineden et al., 2001), adhesin genes *icaA* and *icaD* (Arciola, Baldassarri & Montanaro, 2001), biofilm associated protein gene *bap*, clumping factor a *clfa* (Akineden et al., 2001), accessory regulators genes *agrI*, *agrII*, *agrIII* and *agrIV* (Gilot, Lina, Cochard & Poutrel, 2002), toxic shock syndrome toxin 1 gene *tst* and panton-valentine leukocidin *pvl* (Jarraud et al., 2002).

S. aureus ATCC 25923 was used as an amplification control for *coa*, *spa* and *clfa* genes. *S. epidermidis* ATCC 35984 was used as *icaA* and *icaD* positive control. *S. aureus bap* positive control was kindly provided by Dr. Penadés (Cardenal Herrera University, Valencia, Spain), *agrI*, *agrII*, *agrII* e *agrIV* control strains by Dr. Carmen Torres (Rioja University, Spain), and *tst* and *pvl* positive controls by Dr. Michèle Bes (Centre National de Reference des Staphylocoques, Lyon,Frande).

Evaluation of antibiotic susceptibility and detection of *mecA*

Minimal inhibitory concentrations (MIC) were determined for antibiotics: cefoxitin (Fox), ceftaroline (Cpt), ciprofloxacin (Cip), clindamycin (Cli), doxycycline (Dox), erythromycin (Ery), gentamicin (Gen), linezolid (Lzd), meropenem (Mem) and vancomycin (Van), by placing E-test strips (Biomérieux) on staphylococci inoculated on Mueller Hinton plates, incubated for 24 hours at 37°C. Test performance was monitored using *S. aureus* ATCC 29213.

Detection of *mecA* gene was performed as previously described (Pereira et al., 2010). Amplified products were analysed by electrophoresis with 0.5X Tris-Borate-EDTA (TBE) buffer in a 1.5% agarose gel (Bioline) stained with GreenSafe (NZYTech) and visualized by transillumination under UV (Pharmacia Biotech, Thermal Imaging System FTI-500). NZYDNA

ladder VI (NZYTech) was used as molecular weight marker. MRSA control strain was kindly provided by Dr. Birgit Strommenger (Robert Koch Institute, Germany).

Staphylococci under analysis were defined as Methicillin Resistant Staphylococcus (MRS) if resistant by cefoxitin MIC or if *mecA* positive (Clinical and Laboratory Standards Institute [CLSI], 2013), and as Multi-drug Resistant (MDR) if resistant to three or more antimicrobials belonging to different antibiotic classes and bacterial targets (Magiorakos & Srinivasan, 2012).

Macrorestriction analysis by Pulsed-Field Gel Electrophoresis (PFGE)

Molecular fingerprinting of staphylococci was performed by PFGE using a CHEF-DRIII apparatus (Bio-Rad Laboratories, San Diego, USA). Bacterial cultures were grown overnight on Columbia agar supplemented with 5% sheep blood (BioMérieux) and a cellular suspension of $5x10^9$ CFU/mL incorporated into 1.5% low melting point agarose (BioRad). Discs were immersed into a lysis solution with lysostaphin (Sigma-Aldrich) (50 µg/ml), lysozyme (Merck) (100 µg /ml) and RNase (Roche) (50 µg/ml) at 37 ºC for 3 h. After lysis, discs were incubated with proteinase K (NZYTech, Portugal) (1 mg/ml) for 17 h at 50ºC, followed by overnight digestion with *Sma*I (Takara) at 25ºC. Digested DNA was submitted to electrophoresis in 1% agarose gel (Seakem LE) for 23 h at 14ºC and 6 V/cm with pulse times of five to 35 s. Lambda Ladder PFG Marker (BioLabs) 50 µg/ml was used as molecular weight marker. Agarose gels were stained with ethidium bromide and visualized by transillumination under UV (Pharmacia Biotech, Thermal Imaging System FTI- 500). BioNumerics 7.5 software (Applied Maths, Kortrijk, Belgium) was used to register macrorestriction patterns and clustering analysis was performed using DICE similarity coefficient and the unweighted-pair group method with arithmetic mean (UPGMA).

S. aureus multilocus sequence typing (MLST)

Amplification of seven housekeeping genes, including carbamate kinase *arcC*, shikimate dehydrogenase *aroE*, glycerol kinase *glpF*, guanylate kinase *gmk*, phosphate acetyltransferase *pta*, triosephosphate isomerase *tpi*, and acetyl coenzyme A acetyltransferase *yqiL*, was done according to the already published protocols (Enright et al., 2000). DNA sequencing was performed by Stabvida (Portugal). MLST sequences were analysed using Bionumerics 7.5 software (Applied Maths, Kortrijk, Belgium) and sequence types (ST) assigned according to the *S. aureus* MLST database (http://saureus.mlst.net). The eBURST algor

ithm, available at (http://eburst.mlst.net), was used to classify different ST into clusters or clonal complexes (CC). A minimum spanning tree (MST) constructed with BioNumerics 7.5 software (Applied Maths, Kortrijk, Belgium) using the concatenated seven gene fragments was also used to evaluate the phylogenetic relationships between isolates.

Results

Identification at species level

Among the 53 isolates included in the study, 41 were identified as *S. aureus* and six as *S. epidermidis* by multiplex PCR. The API galleries identified two isolates as *S. haemolyticus*, one as *S. schleiferi*, one as *S. caprae*, one as *S. simulans* and one as *Staphylococcus* sp.

Screening for virulence factors

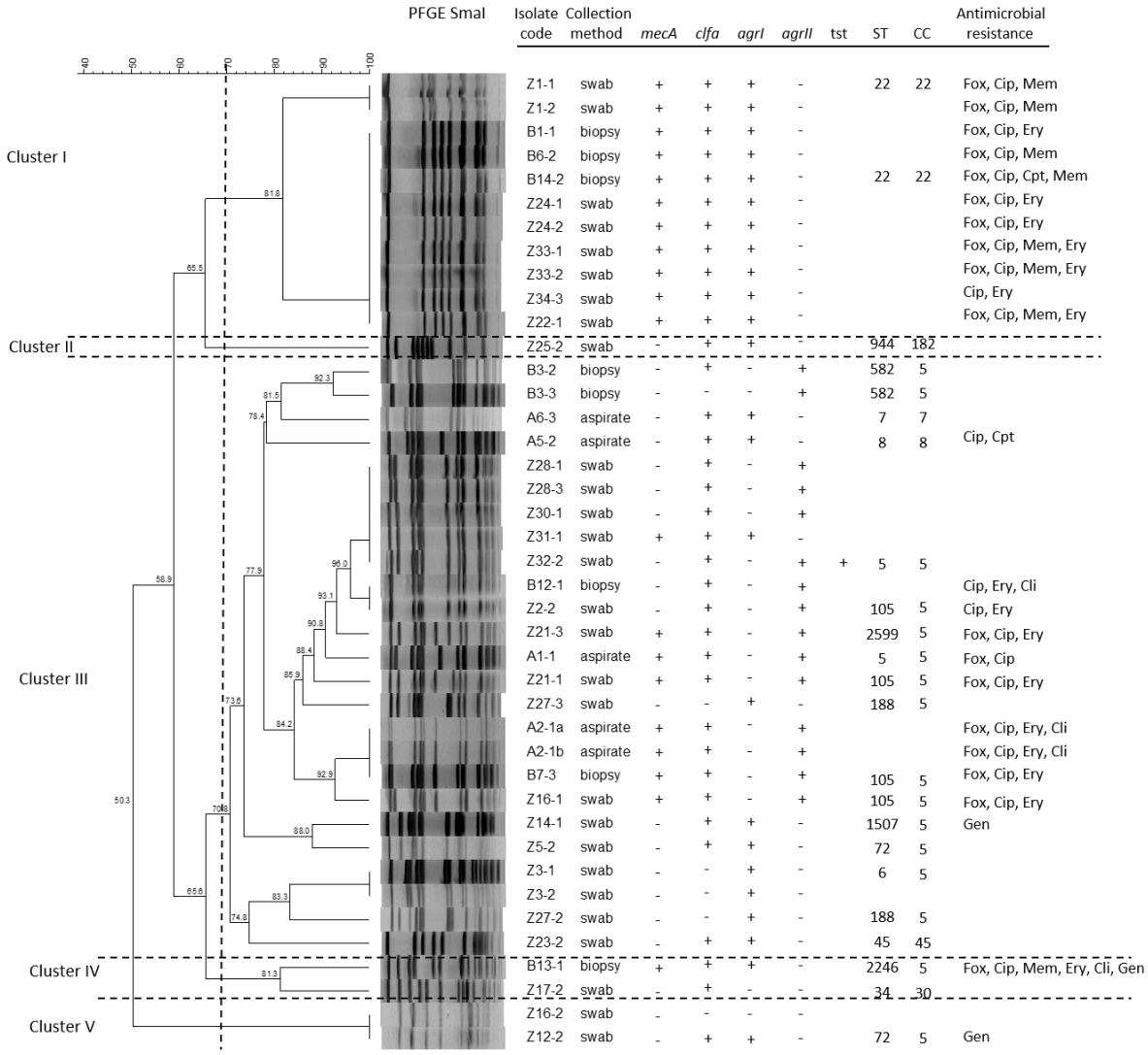
All isolates were positive for *icaA* and *icaD* and negative for *bap* and *pvl*. The *clfa* gene was present in 70% of the isolates (*S. aureus* n=30, *S. epidermidis* n=3 and *S.* sp n=1). The *S. aureus quorum sensing* genes *agrI* and *agrII* were present in 60% and 40% of the *S. aureus* isolates respectively, and no *agrII* or *agrIV* were found. Two *S. aureus* isolates did not harbour *agr*. With the exception of two isolates (one of which also *agr* negative), all *S. aureus* were positive for *spa*. As expected, all *S. aureus* isolates were *coa* positive, whilst only one *S. aureus* was positive for *tst* and it was MSSA (Figure 1).

Evaluation of antibiotic susceptibility and detection of *mecA*

All isolates were considered susceptible to vancomycin (MIC ≤ 2µg/mL) and presented the same susceptibility to linezolid (MIC ≤ 4 µg/mL) and doxycycline (MIC ≤ 4 µg/mL) with the exception of one methicillin-resistant *S. epidermidis* (MRSE) isolate (MIC ≥ 8 µg/mL and MIC ≥ 16 µg/mL for linezolid and doxycycline respectively), which was resistant to six of the antibiotics tested. Ceftaroline MIC values were ≤ 0.5 µg/mL and only two MRSA presented MIC ≥ 4 µg/mL (ceftaroline-resistant). About 90% of isolates obtained MIC values for clindamycin of ≤ 0.25µg/ml and for gentamicin 4 ≤ µg/ml. About 57% of isolates were considered susceptible to ciprofloxacin (≤ 4µg/ml) and eythromycin (≤ 8 µg/ml), presenting a resistance rate of 43%. The percentage of MDR isolates was 36% (Figure 1).

Among the 41 *S. aureus* isolates tested, 20 were classified as MRSA (*mecA* positive) (Figure 1), resulting in a prevalence of 48.7% among *S. aureus* carriers; of these, 14 were cefoxitin resistant. Among the six *S. epidermidis* isolates, five were MRSE (*mecA* positive) and 3 were cefoxitin resistant. The other *Staphylococcus* isolates didn't carry the *mecA* gene and were cefoxitin susceptible. The total prevalence of methicillin-resistant isolates was 47%.

Figure 1. Dendrogram based on *Sma*I-PFGE patterns of the *S. aureus* diabetic foot isolates. The image also displays information regarding sample collection method, presence of virulence genes, ST/CC allocation and antimicrobial resistance profile. Fox - cefoxitin; Cip – ciprofloxacin; Mem – meropenem; Ery – erythromycin; Cpt – ceftaroline; Cli – clindamycin; Gen – gentamicin.



Macrorestriction analysis by Pulsed-Field Gel Electrophoresis -PFGE-

Analysis of the dendrogram displayed in Figure 1 led to the selection of a 70% similarity level for the assignment of PFGE genomic types (pulsotypes). Hence, *Sma*I-macrorestrition analysis revealed 18 distinct genomic patterns among the 41 *S. aureus* isolates examined. Cluster analysis allowed grouping the isolates into five main clusters at approximately 70% similarity with one single member cluster (Figure 1). All isolates included in cluster I were MRSA, *clfa* and *agrI* positives and belonged to ST22 (CC22). They were all resistant to ciprofloxacin and most of them also to erythromycin. Cluster II included only one isolate,

sensitive to all antibiotics tested, *clfa* and *agrI* positive and belonging to ST944 (CC182). The *agrII* positive isolates were located only in cluster III that was the more diverse group because included different genoypes, most of them *clfa* positive belonging to CC5, both MRSA and MSSA. These MRSA isolates showed resistance to ciprofloxacin and erythromycin. The only one MSSA *agrII* isolate that was *tst* positive, belonged to this group. Cluster IV included two different genotypes, one of which stood out (B13-1), being resistant to six of the antibiotics tested. Cluster V included two MSSA isolates, one *clfa-agrI* positive and the other *clfa-agr* negative. Regarding the six *S. epidermidis* isolates, although the number was inferior, four pulsotypes were observed and, noteworthy, two different pulsotypes corresponded to two isolates obtained from the same patient (data not shown).

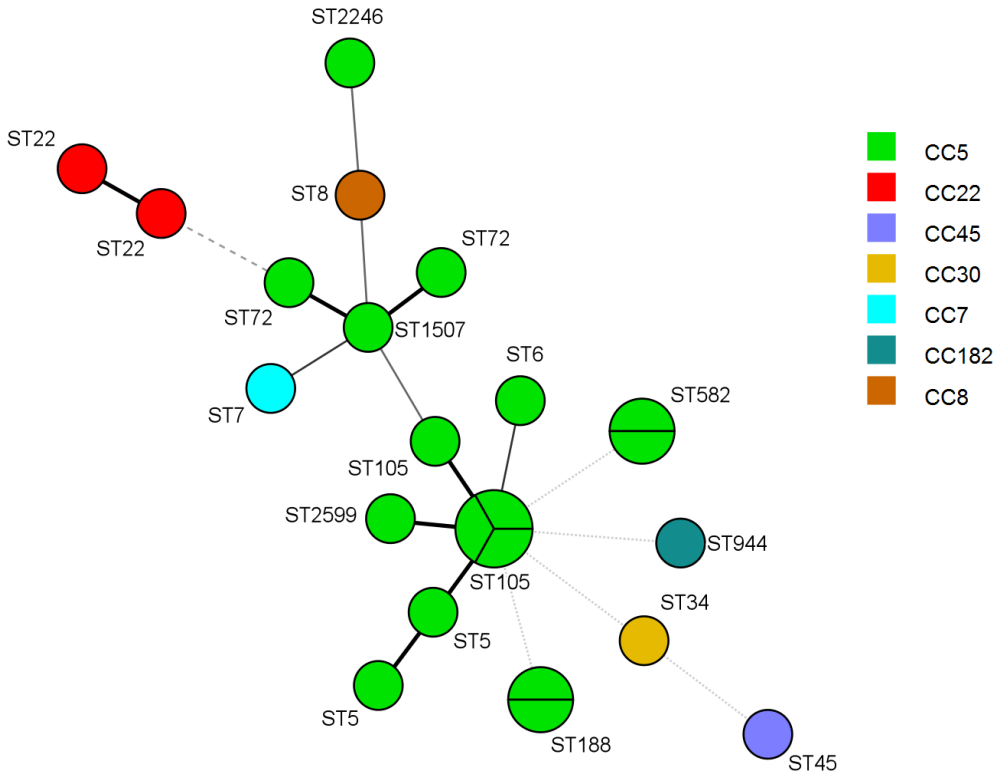
S. aureus multilocus sequence typing (MLST)

High genetic diversity was revealed by MLST, as indicated by the detection of 15 ST among the 23 isolates tested (Figure 1). Briefly, ST105 (n=4), ST5 (n=2), ST22 (n=2), ST188 (n=2 in the same patient with two different pulsotypes), ST582 (n=2 in the same patient with two different pulsotypes), ST6 (n=1), ST7 (n=1), ST8 (n=1), ST34 (n=1), ST45 (n=1), ST 72 (n=1), ST944 (n=1), ST1507 (n=1), ST2246 (n=1), ST2599 (n=1, in a patient with a ST105 also) (Figure 1 and Figure 2). Based on sequence typing, isolates were assigned to seven MLST CC: CC5 (n=17, including the two different pulsotypes found in two patients), CC22 (n=2), CC7 (n=1), CC8 (n=1), CC30 (n=1), CC45 (n=1), CC182 (n=1) (Figure 1). MRSA lineages included ST105 (CC5), ST5 (CC5), ST22 (CC22) and ST2599 (CC5). The only MSSA *tst* positive isolate belonged to ST5. The minimum spanning tree (MST) shows the phylogenetic relationships among diabetic foot staphylococci (Figure 2).

Discussion

Although previous studies reported *Pseudomonas aeruginosa* as the most common isolate in DFU (Shankar et al., 2005; Ramakant et al., 2011), many others authors from the late 1990s have shown that Gram positive cocci are the most predominant agents responsible for DFI, with *S. aureus* being the most commonly isolated pathogen with considerably high rates of MRSA (Tentolouris et al., 2006; Sekhar et al., 2014). According to our results, most isolates were identified as *S. aureus* (77.3%) and 48.7% of them considered MRSA. A study conducted by Sotto et al. (2008) reported a similar MRSA percentage but several studies showed lower rates (Shankar et al., 2005; Gadepalli et al., 2006; Zenelaj et al., 2014). The highest MRSA percentages in DFI, reaching 70% , were found in India (Swarna et al., 2012).

Figure 2. Minimum spanning tree of 23 *S. aureus* representing the 23 different pulsotypes detected amongst the diabetic foot isolates. Nodes indicate sequence type (ST) and their size shows the relative number of isolates for each ST. Every colour represents a distinct clonal complex.



Regarding antimicrobial susceptibility patterns, 34% of all staphylococcal isolates were cefoxitin resistant. However, *mecA* detection is considered the gold standard for methicillin-resistance by the CLSI (2013) and 47% of the studied isolates were *mecA* positive.

Only 10% of all staphylococci showed resistance to meropenem but MRS isolates should be considered resistant to other β-lactam agents, therefore including also meropenem, because most cases of documented MRS infections have responded poorly to β-lactam therapy (CLSI, 2013). Cephalosporins with anti-MRSA activity include ceftaroline, the active metabolite of ceftaroline fosamil (Teflaro®, Forest Laboratories), a cephalosporin with an *in vitro* broad spectrum against MRSA and most enteric organisms (Sader, Pritsche, Kaniga, Ge & Jones, 2005). Ceftaroline, approved by U.S. Food and Drug Administration (FDA) for treatment of acute bacterial skin infections, displayed a very good efficacy in the studied isolates with MIC value ≤ 0.5 μg/mL, with the exception of two resistant MRSA isolates. It is important to refer that one MRSA isolate that was resistant to six antibiotics studied showed sensitivity to ceftaroline. These results are in agreement with some recent studies that have already shown

the excellent activity of ceftaroline, both *in vitro* (Goldstein, Citron, Merriam & Tyrrell, 2013) and *in vivo* (Lipsky, 2015).

Resistance to linezolid and doxycycline was detected only in one MRSE that showed resistance to six antibiotics. Linezolid-resistance in *S. epidermidis* has been already reported (Zhu et al., 2006), possibly linked to a mutation in the V region of the 23S rRNA gene. MIC values for clindamycin and gentamicin showed susceptibility for 90% of the isolates. These results suggest a good efficacy of linezolid, doxycycline, clindamycin and gentamicin for DFI treatment (Citron et al., 2007; Malik et al., 2013). As expected, all *Staphylococcus* tested were susceptible to vancomycin; until today only one case of vancomycin-resistant *S. aureus* was described in Europe, Portugal (Melo-Cristino, Resina, Manuel, Lito & Ramirez, 2013) and few cases worldwide, mostly in the USA (Saravolatz, Pawlak & Johnson, 2012).

About 43% of the isolates were resistant to ciprofloxacin and erythromycin, two antibiotics largely used in clinical practice for these type of infections. Similar rates in ciprofloxacin and erythromycin resistance were found in a study conducted by Gadepalli et al. 2006 (Swarna et al., 2012). With the increasing use of quinolones in clinical practice, the development of resistance mutants has increased (Campion, McNamara & Evans, 2004), pointing out for the importance to their careful administration in clinical settings. Several genes are implicated in macrolide resistance, especially in staphylococci and streptococci (Martineau et al., 2000), explaining the low susceptibility rates of erythromycin in this bacterial genus.

It's important to remember that DFI are generally polymicrobial and the choice of antibiotic therapy often doesn't target specific pathogens. In fact, the present investigation revealed a high rate (36%) of MDR isolates in DFI, which is in accordance with other reports (Gadepalli et al., 2006; Zubair et al., 2011; Sekhar et al., 2014) and should represent a serious warning for the control of this type of infections.

Virulence factors, like surface proteins and extracellular toxins, are widely distributed among staphylococci, potentially causing harmful pathogenic effects to the host (Holmes et al., 2005). In this study two *S. aureus* isolates were *spa* negative. Some studies have already reported the absence of *spa* protein with percentages of 3-5% (Shakeri et al., 2010), that seems to be linked to point mutations. In a recent study we demonstrated that the staphylococcal isolates are able to form biofilm (Mottola et al., 2015) which may explain why all isolates tested were positive for *icaA* and *icaD*. Otherwise, none of the isolates carried the *bap* gene, already described in some *Staphylococcus* spp. isolated from nosocomial infections (Potter et al., 2009).

The *clfA* gene was present in 70% of our isolates including some *S. epidermidis*. The presence of *clfa* in *S. epidermidis* can be justified by the fact that in this species the fibrinogen-binding proteins SdrG or Fbe, associated to adherence to fibrinogen, are highly similar to *S. aureus* clumping factors A and B (Hartford et al., 2001).

The only *tst*-positive isolate was a MSSA, *agrII*-positive belonging to ST5. Jarraud et al. in 2002 reported that most *tst*-positive *S. aureus* strains were associated with both community and hospital-acquired diseases and were all methicillin-sensitive *S. aureus* (MSSA).

None of our isolates was *pvl*-positive. The *pvl* locus is strongly associated to CA-MRSA and often to *agr* group *III* background (Vandenesch et al., 2003). In Portugal, it was related with one case of CA-MRSA in 2012, belonging to the USA300 epidemic clone (ST8-IVa, t008, *pvl* positive), the predominant CA-MRSA clone in USA at present. The USA300 is a rare clone in Europe and its low prevalence in Portugal was expected (Nazareth et al., 2012).

The contribution of the *agr* system to *S. aureus* virulence by gene regulation has already been described, as well as the association of a particular *agr* type in clinical isolates harbouring important virulence factors (Francois et al., 2006). The *agr* group *I* was identified in the majority of the analysed staphylococci, followed by *agrII*, as previously reported in numerous other studies (Jarraud et al., 2002; Machuca, Sosa & González, 2013). Neither *agrIII* nor *agrIV* were observed. Two *S. aureus* isolates were *agr*-negative, but it is known that these variants can occur both *in vivo* and *in vitro* (Traber et al., 2008).

The virulence profile of the studied DFI isolates was more similar to CA-MRS than HA-MRS strains. This is an unusual finding, considering that diabetic patients attend frequently health care facilities and may suggest an increasing lack of barrier between both settings: hospital and community.

Sotto et al. in 2008 demonstrated that the virulence gene profiles of DFI *S. aureus* isolates enables to distinguish the grade of ulcers and to predict its outcome; more knowledge about the virulence features of DFI isolates would be very helpful in establishing a more accurate diagnosis and consequently an adequate therapy.

PFGE genomic typing demonstrated a high diversity of clones, detecting 18 *S. aureus* and four *S. epidermidis* pulsotypes, respectively. According to Tenover et al. 1995 (Tenover et al., 1995), it is highly probable that *S. aureus* isolates grouping in the same pulsotype with 100% similarity belong to the same ST, as determined by MLST. The correlation between PFGE and MLST showed that PFGE cluster I was the most homogeneous cluster, including only MRSA ST22 (CC22) isolates, the most common ST observed in this study. Portugal is the European country with the highest rate of MRSA (54.6%) (ECDC, 2012) and CC22 is a common and widespread clonal group from which different MRSA have emerged, like the pandemic ST22-MRSA-IV (UK-EMRSA 15), present in hospitals as well as in outpatients (Monecke et al., 2011). CC22 represents a major clone in Portugal hospitals since 2001, having replace the Brazilian clone (Aires-de-Sousa et al., 2008), and its prevalence has increased to more than 70% of MRSA, likewise to what is observed in the United Kingdom, where this clone is believed to have originated (Faria et al., 2013). All ST22 isolates were positive for *clfa*, another virulent factor that confers pathogenicity, and presented the quorum sensing *agr I* gene,

already described as being common in ST22 staphylococci (Monecke & Ehricht, 2005; Aires-de-Sousa et al., 2008).

The most common CC isolated in our study was CC5, present in PFGE clusters III, IV and V, and ST5 represented the second most frequent ST, after ST22. CC5 is another common and widespread clonal complex that includes a large number of different MRSA, some of which pandemic (Monecke et al., 2011). Shortly after the emergence of EMRSA-15, the New York-Japan (NY/JP) ST5-II and, more recently, a variant of this clone, ST105-II, appeared as the second most predominant clone in Portuguese hospitals (Tavares et al., 2014). Recently, a high percentage of MRSA (21.6 %) was also found in a community in Portugal, where EMRSA-15 or related clones were the predominant ones (77.2 %), followed by NY/JP or related clones (14.9 %) (Tavares, Miragaia, Rolo, Coelho & De Lencastre, 2013). In this study, isolates belonging to CC5 presented mainly *agr* type *II*, particularly ST5, and included both MRSA and MSSA (Aires de Sousa et al., 2008).

Besides ST5 and ST105, several ST belonging to CC5 were identified, namely MSSA *agrI* ST6, MSSA *agrI* ST72, MSSA *agrI* ST188, MSSA *agrI* ST582, MSSA *agrI* ST1507, MRSA *agrI* ST2246 and MRSA *agrII* ST2599. These less frequent ST have already been described in Portugal (Espadinha et al., 2013; Tavares et al., 2013; Tavares et al., 2014), with the exception of the ST1507 and ST2599, but little information is available regarding these ST. In fact, the only description found in the *S. aureus* MLST database (http://saureus.mlst.net), refers to a MRSA ST1507 isolated in 2006 in South Korea from a foodborne source and a MRSA ST2599 isolated from urine in 2013 in the USA. In our study the patient from which ST2599 (CC5) was recovered, also presented another *S. aureus* belonging to ST105 (CC5), being the only case where it was possible to identify two different ST in the same patient. In the other six cases in which the same patient showed two similar, but not identical pulsotypes, MLST revealed that they belonged to the same ST. Interestingly, some clones belonging to different CC presented a higher PFGE similarity than clones included in the same CC, as already observed (Aires de Sousa, 2005).

Cluster II included only one isolate, MSSA *agrI* ST944 (CC182). MSSA ST944 was described in Switzerland being isolated from nasal swabs of healthy risk-free adult carriers (Sakwinska et al., 2009) and in China, where it was present with high frequency in nasal carriage of healthy children in a kindergarten (Fan et al., 2009). In the *S. aureus* MLST database, a MSSA ST944 has also been described in Norway, related with nasal swab carriage (http://saureus.mlst.net).

Cluster III was the most heterogeneous cluster, including mainly MSSA *agrI* isolates, belonging to the following ST: ST7 (CC7), ST8 (CC8), ST34 (CC30) and ST45 (CC45). In fact, a previous study concerning the population structure of MSSA in Portugal showed that these CC were, among others, the most predominant clonal types found between 1992 and 2011, both in the community and hospitals settings (Tavares et al., 2014).

Patients with DFI constantly attend clinical centres for wounds healthcare, which may explain the high diversity of pulsotypes and ST found, including the main hospital-acquired clones present in Portugal (CC5 and CC22). It is important to refer that several less frequent clones, seldom described in literature and MLST database, were also found in this study. Therefore, diabetic patients can be important vehicles for clonal dissemination from the hospitals into the community and contrariwise, including less common clones.

Conclusions

To our knowledge this is one of the few reports of staphylococci isolated from DFI that include information about the isolates origin, virulence factors and antimicrobial resistance profiles. Studies in DFI microbiology are scarce, as described recently by Zenelaj et al. (2014), and further investigation of diabetic foot infections is urgent, allowing to adapt the therapeutic approach of these patients to the microbiological characteristics of the microorganisms involved.

CHAPTER IV

Susceptibility patterns of *Staphylococcus aureus* biofilms in diabetic foot infections

4.1. Susceptibility patterns of *Staphylococcus aureus* biofilms in diabetic foot infections

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* The author participated in the conception and design of the study, conducted the experiments, performed the data analysis process and drafted the manuscript.

Abstract

Background: Foot infections are a major cause of morbidity in people with diabetes and the most common cause of diabetes-related hospitalization and lower extremity amputation. *Staphylococcus aureus* is by far the most frequent species isolated from these infections. In particular, methicillin-resistant *S. aureus* (MRSA) has emerged as a major clinical and epidemiological problem in hospitals. MRSA strains have the ability to be resistant to, most β-lactam antibiotics, but also to a wide range of other antimicrobials, making infections difficult to manage and very costly to treat. To date, there are two fifth-generation cephalosporins generally efficacious against MRSA, ceftaroline and ceftobripole, sharing a similar spectrum. Biofilm formation is one of the most important virulence traits of *S. aureus.* Biofilm growth plays an important role during infection by providing defence against several antagonistic mechanisms. In this study, we analysed the antimicrobial susceptibility patterns of a group of biofilm-producing *S. aureus* strains isolated from diabetic foot infections.

Methods: The antibiotic minimum inhibitory concentration (MIC) was determined for ten antimicrobial compounds, along with the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC), followed by PCR identification of the genetic determinants of biofilm production and antimicrobial resistance.

Results: results demonstrate that very high concentrations of the most used antibiotics in treating diabetic foot infections (DFI) are required to inhibit *S. aureus* biofilms *in vitro,* which may explain why monotherapy with these agents frequently fails to eradicate biofilm infections. In fact, biofilms were resistant to antibiotics at concentrations 10-1000 times greater than the ones required to kill free-living or planktonic cells. The only antibiotics able to inhibit biofilm eradication on 50% of isolates were ceftaroline and gentamicin.

Conclusions: The results suggest that the antibiotic susceptibility patterns cannot be applied to biofilm established infections. Selection of antimicrobial therapy is a critical step in DFI and should aim at overcoming biofilm disease in order to optimize the outcomes of this complex pathology.

Keywords: *Staphylococcus aureus*, Diabetic foot infections, MBIC, MBEC, Resistance genes.

Background

Foot infections are a major cause of morbidity in diabetes patients and the most common cause of diabetes-related hospitalization and lower limb amputation (Spichler et al., 2015). The physiopathology of diabetic foot infections (DFI) is complex, but its severity and prevalence are a consequence of host-related disorders and pathogens-factors, as virulence and antibiotic resistance traits (Spichler et al., 2015). DFI are mostly polymicrobial and *Staphylococcus aureus* is by far the more frequent species involved, either alone or as a component of mixed infections (Mendes et al., 2012; Lipsky et al., 2013).

S. aureus is an important nosocomial pathogen that can cause several infections such as: bacteraemia, osteomyelitis, skin infections, pneumonia, meningitis and endocarditis. In particular, MRSA has emerged as a major clinical and epidemiological problem in hospitals since the 1980s (Petrelli et al., 2008). MRSA strains have the ability to resist to all β-lactam antibiotics and also to a wide range of other antimicrobials, making MRSA infections costly and difficult to manage (Rice, 2006). Ceftaroline and ceftobiprole are, to date, the only anti-MRSA cephalosporins that inhibit PBP2a at therapeutically concentrations. Ceftobiprole, already evaluated in clinical trials, access the active site of PBP2a by its R2 group, whereas ceftaroline causes an allosteric change in PBP2a (Chan et al., 2015). Ceftaroline is FDA approved for treatment of skin and skin structure infections, including those caused by MRSA (Chan et al., 2015).

Several structural and secreted virulence factors play a role in *S. aureus* infections, which are multifactorial and depend on bacterial adherence and biofilm formation. In the beginning of an infection, *S. aureus* produces numerous surface proteins, called “microbial surface components recognizing adhesive matrix molecules” (MSCRAMM) that mediate adherence to host tissues. Once *S. aureus* adheres to host tissues, it can form biofilms, which enable its persistence by allowing bacteria to evade host defences, impeding access to certain types of immune cells, such as macrophages, which display incomplete penetration into the biofilm matrix and “frustrated phagocytosis” (Scherr, Heim, Morrison & Kielian, 2014) . Additionally, biofilm cells display increased tolerance to antibiotics (Gordon & Lowy, 2008).

In contrast to heritable antibiotic resistance mechanisms, biofilm-associated tolerance is a transient state in which normally susceptible bacteria display an altered physiology that decreases sensitivity. When these cells disperse and re-enter a planktonic state, they present their normal susceptibility profile (Lister & Horswill, 2014). Bacteria embedded within a biofilm are difficult to eradicate due to a wide variation of nutrient gradients that slow or arrest bacterial growth, protein synthesis, and other physiologic activities; bacteria sequestered in biofilms are less susceptible to antibiotics by virtue of their reduced growth rates (Kaplan, 2011). Other factors that contribute to biofilm-mediated antimicrobial resistance include inefficient diffusion or sequestering of the agent within the biofilm matrix, the presence of “persister” cells, and other unknown phenotypic differences (Kaplan, 2011).

Various genes have been implicated in the onset and maintenance of biofilms by staphylococci. Among these, the most extensively studied are *icaA* and *icaD* (intercellular adhesion *A* and *D*), products of a gene locus composed by the genes *icaR* (intercellular adhesion regulator) and *ica A, B, C,* and *D* (intercellular adhesion *ABCD*), responsible for the synthesis of the polysaccharide intercellular adhesin (PIA), which contains N-acetylglucosamine, a major component of the exopolysaccharide matrix that surrounds bacterial cells in the biofilm (Rohde, 2007). Also, the products of *pls* (plasmin sensitive) which encodes a surface protein, and *atl* (autolysin), which encodes an autolysin, have been implicated in the formation and structuring of biofilms. The *atl* is the most predominant peptidoglycan hydrolase in staphylococci, and was also identified as an adhesin involved in primary attachment of cells to polystyrene surfaces (Biswas et al., 2006). The *pls* is a homologue of the serine-aspartate repeat (Sdr) surface protein family, of which *ClfA* is the best-characterized member, that reduces adhesion to host proteins and cellular invasiveness (Hussain et al., 2009).

In this study, a collection of *S. aureus* strains isolated from DFI was characterized in terms of their planktonic and biofilm susceptibility patterns, and presence of biofilm and antibiotic resistance genes. The antibiotic minimum inhibitory concentration (MIC) was determined, along with the minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC), followed by PCR identification of the genetic determinants of biofilm production and antimicrobial resistance.

Methods

Strains

A total of 53 staphylococci clinical isolates from diabetic foot ulcers (DFU), obtained from 49 samples, were collected in a previous epidemiological survey, as described by Mendes et al. in 2012. From this collection, twenty-three (n=23) representative biofilm-producing *S. aureus* isolates were selected, based on Pulse Field Gel Electrophoresis (PFGE) and Multilocus Sequence Type (MLST) analysis, previously performed by our research team (Mottola et al., 2016). A reference strain, *S. aureus* ATCC 29213, a known biofilm producer, was also included in this study.

Antimicrobial agents

The antibiotics cefoxitin (FOX), ciprofloxacin (CIP), clindamycin (CLI), doxycycline (DOX), erythromycin (ERY), gentamicin (GEN), linezolid (LZD), meropenem (MEM) and vancomycin (VAN) were obtained from Sigma-Aldrich (Portugal). AstraZeneca (Portugal) generously provided ceftaroline (CPT). All antibiotics were prepared according to CLSI guidelines (2013).

Minimum inhibitory concentrations

MIC were determined for all antibiotics to all strains; E-test was performed according to the manufacturer's recommendations (Biomérieux). Test performance was monitored using *S. aureus* ATCC 29213.

Biofilm susceptibility tests

A modified version of the Calgary Biofilm Pin Lid Device (CBPD) (Ceri, Olson & Stremick, 1999) was used to determine the antimicrobial susceptibility of bacteria embedded in a 24 hours biofilm, in order to determine the MBIC and MBEC (Ceri et al., 1999; LaPlante & Mermel, 2009). Briefly, a starting inoculum of 5 x 10^5 CFU/mL in Mueller Hinton Broth (MHB, Liofilchem Italy) was distributed in 96-well flat-bottom microtiter plates (Nunc, Roskilde, Denmark), covered with a 96-peg lid (Imuno TSP; Nunc, Roskilde, Denmark) and statically incubated for 24 hours at 35°C, to allow biofilm formation on the pegs (Pettit et al., 2005; Cafiso, Bertuccio, Spina, Purrello & Stefani, 2010). The peg lid was then rinsed three times in 1 X sterile PBS to remove planktonic bacteria, placed on a new plate filled with 200 µL of fresh broth containing serial dilutions of antibiotics, from 1024 µg/ml to 0.5 µg/ml, and incubated for 24 hours at 37°C (LaPlante & Mermel, 2009; Pettit et al., 2005; Cafiso et al., 2010; Coraça-Huber, Fille, Hausdorfer, Pfaller & Nogler, 2012). After incubation, the peg lid was removed and the MBIC value was recorded and defined as the last well in which there was no visible growth after incubation (LaPlante & Mermel, 2009; Pettit et al., 2009). Next, to determine the MBEC value, the peg lid was rinsed three times in 1 X sterile PBS, placed in a new plate filled with 200 µL of fresh MHB and sonicated at 45-60 Hz during 10 minutes (LaPlante & Mermel, 2009; Harrison et al., 2010), in order to disperse the bacteria from the peg surface. After sonication, the peg lid was discarded and the plate was covered with a normal lid and incubated for 24 hours at 37°C. After incubation, the quantification of biofilm formation was conducted according with a previously described colorimetric microtiter plate, using Alamar Blue (Mottola et al., 2015). Briefly, 5 µl of resazurin (Alamar Blue, AB, ThermoScientific, Spain) was added to the wells and the plates were incubated for one hour at 37°C. After incubation, absorbance (A) values at 570 nm and 600 nm were recorded in a microplate reader (BMG LABTECH GmbH, Germany). Controls included media plus Alamar Blue (control one) and bacterial cells plus media plus AB without antibiotic (control two). The MBEC was defined as the lowest drug concentration resulting in ≤ 1/2 the absorbance values when compared to control two. Assays were performed at least twice and the average absorbance values used to determine the MBEC values.

PCR screening of biofilm associated and antibiotic resistance genes

All strains were investigated to detect the presence of genes associated with biofilm formation, namely: *icaA*, *icaD*, *atl* and *pls*. Genes associated with antibiotic resistance were also

screened, namely: *blaZ* (penicillin resistance); *mecA* and *mecC* (oxacillin resistance); *tetK*, *tetL*, *tetM* and *tetO* (tetracycline resistance); *msrA*, *ermA*, *ermB* and *ermC* (erythromycin resistance); *aac(6')-aph(2")* (gentamicin resistance) and *norA* (ciprofloxacin resistance).

Detection of *mecA* gene and its homologous *mecC* were performed by multiplex PCR [24]. Oligonucleotide primer sequences are described in Table 1. All amplification reactions were prepared with a mixture containing: 12.5 µl of Supreme NZYTaq 2x Green Master Mix (Nzytech®), 1 µl of each primer (forward and reverse) (STABVIDA®) and 5.5 µl of sterile water (water for molecular biology, Nzytech®). To this mixture, 1 µl of the previous extracted DNA was added, resulting in a total reaction volume of 25 µl. PCR amplification was performed in a thermal cycler (MyCycler Thermal Cycler, BioRad®) using conditions described in the references reported in Table 1. Positive controls for the tested genes were gently provided by: Dr. Mark Holmes, University of Cambridge, Dr. Penadés (Cardenal Herrera University, Valencia, Spain) and Dr. Birgit Strommenger, Robert Koch Institute.

Minimum inhibitory concentration

All isolates were considered susceptible to vancomycin, linezolid and doxycycline, with MIC values ≤ 1 µg/ml, ≤ 4 µg/ml and ≤ 0.5 µg/ml, respectively. Ceftaroline MIC values were ≤ 0.5 µg/ml, and only two isolates presented MIC ≥ 4 µg/ml (ceftaroline-resistant). All isolates, except for one, originated MIC values for clindamycin of ≤ 0.5 µg/ml. Gentamicin MIC values were ≤ 1 µg/ml, except for three resistant isolates. About 57% of isolates were considered susceptible to ciprofloxacin with MIC ≤ 2 µg/ml, and 65% were erythromycin susceptible with MIC ≤ 0.5 µg/ml. Eight isolates (35%) were cefoxitin-resistant, with MIC values ≥ 8 µg/ml (Table 2 and Table 3).

Results

Biofilm susceptibility tests

MBIC and MBEC concentration values are summarized in Table 2. For MRSA isolates, cefoxitin MBIC concentrations ranged from two to five dilutions higher than MIC values, reaching values of 256 to 1024 µg/ml, while MBEC values were even higher (from 2 to ≥ 1024 µg/ml). Instead, for methicillin-susceptible *S. aureus* (MSSA) isolates MBIC and MBEC values for cefoxitin were the same as MIC values, with the exception of four isolates, for which MBIC and MBEC values were two times higher when compared to MIC. In 65% of the isolates (n=15), MBIC and MBEC values for linezolid were the same, and one thousand times higher when compared with MIC values (Figure 1).

Other antibiotics originated different results. All MBIC and MBEC values were much higher than the respective MIC values, and MBEC values were at least twice higher than the MBIC ones. Ceftaroline MBIC values, were four to sixteen times higher than MIC values, ranging from 0.5 to 8 µg/ml, except for one MRSA isolate that reached 1024 µg/ml, and the MBEC

concentrations reached 1024 µg/ml, including four of the eight MRSA isolates. Regarding ciprofloxacin, MBIC values achieved an average of eight times higher compared with MIC, ranging from 0.5 to 512 µg/ml, except one isolate that reached 1024 µg/ml; and for all isolates, except for two, MBEC values increased to 256 to 512 µg/ml. Similar results were obtained for clindamycin, but the gap between the MIC and MBIC values was higher, with MIC of 0.03 to MBIC of 0.5-128 µg/ml, except for one isolate that reached a MBIC value of 1024 µg/ml. MBIC values for doxycycline were even higher, as half of the isolates reached a MBIC concentration of 0.5 to 2 µg/ml, three hundreds to one thousand times higher than MIC. The other half showed MBIC values of 32 to 512 µg/ml, one thousand times higher than the respective MIC. Doxycycline MBEC values ranged from 64 to 128 µg/ml (Figure 1).

The biofilm inhibition concentrations for erythromycin increased about four times in comparison with the values for MIC, from 0.12 - > 256 µg/ml to 0.5 - 256 µg/ml. For MRSA, MIC and MBIC values were the same and equal to > 256 µg/ml. These isolates reached MBEC concentrations of 64 - >1024 µg/ml, representing an increase of five hundred to one thousand times compared with MIC and MBIC values (except for the resistant isolates). In the case of gentamicin, it was observed an increase from MIC to MBIC of two to five hundred times higher, reaching values of 0.5 - > 128 µg/ml (except for one MRSA isolate, that showed MBIC value of 1024 µg/ml); MBEC reached 256 µg/ml (including six of the eight MRSA isolates). Meropenem and vancomycin produced the major increase regarding MBEC values, being one thousand times higher than the value of MIC, and five hundred times higher than the MBIC values (0.5 to > 1024 µg/ml and 8 to > 1024 µg/ml for meropenem and vancomycin, respectively). The MBIC values ranged from 0.5 to 32 µg/ml for meropenem and from 1 to 16 µg/ml for vancomycin, except for one isolate that reached 1024 µg/ml (Figure 1).

PCR screening of biofilm associated and antibiotic resistance genes

All isolates were positive for the biofilm associated genes *icaA*, *icaD* and *atl,* and negative for *pls.* Eight isolates (35%) were MRSA harbouring the *mecA* gene and were resistant to cefoxitin. None of the isolates presented the *mecC* gene. Three isolates presented the *blaZ* gene, one of which was MRSA and resistant to six of the antibiotics tested (Table 3).

Regarding the *tet* genes, none of the isolates were positive for *tetL* and *tetO*, one MRSA isolate was positive for *tetM* (and also *blaZ* positive), and *tetK* was found in three MSSA isolates. With the exception for two isolates, the erythromycin-resistant isolates were positive for *erm* genes, namely six for *ermA* (five MRSA and one MSSA) and three for *ermC* (two MRSA and one MSSA); contrariwise, none of these isolates was positive for *ermB* and *msrA* genes. The *aac(6')-aph(2'')* gene was found in three isolates, one MRSA and two MSSA. Nineteen isolates (82%) presented the *norA* gene, six of which were MRSA and thirteen MSSA (Table 3).

Table 1. PCR target genes and primers used in this work.

Gene	Primer Sequence (5'→3')	Reference
icaA	TCTCTTGCAGGAGCAATCAA AGGCACTAACATCCAGCA	Arciola *et al.* (2001) [46]
icaD	ATGGTCAAGCCCAGACAGAG CGTGTTTTCAACATTTAATGCAA	Arciola *et al.* (2001) [46]
atl	CTTCAGCACAACCAAGATC GGTTACCGACTGCACCGTCAC	Petrelli *et al.* (2008) [4]
pls	GTAATACAACAGGAGCAGATGG GTAGCTTTCCATGTTTTTCCTG	Petrelli *et al.* (2008) [4]
blaZ	ACTTCAACACCTGCTGCTTTC TGACCACTTTTATCAGCAACC	Martineau *et al.* (2000) [38]
mecA	TCCAGATTACAACTTCACCAGG CCACTTCATATCTTGTAACG	Stegger *et al.* (2012) [24]
mecC	AAAAAAAGGCTTAGAACGCCTC GAAGATCTTTTCCGTTTTCAGC	Stegger *et al.* (2012) [24]
tetK	TCGATAGGAACAGCAGTA CAGCAGATCCTACTCCTT	Ng *et al.* (2001) [47]
tetL	TCGTTAGCGTGCTGTCATTC GTATCCCACCAATGTAGCCG	Ng *et al.* (2001) [47]
tetM	GTGGACAAAGGTACAACGAG CGGTAAAGTTCGTCACACAC	Ng *et al.* (2001) [47]
tetO	AACTTAGGCATTCTGGCTCAC TCCCACTGTTCCATATCGTCA	Ng *et al.* (2001) [47]
msrA	TCCAATCATTGCACAAAATC AATTCCCTCTATTTGGTGGT	Martineau *et al.* (2000) [38]
ermA	TATCTTATCGTTGAGAAGGGATT CTACACTTGGCTTAGGATGAAA	Martineau *et al.* (2000) [38]
ermB	TATCTGATTGTTGAAGAAGGATT TTTACTCTTGGTTTAGGATGAAA	Martineau *et al.* (2000) [38]
ermC	CTTGTTGATCACGATAATTTCC ATCTTTTAGCAAACCCGTATTC	Martineau *et al.* (2000) [38]
aac(6')–aph(2'')	TTGGGAAGATGAAGTTTTTAGA CCTTTACTCCAATAATTTGGCT	Martineau *et al.* (2000) [38]
norA	TTCACCAAGCCATCAAAAAG CTTGCCTTTCTCCAGCAATA	Pourmand *et al.* (2014) [48]

Discussion

The diversity of bacterial populations in chronic wounds, such as diabetic foot ulcers, and the biofilm mode of growth of the infecting organisms, may be important contributors to the chronicity of wounds (Neut et al., 2011). All isolates carried genes *icaA*, *icaD* and *atl*, as expected, due to their virulence profile and ability to form biofilm. None of the isolates were positive for *pls* gene, and this may suggest the adhesion and cellular invasiveness properties of the studied isolates, considering that the MRSA surface protein *pls* reduces these virulence features (Savolainen et al., 2001; Hussain et al., 2009).

Biofilm formation, as widely described in literature, represents a big obstacle for the clinical efficacy of antibiotics, and the results of antimicrobial susceptibility testing cannot be directly applied to bacterial biofilm infections, due to the higher probability of failure (Patel, 2005). Biofilm can resist even to antibiotic concentrations 10-10000 times higher than the ones needed to kill planktonic cells (Kaplan, 2011). In this study, antibiotic concentrations required to inhibit or eradicate biofilm were much higher than the respective MIC values and should not be clinically applied. Furthermore, MBEC values were often several times higher than MBIC values.

Table 2. *In vitro* MIC, MBIC and MBEC values for the antibiotics tested against *S. aureus* DFU isolates (*CLSI range susceptibility).

	Antimicrobial agents									
	FOX	CPT	CIP	CLI	DOX	ERY	GEN	LZD	MEM	VAN
MIC range	1.5-256	0.064-38	0.06->32	0.015-0.06	0.064-0.125	0.12->256	0.06-64	1-2	0.015-16	0.25-1
MBIC range	2-256	0.5-8	0.5-512	0.5-128	0.5-512	0.5->256	0.5->128	1->1024	0.5-32	1-16
MBEC range	2-1024	0.5-1024	256-512	64->1024	64-128	64->1024	1->256	4->1024	0.5->1024	8->1024

FOX, cefoxitin (≤ 4 µg/ml*); CPT, ceftaroline (≤ 0.5 µg/ml *); CIP, ciprofloxacin (≤ 4 µg/ml *); CLI, clindamycin (≤ 0.25 µg/ml*); DOX, doxycycline (≤ 4 µg/ml*); ERY, erythromycin (≤ 8 µg/ml*); GEN, gentamicin (≤ 4 µg/ml*); LZD, linezolid (≤ 4 µg/ml*); MEM, meropenem (≤ 4 µg/ml*); VAN, vancomycin (≤ 2 µg/ml*).

Although all MRSA isolates should be considered as resistant to β-lactams *in vivo* (CLSI, 2013), almost all isolates were susceptible to meropenem. MBIC values for this antibiotic were thirty to one thousand times higher than MIC, being still in the range of susceptibility, however meropenem was unable to eradicate biofilm. The results suggested that cefoxitin is able to inhibit and eradicate *S. aureus* biofilms formed by MSSA isolates.

PCR amplification of *mecA* is considered the “gold standard” technique for detection of methicillin resistance among *S. aureus* (Siripornmongcolchai, Chomvarin, Chaicumpar, Limpaiboon & Wongkhum, 2002). However, the discovery of a new *mecA* homologous gene, *mecC*, determined the need to establish new detection protocols (Stegger et al., 2012), although normally the screening of the homologous gene is only performed in oxacillin-resistant *mecA* negative isolates (García-Álvarez, 2011). In this study, a multiplex assay was applied for the screening of *mecA* and *mecC* in all isolates, being possible to detect the *mecA* gene in 35% of the *S. aureus* DFU isolates (n=8). By the contrary, *mecC* was not detected in any isolates, which is not surprising because MRSA isolates harbouring *mecC* are currently

rare, and have only been reported in 13 European countries to date, not including Portugal (Paterson, Harrison & Holmes, 2014). The presence of *mecA* positive strains among the study isolates can be associated with the increasing prevalence of antibiotic-resistant bacteria, particularly MRSA, in DFU isolates, as described by Bowling et al. (2009). Also, Djahmi et al. (2013) suggested that MRSA prevalence may be related with the increase in antimicrobial treatment required, considering the high frequency of recurrent ulcers. Nowadays, penicillin resistance is present in about 90% of human *S. aureus* isolates. Two mechanisms are involved: the production of β-lactamases encoded by the *blaZ* gene and an altered penicillin-binding protein, PBP2a, encoded by *mecA* (El Feghaly, Stamm, Fritz & Burnham, 2014; Pereira, Harnett, Hodge, Cattell & Speers, 2014). In our study, only three isolates were positive for *blaZ*. This may be due to the primers used, because multiple polymorphisms within the *blaZ* gene have already been identified and the results can vary when different regions of the gene are targeted (El Feghaly et al., 2014), or may also be due to the fact that the isolates express penicillin resistance encoded by *mecA*.

Table 3. Antibiotic resistance phenotypes and genotypes of the *S. aureus* DFU isolates.

	Phenotype										Gene												
	FOX	CIP	CPT	CLI	DOX	ERY	GEN	LZD	MEM	VAN	*blaZ*	*mecA*	*mecC*	*ermA*	*ermB*	*ermC*	*msrA*	*norA*	*tetK*	*tetL*	*tetM*	*tetO*	*aac(6')-aph(2")*
I01	R	R	S	S	S	S	S	S	I	S	-	+	-	+	-	-	-	+	-	-	-	-	+
I02	S	R	R	S	S	S	S	S	S	S	-	-	-	-	-	-	-	+	-	-	-	-	-
I03	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	+	-	-	-	-	-
I04	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	+	-	+	-	-	-	-	-
I05	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	+	-	-	-	-	-
I06	R	R	S	S	S	R	S	S	S	S	-	+	-	+	-	-	-	+	-	-	-	-	-
I07	R	R	S	R	S	R	R	S	R	S	+	+	-	-	-	-	-	+	-	-	+	-	-
I08	R	R	R	S	S	R	S	S	I	S	-	+	-	-	-	+	-	-	-	-	-	-	-
I09	R	R	S	S	S	R	S	S	R	S	-	+	-	-	-	+	-	-	-	-	-	-	-
I10	S	R	S	S	S	R	S	S	R	S	-	-	-	+	-	-	-	+	-	-	-	-	-
I11	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	+	-	-	-	-	-
I12	S	S	S	S	S	S	S	S	S	S	+	-	-	-	-	-	-	+	-	-	-	-	-
I13	S	I	S	S	S	S	R	S	S	S	-	-	-	-	-	-	-	+	+	-	-	-	+
I14	S	S	S	S	S	S	R	S	S	S	+	-	-	-	-	-	-	+	+	-	-	-	+
I15	R	R	S	S	S	R	S	S	S	S	-	+	-	+	-	-	-	+	-	-	-	-	-
I16	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	-	+	-	-	-	-
I17	R	R	S	S	S	R	S	S	S	S	-	+	-	+	-	-	-	+	-	-	-	-	-
I18	R	R	S	S	S	R	S	S	S	S	-	+	-	+	-	-	-	+	-	-	-	-	-
I19	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	-	-	-	-	-	-
I20	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	+	-	-	-	-	-
I21	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	+	-	-	-	-	-
I22	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	+	-	-	-	-	-
I23	S	S	S	S	S	S	S	S	S	S	-	-	-	-	-	-	-	+	-	-	-	-	-

R, resistant; S, susceptible; I, intermediate; +, positive in specific PCR; -, negative in specific PCR. FOX, cefoxitin; CIP, ciprofloxacin; CPT, ceftaroline; DOX, doxycycline; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; MEM, meropenem; VAN, vancomycin. *blaZ*, penicillin resistance; *mecA* and *mecC*, oxacillin resistance; *ermA*, *ermB*, *ermC*, *msrA*, erythromycin resistance; *norA*, ciprofloxacin resistance; *tetK*, *tetL*, *tetM*, *tetO*, tetracycline resistance; *aac(6')-aph(2)"*, gentamicin resistance.

Antibiotic susceptibility tests showed that gentamicin and ceftaroline were the most potent agents against *S. aureus* biofilms, reaching clinical concentrations that can be applied to inhibit and eradicate biofilms. This was observed even for the MRSA isolates, since ceftaroline and gentamicin were effective in inhibiting biofilm production by seven of the eight MRSA isolates, while ceftaroline was effective in eradicating biofilm production by half of the MRSA isolates and gentamicin was effective in eradicating biofilm production by two isolates MRSA isolates. Today ceftaroline represents a good alternative to treat infections by *S. aureus* with reduced susceptibility to current agents, as recent studies have proven its efficacy against biofilm, applied alone or in combination (Barber, Werth, McRoberts & Rybak, 2014; Barber et al., 2015).

In 1999, Ceri et al. (1999) already described the efficacy of gentamicin against *S. aureus* biofilms, as well as other studies that followed (Kotulová & Slobodníková, 2010; Coraça-Huber et al., 2012). The *aac(6')-aph(2')* is the gene coding for the most frequently encountered aminoglycoside modifying enzyme (AME) in Gram-positive bacteria, which inactivates a broad range of clinically useful aminoglycosides, especially gentamicin and tobramycin; this enzyme is bifunctional because it catalyses both acetyltransferase and phosphotransferase reactions (Martineau et al., 2000). The *aac(6')-aph(2')* gene was found in three isolates, one MRSA and two MSSA, with no discrepant results with the resistance phenotypes obtained by e-test. These findings are in agreement with other studies, which reported that all aminoglycoside-resistant strains carried the *aac(6')-aph(2')* gene (Martineau et al., 2000).

Linezolid lacked activity against staphylococci biofilms because it didn't inhibit or eradicate biofilms, as already reported in other studies (Pettit et al., 2005; Smith, Perez, Ramage, Gemmell & Lang, 2009; Coraça-Huber et al., 2012). Clindamycin, doxycycline and vancomycin were effective against planktonic cultures and inhibited biofilm produced by most isolates; however, these antibiotics showed no ability to eradicate biofilms. This may suggest that these agents, although effective against bacteria in suspensions, may not be the most suitable antibiotics for treating biofilm related infections. Previous studies have shown that these antibiotics lack activity against staphylococci grown in biofilms (Pettit et al., 2005; Cafiso et al., 2010; LaPlante & Mermel, 2009; Rose & Poppens, 2009; Smith et al., 2009;).

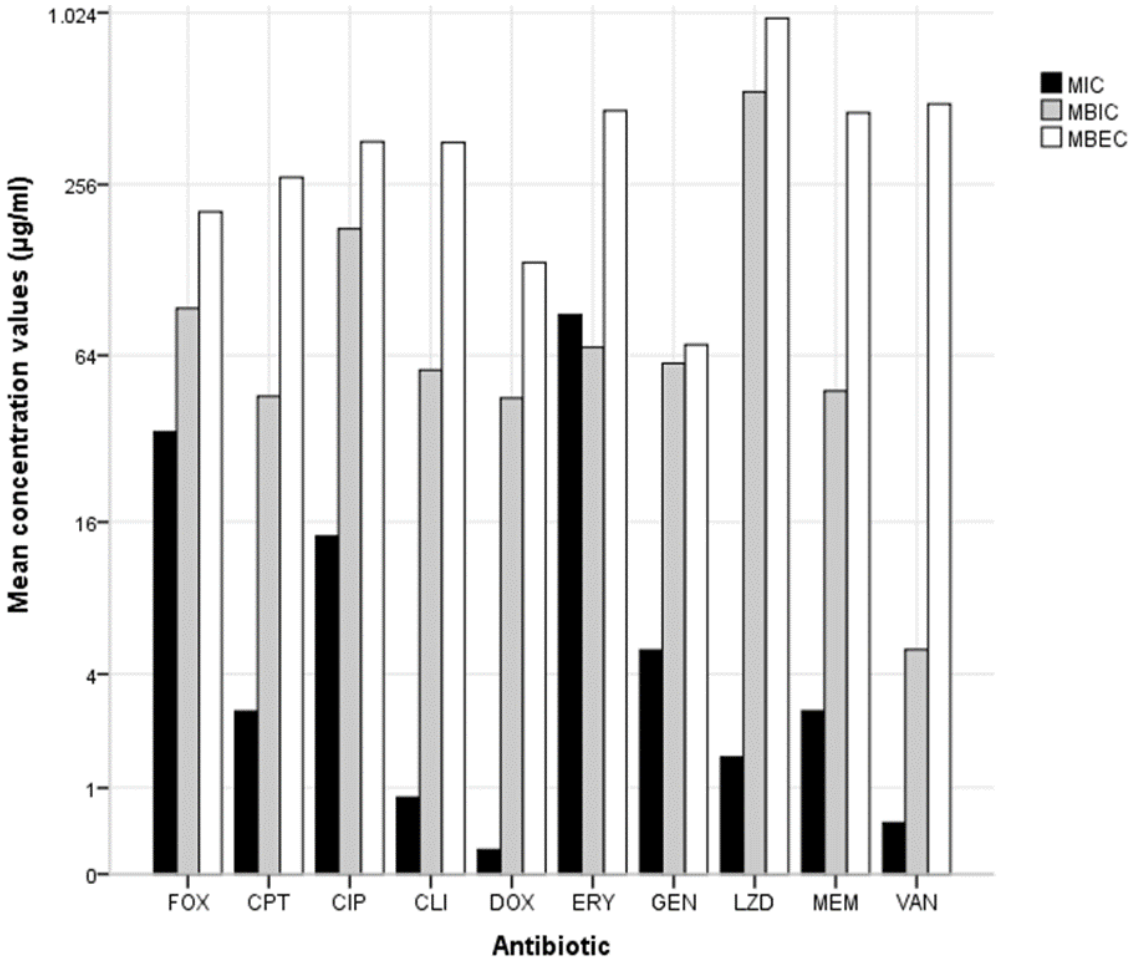
Although isolates presented a high rate of resistance to ciprofloxacin and erythromycin, their MBIC concentrations were about eight times higher than MIC values but were still clinically adequate. They were not able to eradicate biofilm, as previously described for ciprofloxacin in other studies (Pettit et al., 2005; Ceri et al., 2009). Several genes are implicated in erythromycin resistance, especially in staphylococci and streptococci. The gene *ermA* is located on the transposon Tn554 and has a single specific site for insertion into the *S. aureus* chromosome; the *ermB* gene is located on the transposon Tn551 of a penicillinase plasmid; the *ermC* gene is generally located on small plasmids and is responsible for constitutive or inducible resistance to erythromycin (Martineau et al., 2000). Staphylococcal strains resistant

to macrolides and type-B streptogramins also frequently harbor *msrA,* which encodes an ATP-dependent efflux pump (Martineau et al., 2000). In this study, *ermA* was found in five resistant and one sensible erythromycin staphylococci. Regarding *ermC*, this gene was less frequently found than *ermA,* namely in two resistant and one sensible erythromycin staphylococci. These results are according to previous reports, in which *ermA* was the most prevalent *erm*-gene followed by *ermC* (Martineau et al., 2000; Werner, Cuny & Schmitz, 2001; Strommenger, Kettlitz & Werner, 2003). The discrepance between the erythromycin susceptible isolates and their *erm* positivity, was already described (Martineau et al., 2000). Numerous factors could explain the sensitive phenotype in these strains, including regulation of *erm* genes and absence of host factors associated with the expression of erythromycin resistance. These factors can also explain the cefoxitin-susceptible *S. aureus* isolates carrying the *mecA* gene.

The increasing prevalence of MRSA has led to a new interest in the usage of macrolide-lincosamide-streptogramin B (MLS_B) antibiotics to treat *S. aureus* infections, with clindamycin being the preferred agent due to its excellent pharmacokinetics. However, this increased application promoted a raise in resistance to MLS_B antibiotics. Clindamycin resistance is commonly caused by a one target site modification mediated by *erm* genes, difficult to detect *in vitro*, as they appear erythromycin resistant and clindamycin sensitive (Jv, Janakiram & Vijaya, 2015).

Tetracycline resistance determinants are widespread among bacterial species, consisting in active efflux pumps that result from acquisition of plasmid-located genes, *tetK* and *tetL*, and in ribosomal protection mediated by transposon or chromosomal located genes *tetM* or *tetO* (Werner et al., 2001; Paterson et al., 2014). MRSA isolates typically show *tetM* or *tetKM* genotype; *tetK* is the most frequent genotype found in S. aureus, followed by *tetM* (Smith et al., 2009; Andersen et al., 2015; Jv et al., 2015). The same was observed in this study, in which three MSSA isolates were *tetK* positive and one MRSA was *tetM* positive; *tetL* and *tetO* were not found. Tet -positive isolates were sensible to doxycycline. In *tetK* positive isolates, this gene confers high resistance to tetracycline, oxytatracycline, chlortetracycline but low resistance to monocycline, 6-demethyl-6-deoxytetracycline and doxycycline (Andersen et al., 2015). Surprisingly, the only *tetM* positive isolate was doxycycline sensible. Since this gene is believed to confer resistance to all drugs of tetracycline group (Trzcinski, Cooper, Hryniewicz & Dowson, 2000), it may be suggested that prevalence of resistance to tetracyclines in *S. aureus* is underestimated, or, as demonstrated by Trzcinski et al. (2000), recognition of tetracycline resistance in *S. aureus* strains often depends on the different interpretation guidelines used.

Figure 1. Minimum inhibitory concentration (MIC), minimum biofilm inhibitory concentration (MBIC) and minimum biofilm eradication concentration (MBEC) of *S. aureus* DFU isolates as determined by a modified version of the Calgary Biofilm Pin Lid Device. FOX, cefoxitin; CIP, ciprofloxacin; CPT, ceftaroline; DOX, doxycycline; ERY, erythromycin; GEN, gentamicin; LZD, linezolid; MEM, meropenem; VAN, vancomycin.



In recent years, an increase in fluoroquinolone resistance in S. aureus, including MIC strains, has been spreading worldwide. Resistance mechanisms to these antibiotics involve mutations within the *gyrA* and *gyrB* genes, which encode for subunits of DNA gyrase, an established target of fluoroquinolones; analogous mutations in *grlA* and *grlB*, which encode for subunits of DNA topoisomerase IV; and the increased expression of the *norA* gene, which encodes for a drug efflux protein, *norA*, and mutations in the *norA* coding region (Trzcinski et al., 2000; Arciola et al., 2001). *NorA* is a membrane protein, which actively transports norfloxacin and other hydrophilic fluoroquinolones out of the bacterial cell, thus effectively decreasing the intracellular concentration of the drugs (Trzcinski et al., 2000). Nineteen isolates were positive for *norA* gene but eleven of them were susceptible to ciprofloxacin.

From a clinical perspective, the discrepancy between genotype and phenotypic resistance expression suggest that a susceptible strain harbouring, but not expressing, an antibiotic resistance gene should be regarded as potentially resistant to that antibiotic. Overall, we did not detect a significant presence of antibiotic resistance genes, compared to the great biofilm resistance of the isolates, even when using high antimicrobial concentrations.

Conclusions

To our knowledge, this is the first study on antibiotic susceptibility tests targeting biofilm-producing *S. aureus* isolates from diabetic foot infections. It was found that only very high concentrations of the most used antibiotics in DFU were capable to inhibit *S. aureus* biofilms in vitro, which may explain why monotherapeutics frequently fail to eradicate biofilm infections. Biofilms were resistant to antibiotics concentrations 10 to 1000 times higher than the concentrations needed to kill free-living or planktonic cells. This high level of resistance in biofilms makes chronic infections, like DFI, extremely difficult to eradicate using conventional antimicrobial therapy.

MIC values were not always predictive of the MBIC and MBEC values. Only gentamicin and ceftaroline proved to be effective in eradicating biofilms, formed by half of the isolates at clinical drug concentrations, while the other tested drugs were only able to inhibit adherent cells. In particular, ceftaroline showed a very good potential for inhibiting and eradicating biofilms produced by MRSA isolates. It is clear that antibiotic susceptibility values for planktonic populations are not necessarily applicable to effective treatment of infections by the same organism, once a biofilm has been established. These differences may be an important factor in the selection of antimicrobial therapy for most of DFI, for *S. aureus* is the main virulent organism involved, rendering important the investigation of antibiotic susceptibility of biofilm infections.

CHAPTER V

Characterization of multidrug resistant diabetic foot ulcer enterococci

5.1. Characterization of multidrug-resistant diabetic foot ulcer enterococci

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* These authors contributed equally to the conception and design of the study, the conduction of the experiments, the performance of the data analysis process and the draft of the manuscript.

Abstract

Background: Diabetes *mellitus* is a highly prevalent chronic progressive disease with complications that include diabetic-foot ulcers.

Methods: Enterococci isolated from diabetic foot infections were identified, evaluated by macrorestriction analysis, and screened for virulence traits and antimicrobial resistance.

Results: All isolates were considered multidrug-resistant, cytolysin and gelatinase producers, and the majority also demonstrated the ability to produce biofilms.

Conclusions: These results indicate the importance of enterococci in diabetic foot infection development and persistence, especially regarding their biofilm-forming ability and resistance to clinically relevant antibiotics.

Keywords: Diabetic foot infections, *Enterococcus*, Multi-drug-resistant (MDR), Virulence factors, Biofilms.

Background

Diabetes *mellitus* is a serious health problem in rapid expansion worldwide (Zhang et al., 2015). One the most frequent diabetes complications being the development of diabetic foot infections - DFI, which represent a major cause of morbidity and mortality among patients. Antibiotherapy continues to be the most important approach to solve or control such infections, however, increasing bacterial resistance to a growing number of antimicrobial agents, frequently results in treatment failure. Previous reports (Mendes et al., 2012) point towards gram-positive cocci as the most common pathogens in DFI samples, contributing to the persistence/severity of the disease and leading to higher morbidity and mortality rates. Members of the *Enterococcus* genus are known to be among such bacteria. In this context, the present study aimed to evaluate the diversity, antimicrobial drug resistance, biofilm forming ability and virulence patterns of enterococci isolated from diabetic foot infections.

Methods

Bacterial isolates

The study was conducted in 4 clinical centers (2 outpatient clinics, 1 general surgery ward and 1 vascular surgery ward) in Lisbon from January 2010 to June 2010. Specimens (aspirates, biopsies and swabs) were obtained from patients with diabetes *mellitus* and clinically infected foot ulcers, as advised by current clinical guidelines (NICE, 2011). After collection, forty-nine clinical samples were screened for the presence of *Enterococcus* spp. using conventional microbiological procedures (Mendes et al., 2012).

Molecular characterization

Following DNA isolation by boiling lysis, genus and species allocation were performed according to methodologies described elsewhere (Jackson, Fedorka-Cray & Barrett, 2004). Macrorestriction analysis by Pulsed-Field Gel Electrophoresis - PFGE - was applied as previously reported (Turabelizde, Kotetishvili, Kreger, Morris & Sulakvelidze, 2000) and data generated were analyzed using the BioNumerics 6.6 software (Applied Maths, Kortrijk, Belgium).

Virulence factors

Application of previously described protocols (Semedo-Lemsaddek et al., 2012) included screening for genes coding for aggregation substance – *agg*, the *E. faecalis* antigen A – *efaAfs*, the enterococcal surface protein – *esp*, gelatinase – *gelE*, the cytolysin activator – *cylA* and plate assays for the evaluation of hemolytic and gelatinolytic phenotypes.

Evaluation of biofilm forming ability was by Fluorescent in Situ Hybridization – FISH

Biofilm production was evaluated in vitro as described elsewhere (Oliveira et al., 2007).

Antibiotic susceptibility tests

Susceptibility to sixteen antimicrobial agents, representing distinct classes, was evaluated by the disk diffusion method, using previously established breakpoints of resistance (CLSI, 2013). MICs for vancomycin were further determined using E-test.

Table 1. Data regarding enterococcal isolates and DFI patients.

				Patient information				
Isolate	**Enterococcal species**	**Sample**	**Hospital***	**Age**	**Gender**	**Years of diabetes**	**Previous ulcers**	**Amputation**
A5-3	*Enterococcus faecalis*	aspirate	APDP	53	M	47	4	1
B4-2	*Enterococcus galinarum*	biopsy	HSAC	70	M	20	0	0
B4-3	*Enterococcus galinarum*							
B8-2	*Enterococcus faecalis*	biopsy	HSAC	66	F	20	0	0
B10-2	*Enterococcus faecalis*	biopsy	HSAC	76	F	20	3	0
Z5-4	*Enterococcus faecalis*	swab	HCC	69	M	15	1	0
Z8-2	*Enterococcus faecalis*	swab	HCC	67	M	20	1	1
Z11-2	*Enterococcus faecalis*	swab	HCC	47	M	30	1	1
Z11-3	*Enterococcus faecalis*							
Z15-1	*Enterococcus faecalis*	swab	HCC	61	M	50	0	0
Z22-2	*Enterococcus faecalis*	swab	APDP	26	M	26	3	0
Z24-3	*Enterococcus faecalis*	swab	HCC	77	M	13	4	1

Note: The present study was approved by the Faculty of Medicine of the University of Lisbon Research Ethics Committee and the Portuguese Data Protection Authority, and written informed consent was obtained for every patient.

* APDP - Associação Protectora dos Diabéticos de Portugal; HCC - Hospital Curry Cabral (Centro Hospitalar Lisboa Central EPE) and HSAC - Hospital Santo Antonio dos Capuchos (Centro Hospitalar Lisboa Central EPE).

Results and Discussion

The pathogenesis of foot ulceration is complex, the mortality is high and healed ulcers often recur, resulting in severe chronic foot infections. Additionally, the indiscriminate misuse and abuse of antibiotics for DFI treatment has triggered an increase in the development of multidrug-resistances, leading to serious public health issues due to treatment failure. Most DFI have a polymicrobial etiology, enterococcal strains being part of the complex diabetic foot microbiota (Zhang et al., 2010; Mendes et al., 2012). The present study screened forty-nine samples from DFI for the presence of *Enterococcus* spp. Twelve enterococci were recovered (see Table 1 for further details) and identified as *E. faecalis* (Magiorakos et al., 2011) and *E. gallinarum* (Mendes et al., 2012). The higher prevalence of *E. faecalis* among the diabetic foot ulcer enterococci corresponds to the expected, as this species is considered the most

pathogenic of this genus, being commonly associated with clinical samples (Higuita & Hui, 2014).

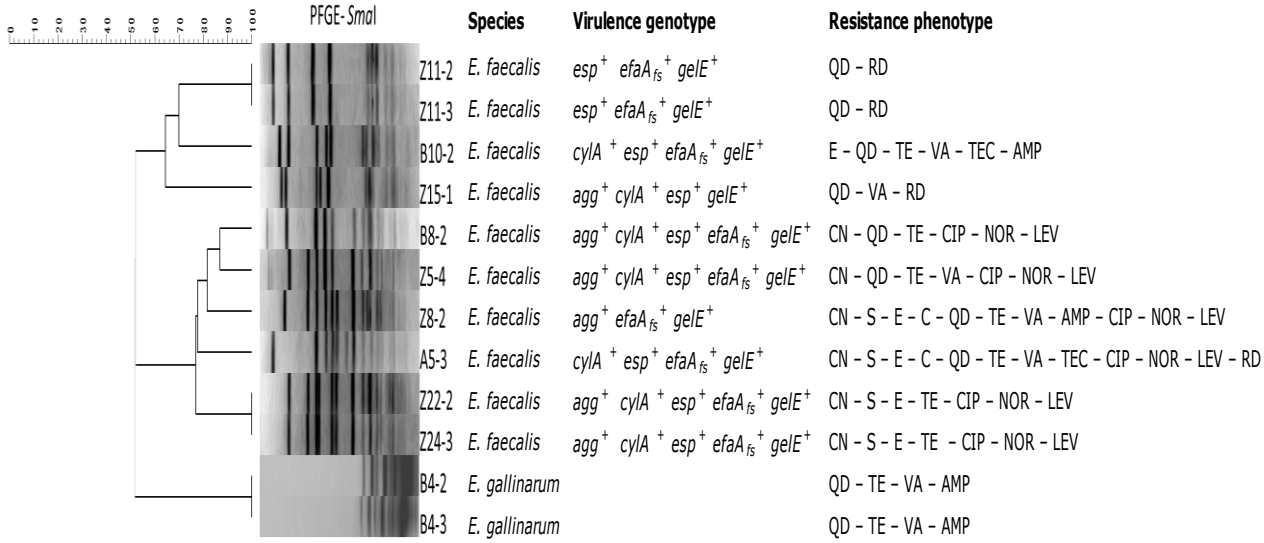
Macrorestriction analysis was the methodology chosen to assess the genomic diversity of the enterococci under study. The dendrogram built based on macrorestriction patterns (pulsotypes) allocated the enterococcal isolates into nine genomic groups (Figure 1). Results demonstrated that no single enterococci was present in all the samples under analysis, although high similarity levels could be observed between distinct isolates, revealing their clonal relationships. Briefly, the two *E. gallinarum* share the same pulsotype; since they were isolated from the same patient it can be established that they are identical, or highly related. Similar conclusions can be drawn for *E. faecalis* Z11-2 and Z11-3 (swab samples from the same patient), as well as for Z22-2 and Z243, obtained from DFI of patients attending distinct healthcare units, which apparently harbor the same enterococcal isolate. However, to further prove the persistence of specific enterococci in DFI, additional sample collection should be performed over the years in the same patients.

Concerning the screening for virulence features (see Figure 1), all DFI enterococci present hemolytic and gelatinolytic abilities and the *E. faecalis* DFI isolates harbor distinct virulence determinants. Since the screened virulence traits are considered among the most relevant for enterococcal pathogenicity mechanisms, often detected in clinical isolates and correlated with the persistence and severity of infection (Higuita & Huy, 2014) these results constitute important indicators for the putative pathogenicity of the DFI enterococci under study.

Furthermore, analysis of phenotypic biofilm expression revealed 83% (10/12) of biofilm producers at 48h, with negative results being associated only with the non-*E. faecalis* DFI enterococci. Due to the known importance of biofilms in the persistence of human infections, such as DFI, the biofilm forming ability demonstrated by the enterococci further demonstrates their putative contribution for the chronicity of infection.

Regarding antibiotic resistance, all isolates were simultaneously resistant to several antibiotics, representing distinct drug classes and directed towards various bacterial targets (Figure 1). Considering as multidrug-resistant -MDR- the enterococci non-susceptible to more than 3 antibiotics representing distinct classes and bacterial targets,10 the majority of the isolates under analysis fall into the MDR category. Although vancomycin MIC determination showed that none of the isolates are resistant to this drug (MIC ≤ 4 μg/ml), the MDR status attributed to the majority of the enterococci continues to be highly relevant, especially in chronic severe infections such as DFI, since antimicrobial resistance often results in treatment failure.

Figure 1. Dendrogram based on SmaI-PFGE patterns. The BioNumerics 6.6 software (Applied Maths, Kortrijk, Belgium) was used to register macrorestriction patterns and clustering analysis was performed using Dice similarity coefficient and the unweighted-pair group method with arithmetic mean (UPGMA).



Samples: A – aspirate, B – biopsy, Z – swab. Virulence determinants: agg – aggregation substance, cylA – cytolysin activator, efaAfs – cell wall adhesin, esp – cell wall-associated protein, gelE – gelatinase. Antibiotics: AMP – ampicillin, C – chloramphenicol, CIP – ciprofloxacin, QD – quinupristin-dalfopristin, E – erythromycin, CN – gentamicin-120, LEV – levofloxacin, LZD – linezolid, F – nitrofurantoin, NOR – norfloxacin, RD – rifampicin, S – streptomycin-300, TEC – teicoplanin, TE – tetracycline, VA – vancomycin.

Overall, the present study demonstrated that DFI enterococci harbor virulence determinants, which are associated with biofilm-forming ability and resistance to medically important antibiotics, suggesting their contribution to the persistence and severity of diabetic foot infections. The presence of multidrug-resistant diabetic foot ulcer enterococci is of major importance also due to the possibility of transmitting those multi-drug resistances to other microorganisms sharing the same ecological niche, highly impairing the implementation of successful antibiotic treatment. Since DFI are one of the most frequent diabetes complications, which represent a major cause of morbidity and mortality among patients, further studies directed towards the evaluation of the role of enterococci, during the establishment and persistence of infection, are fundamental.

CHAPTER VI

In vitro design of a novel lytic bacteriophage cocktail with therapeutic potential against organisms causing diabetic foot infections

6.1. *In vitro* design of a novel lytic bacteriophage cocktail with therapeutic potential against organisms causing diabetic foot infections

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* The author participated in the conception and design of the evaluation of combined bacteriophage activity against established biofilms, performed the corresponding data analysis and helped to draft the manuscript.

Abstract

Background: In patients with diabetes mellitus, foot infections pose a significant risk. These are complex infections commonly caused by *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, all of which are potentially susceptible to bacteriophages.

Methods: Here, we characterized five bacteriophages that we had determined previously to have antimicrobial and wound-healing potential in chronic *S. aureus*, *P. aeruginosa* and *A. baumannii* infections.

Results: Morphological and genetic features indicated that the bacteriophages were lytic members of the family Myoviridae or Podoviridae and did not harbour any known bacterial virulence genes. Combinations of the bacteriophages had broad host ranges for the different target bacterial species. The activity of the bacteriophages against planktonic cells revealed effective, early killing at 4 h, followed by bacterial regrowth to pre-treatment levels by 24 h. Using metabolic activity as a measure of cell viability within established biofilms, we found significant cell impairment following bacteriophage exposure. Repeated treatment every 4 h caused a further decrease in cell activity. The greatest effects on both planktonic and biofilm cells occurred at a bacteriophage : bacterium input multiplicity of 10.

Conclusions: These studies on both planktonic cells and established biofilms allowed us to better evaluate the effects of a high input multiplicity and a multiple-dose treatment protocol, and the findings support further clinical development of bacteriophage therapy.

Keywords: Diabetic foot infections, Bacteriophage therapy, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*.

Background

Diabetes *mellitus* affects an estimated 171 million patients worldwide (Wild et al., 2004) and has become a major world epidemic. Even with the best preventative care, 9% of patients will develop a diabetic foot infection (DFI), which brings the consequent risk of amputation (Lavery et al., 2006). Qualitative and quantitative aspects of wound microbiology are critical determinants of the wound outcome. Gram-positive microorganisms are the first to colonize and acutely infect breaks in the skin, whereas chronic wounds develop a more complex polymicrobial microbiology, including aerobic Gram-negative rods (Lipsky et al.,2004).

These microorganisms aggregate in communities encased within extracellular polymeric substances on the wound surface. Such an entity is defined as a biofilm, and shows increased resistance to immunological and antimicrobial attack (Percival et al., 2012). In current clinical practice, DFI treatment includes debridement and systemic antibiotics (Lipsky et al., 2004). The increased incidence of antibiotic- resistant bacterial strains, such as methicillin-resistant *Staphylococcus aureus* and pan-drug-resistant, non-fermenting, Gram-negative bacilli, threatens the efficacy of antimicrobial therapy (Mendes et al., 2012). Thus, it is necessary to identify new therapeutic strategies for DFI.

Bacteriophages are viruses that consist of a genome contained within a protein coat and that specifically infect bacteria. In contrast to filamentous bacteriophages, the multiplication of tailed bacteriophages and release of the newly formed virus particles always involves lysis of the host bacterial cell. However, among tailed bacteriophages, some may not immediately follow this lytic pathway. The genome of these so-called temperate bacteriophages may instead reside in the host cell (integrated in the bacterial chromosome or in a plasmid-like form in the cytoplasm) and be propagated for several bacterial generations without lysis. In contrast, strictly lytic phages do not have this option and usually undergo the lytic pathway once inside the bacterial host (Ansaldi, 2012). Bacteriophage therapy (BT) is the use of lytic bacteriophages to reduce or eliminate pathogenic bacteria. BT has become a broadly relevant technology for veterinary, agricultural and food microbiological applications; however, the treatment of human infections with BT attracts the greatest interest (Kutter et al., 2010).

The use of bacteriophages as antibacterial agents for suppurative infections began shortly after the discovery of bacteriophages. Bruynoghe and Maisin first demonstrated BT, using bacteriophages to treat S. aureus skin infections (Bruynoghe & Maisin, 1921). However, following the discovery and general application of antibiotics, interest in the therapeutic uses of bacteriophages waned. Recently, the increase in antibiotic-resistant bacterial strains has reinvigorated enthusiasm about these bacteria-specific viruses (Chopra, Hodgson, Metcalf & Poste, 1997). This interest is particularly true in cases in which bacteriophages can be applied externally (topical application), as is the case for DFI.

The development of an effective BT is a multistep process consisting of: (i) bacteriophage isolation and assessment for antibacterial activity against specific bacterial strains; (ii)

bacteriophage characterization and screening for un- desirable traits; (iii) in vitro posology and dosage regimen design; (iv) pre-clinical animal efficacy and toxicology studies; and (v) regulated human clinical trials. Although the use of bacteriophages to treat DFIs is promising, difficulties in any of these steps can hinder widespread clinical application (Abedon, 2010).

Recently, we demonstrated the antimicrobial activity and wound-healing capability of a topically delivered bacteriophage suspension against wounds chronically infected with chronic *S. aureus*, *Pseudomonas aeruginosa* and *Acinetobacter baumannii* in two animal models of diabetes mellitus (Mendes et al., 2013). In the current study, we present a characterization of the bacteriophages used in this previous study. We examined their spectrum of activity, genetic and morphological structures, and activity against planktonic cells and established biofilms. Collectively, the findings justify the posology and dosage regimen used in the animal studies.

Methods

Bacterial strains

The *S. aureus* 743/06, *P. aeruginosa* 433/07 and *A. baumannii* 1305/05 host strains were isolated from human clinical samples that were collected and identified in hospitals in the Lisbon area. The three strains were characterized previously as biofilm producers (Mottola et al., 2013). Bacterial clinical isolates used for bacteriophage host-range investigation included *S. aureus* (n5132), *P. aeruginosa* (n593) and *A. baumannii* (n5103) from wound specimens. Of these isolates, 44 were from DFI. The epidemiology, clinical details and specific microbiology of our collection of DFI isolates have been described previously (Mendes et al., 2012). All isolates were stored in tryptone soy broth (TSB; Biokar Diagnostics) with 15% glycerol (w/v) at - 70°C until needed. For the experiments, single bacterial colonies were grown in TSB at 37°C. After a 24h incubation, the bacterial cells were suspended in saline and adjusted to McFarland's scale 0.5 (bioMérieux), producing a final working suspension of approximately 5.0 x 10^8 c.f.u. ml^{-1}.

Bacteriophage isolation, amplification and purification

S. aureus F44/10 and F125/10, *P. aeruginosa* F770/05 and F510/08, and *A. baumannii* F1245/05 bacteriophages were isolated from environmental water samples from the Lisbon area. Standard methods for bacteriophage isolation (Adams, 1959) were employed for all five bacteriophages using the host strains described above. The obtained bacteriophage plaques were purified by repeated single plaque isolation to ensure that each contained only one type of bacteriophage.

To produce bacteriophage stocks in sufficient quantities for the experiments, a previously described protocol of amplification, concentration by high-speed centrifugation and purification on a CsCl gradient (Miller, 1987) was used for all five bacteriophages. Briefly, a final lysate of

each bacteriophage was centrifuged at 10.000 g for 20 min at 4°C. The pellet was discarded, and the supernatant fraction was concentrated overnight at 8000 r.p.m. (JA-14 rotor; Beckman Coulter). The bacteriophage pellet was resuspended in SM buffer (5.8 g NaCl l^{-1}, 2 g $MgSO_4.7$ H_2O l^{-1}, 50 ml 1 M Tris/HCl, pH 7.5). This concentrated bacteriophage suspension was loaded onto a discontinuous CsCl gradient and centrifuged at 30.000 r.p.m. for 5h at 4°C in a Beckman L-90 ultracentrifuge with an SW41Ti rotor (Beckman Coulter). The banded bacteriophage particles were collected and thoroughly dialysed against SM buffer. Final bacteriophage titres were determined using double agar overlay plaque assays (Kropinski, Mazzocco, Waddell, Lingohr & Johnson, 2009). Purified bacteriophages were stored at 4°C and further diluted in SM buffer to achieve a working suspension of approximately 2 x 10^{10} p.f.u. ml^{-1} prior to the assays.

Morphology of bacteriophages

The morphology of each of the five bacteriophages was analysed by transmission electron microscopy at the Félix d'Hérelle Reference Center for Bacterial Viruses, Laval University, Québec, Canada. Briefly, a 200-mesh Formvar carbon-coated copper grid (Pelco International) was deposited face down on 10 ml staining suspension (2% uranyl acetate, pH 7.0, for all bacteriophages except for F770/05, which was stained with 2% phosphotungstic acid, pH 7.0). After 30 s, 10 ml bacteriophage suspension was mixed with the stain. After 2-3 min, the grid was deposited face up on blotting paper. The grid was dried for 5 min and then observed at 80 kV using a JEOL 1230 transmission electron microscope. These data were integrated with the genomic analysis, and the bacteriophages were classified according to the Ackermann (2009) classification.

Genomic analysis of bacteriophages

The DNA of all five bacteriophages was isolated using a standard phenol/chloroform extraction and DNA precipitation protocol (Sambrook, Fritsch & Maniatis, 1989). The purified nucleic acid was sent to Macrogen for commercial sequencing. In brief, pyrosequencing of the sample DNA was performed using a GS FLX Titanium General Library Preparation kit (Roche 454 Company) according to the manufacturer's instructions. The assembly of quality-filtered reads was performed using Genome Sequencer De novo Assembler software (Newbler) version 2.5.3. An extensive bioinformatics evaluation was conducted to analyse the sequences and identify regions of similarity with entries in databases, which yield clues about structure and function. Each genome sequence was scanned using the National Center for Biotechnology Information (NCBI) BLASTN and BLASTX bioinformatics tools (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Prediction of ORFs was performed by integrating the results obtained by the programs GeneMark.hmm (http://exon.gatech.edu/genemark/eukhmm.cgi) and MetaGeneAnnotator

(http://metagene.cb.k.u-tokyo.ac.jp). Protein homology searches were performed with the BLASTP program (http://blast. ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins) using the NCBI non- redundant protein sequence database. The genome sequences were deposited in the patent division of GenBank (specific patent nos: WO2010090542 and WO2012036580).

Bacteriophage host range

The five bacteriophages were tested against a panel of clinical isolates using the bacteriophage spot-test procedure (Armon & Kott, 1993). Briefly, 3 ml top-0.7% tryptone soy agar (TSA; Biokar Diagnostics) was added to 200 ml overnight culture of each clinical isolate and poured over TSA. The agar was allowed to solidify, after which 5 ml each bacteriophage suspension (approx. 10^8 p.f.u.) was spotted on the bacterial lawn of each different isolate. The drop was allowed to dry, and the plates were incubated overnight at 37°C. Specific bacteriophage-sensitive isolates showed clear areas where the bacteriophage suspensions had been spotted.

Bacteriophage activity against planktonic cells

To determine the activity of the bacteriophages against planktonic cells *in vitro*, a kinetic time-kill assay (National Committee for Clinical Laboratory Standards [NCCLS], 1999) was performed using a modified protocol. Briefly, 1 ml host bacterial suspension (5 x 10^8 c.f.u.) was diluted in 9 ml TSB, yielding a final concentration of 5 x 10^7 c.f.u. ml^{-1}. For single-bacteriophage studies, 100 ml (5 x 10^9 p.f.u.) specific bacteriophage was added, yielding a final concentration of 5 x 10^8 p.f.u. ml^{-1} [input multiplicity (IM) of bacteriophage : bacterium of 10]. For combination studies, 100 ml (5 x 10^9 p.f.u.) each bacteriophage suspension was added, resulting in a final concentration of 5 x 10^8 p.f.u. ml^{-1} (IM of 10) for each of the bacteriophages. An additional kinetics assay was performed for *P. aeruginosa* 433/07, in which 10 ml (5 x 10^8 p.f.u.) bacteriophage F770/05 suspension was added (yielding an IM of 1), alone or in combination with the bacteriophage F510/08 at an IM of 10. Control experiments were performed in parallel using bacteriophage buffer instead of a bacteriophage suspension. All mixtures were incubated at 37°C with agitation, and 100 ml aliquots were collected at 0, 1, 3, 5 and 24 h post-infection (p.i.). Bacterial quantification was performed using a 10-fold serial dilution method (Murray, Baron, Jorgensen, Pfaller & Yolken, 2003). All experiments were conducted in triplicate. The results are presented as the mean±SD and are expressed as log- transformed values [log (c.f.u. ml^{-1})] over time.

Combined bacteriophage activity against established biofilms

The activity of the bacteriophages against established biofilms was examined using a modification of previously described protocols (Cerca et al., 2005; Pettit et al., 2005). Briefly, 1 ml of each of the host bacterial suspensions (5 x 10^8 c.f.u.) was diluted in 9 ml TSB, and 100

ml of this dilution (5 x 10^6 c.f.u.) was added to a 96-well flat-bottomed polystyrene microtitre plate (Orange Scientific) and incubated at 37°C for 24h to allow biofilm formation. After incubation, the planktonic bacteria were removed carefully with a sterile pipette. The number of biofilm cells at 24h has been demonstrated previously to be approximately 10^7 c.f.u. per well for all bacterial species (Mottola et al., 2013). Next, 150 ml bacteriophage suspension (IMs of 10 and 100) diluted in TSB was added to the wells. The following bacteriophage suspensions were used for each bacterium: for *S. aureus*, a 1 : 1 combination of F44/10 and F125/10; for *P. aeruginosa*, a combination of F770/05 and F510/08 at a 1 : 10 ratio; and for *A. baumannii*, F1245/05 alone. Biofilms treated with TSB alone served as positive controls in measurements of cell metabolic activity (see below).

The microplates were incubated at 37°C for 4 or 24h. At each time point, the wells were processed according to a previously described protocol (Pettit et al., 2005) using alamarBlue (AB; Thermo Scientific), and their absorbance at 570 and 600 nm was measured using a SpectraMax 340PC microplate reader (Molecular Devices). A second assay was performed in which, after biofilm formation, planktonic bacteria were removed from the wells and replaced with a bacteriophage suspension every 4h over a 24h incubation period. In the positive-control group, planktonic bacteria were removed from the wells and replaced with TSB every 4h. These plates were then processed as described previously (Pettit et al., 2005).

Biofilm susceptibility experiments were performed a minimum of three times. All results are presented as the percentage variation of AB±SD. This value was calculated using the manufacturer's formula, with one exception: the medium-only negative control in the formula was replaced by a more robust negative control that consisted of medium plus bacteriophage at each IM (i.e. IMs of 10 and 100). Strong antimicrobial suppression was defined as a ≥ 50% reduction in AB compared with the positive control.

Statistical analysis

For all datasets, comparisons between groups were performed using two-tailed Student's t-tests, and values of $P<0.05$ were considered significant. All data were entered into a spreadsheet program (Excel; Microsoft) for statistical analysis. Analytical statistics were performed using Analyse-it, version 2.21 Excel 12+ (Analyse-it Software), a statistical add-in program for Excel.

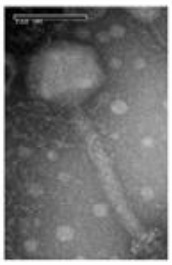
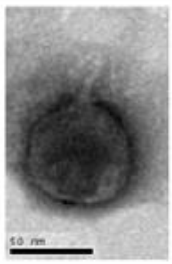
Results

Bacteriophage features

After purification, the selected bacteriophages were initially characterized according to plaque morphology. The *S. aureus* F44/10 and F125/10, *P. aeruginosa* F770/05 and F510/08, and *A. baumannii* F1245/05 bacteriophages produced clear lytic plaques ranging from 1.5 to 5 mm in diameter. Plaques produced by the bacteriophages F770/05, F510/08 and F1245/05 were

surrounded by growing opaque halo zones. The morphological and genomic characteristics of the five bacteriophages are presented in Figure 1.

Figure 1. Morphological and genomic characteristics of the bacteriophages used for bacteriophage therapy. Five bacteriophages previously shown to successfully treat infections in vivo were characterized using transmission electron microscopy. Representative images are shown. The genomes were sequenced by pyrosequencing and analyzed extensively using BlastN, BlastX, GeneMark.hmm, MetaGeneAnnotator, and BlastP. ICTV = International Committee on Taxonomy of Viruses.

		Staphylococcus aureus		*Pseudomonas aeruginosa*		*Acinetobacter baumannii*
		F44/10	**F125/10**	**F770/05**	**F510/08**	**F1245/05**
Morphology	**ICTV classification**	*Myoviridae*		*Podoviridae*		
	electron micrograph					
		capsid 100±8 nm tail: 225±12 nm	capsid: 92±7 nm tail: 234±11 nm	capsid: 69±3 nm tail: 48±8 nm	capsid: 68±3 nm	capsid: 63±3 nm
Genome	**nucleic acid**	linear dsDNA	linear dsDNA	linear dsDNA	linear dsDNA	linear dsDNA
	size	137,360 bp	144,994 bp	72,177 bp	43,313 bp	43,016 bp
	homology group	*K-like*	*K-like*	*N4-like*	*ϕKMV-like*	undefined

Morphology

To classify the purified bacteriophages based on their virion morphology, we used transmission electron microscopy. The staphylococcal bacteriophages F44/10 and F125/10 appeared to be composed of a contractile tail and an isometric head, with a baseplate structure also discernible at the tip of the F44/10 tail. These features, along with their genomic properties (see below), allowed us to classify F44/ 10 and F125/10 as members of the family Myoviridae. The Pseudomonas bacteriophages F770/05 and F510/08 and the Acinetobacter bacteriophage F1245/05 had short tails and were classified as members of the family Podoviridae. The family Podoviridae consists of different subgroups. Although there are certain morphological similarities between the bacteriophages F510/08 and F1245/05 and the

wKMV-like group (Chang et al., 2011; Lammens et al., 2009), definite morphological assignment of F1245/05 could not be performed due to the uncharacteristic morphology of these virion particles.

Genomic analysis

The bacteriophages were characterized at the genomic level by determining and analysing their genome sequences. The bacteriophages F44/10 and F125/10 had the largest genomes and hence a greater number of putative genes and ORFs, which is in agreement with the characteristic features of viruses belonging to the family Myoviridae (Lavigne et al., 2009). The genomes of the bacteriophages F44/10 and F125/10 displayed high similarity (up to 98% nucleotide sequence identity, 80–90% genome coverage) to those of a group of highly related staphylococcal myoviruses, which includes bacteriophages K (O'Flaherty et al., 2004), A5W (GenBank accession no. EU418428) and GH15 (Gu et al., 2012). The bacteriophages F510/08 and F770/05 shared high sequence identity (up to 98% nucleotide sequence identity, 83-98% genome coverage) with *Pseudomonas* wKMV-like and N4-like viruses, respectively (Ceyssens et al., 2010). Examples of wKMV-like viruses are the bacteriophages wKMV and LUZ19 (Ceyssens et al., 2006; Lavigne et al., 2003) and of N4-like viruses are LIT1 and LUZ7 (Ceyssens et al., 2010). The bacteriophage F1245/05 presented no significant similarity at the DNA level with any known bacteriophage in the databases, except for a few short segments with up to 4% nucleotide sequence identity and 81% genome coverage.

The deduced products of the predicted genes of all bacteriophages were compared with sequences in the NCBI non-redundant protein sequence database using BLASTP. No significant similarity with known virulence or toxin proteins or with elements typically associated with lysogeny (integrases, repressors and antirepressors) could be found. Finally, the protein similarity searches did not reveal potential exopolysaccharide depolymerase genes.

Bacteriophage host range

To gain insight into the host range of selected bacteriophages, the susceptibility of three panels of clinical isolates of *S. aureus* (n=132), P. *aeruginosa* (n=93) and *A. baumannii* (n=103) was tested for each species-specific bacteriophage. There was a degree of variability in the host range of each bacteriophage (Table 1). All tested staphylococcal strains were susceptible to both *S. aureus* bacteriophages (F44/10 and F125/10). In contrast, when examined individually, the *P. aeruginosa* bacteriophages F770/05 and F510/08 lysed only 63.4 and 68.8% of the tested isolates, respectively. However, when these results were considered together, we observed that 80.6% of the *P. aeruginosa* isolates were infected by at least one of the bacteriophages, whereas 51.6% were susceptible to both bacteriophages. Finally, of the tested *A. baumannii* strains, 74.8% were susceptible to the bacteriophage F1245/05.

Bacteriophage activity against planktonic cells

To evaluate the activity of the selected bacteriophages against planktonic cells, liquid cultures of the different bacterial hosts were exposed to the corresponding bacteriophages, both individually and in combination, and cell growth/viability was monitored over time with constant agitation. The time-kill curves are presented in Figure 2. *S. aureus* 743/06, when challenged with either F44/10 or F125/ 10 at an IM of 10, showed impaired growth, with reductions in cell counts of 2.3±0.3 and 2.2±0.2 log (c.f.u. ml^{-1}), respectively, at 3h p.i. However, after 24h, the cultures recovered to near-control levels. The reduction in the number of viable cells observed at 3h was significantly enhanced when the two bacteriophages were used in combination [3.4±0.2 log (c.f.u. ml^{-1}); $P<0.01$]. Nevertheless, there was no difference in the recovery of growth at 24h.

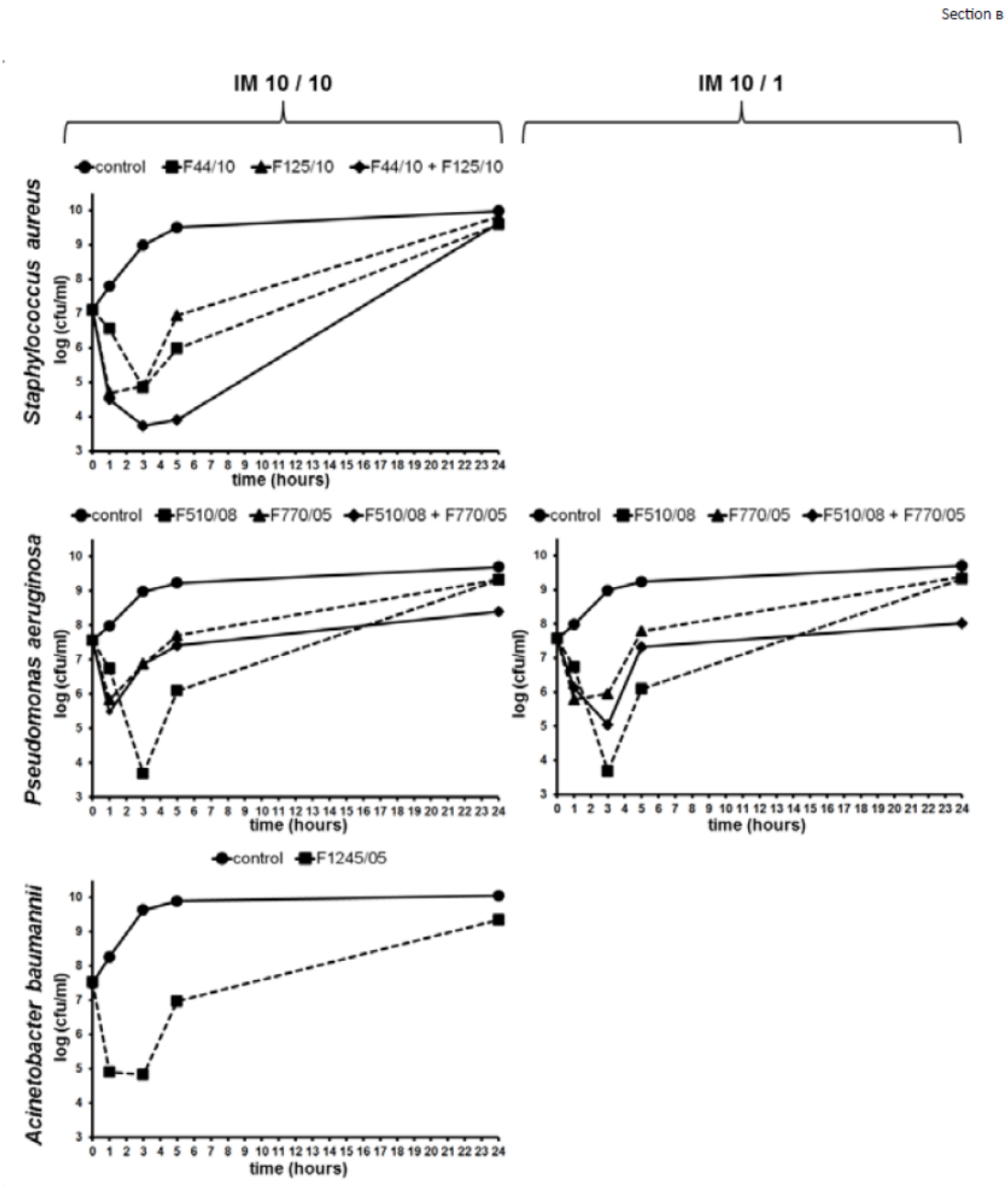
At an IM of 10, the *P. aeruginosa* bacteriophage F510/08 caused a 3.9±0.4 log (c.f.u. ml^{-1}) reduction in the viability of *P. aeruginosa* 433/07 at 3 h p.i. This reduction was more modest [0.7±0.4 log (c.f.u. ml^{-1})] for F770/05 at the same IM and time point. When the two bacteriophages were combined, the kill curve was not different from that of F770/05 for the first 5h; however, the combination provided a statistically significant reduction relative to the control at 24h [1.3±0.3 log (c.f.u. ml^{-1}); $P<0.01$]. When the IM of F770/05 was reduced to 1, combined with F510/08 at an IM of 10, the initial 3 h reduction was more pronounced [2.5±0.4 log (c.f.u. ml^{-1}); $P<0.01$]. Similarly, this combination caused a statistically significant reduction [1.7±0.3 log (c.f.u. ml^{-1}); $P<0.01$] relative to the control at 24h. *A. baumannii* 1305/05 suffered an initial 2.7±0.2 log (c.f.u. ml^{-1}) reduction at 3h after single-bacteriophage (F1245/05) challenge. Although this bacterial strain recov- ered by 24h, it did not reach the control levels of viability.

Table 1. Susceptibility of wound bacterial isolates to candidate bacteriophages for BT

	S. aureus		*P. aeruginosa*			*A. baumannii*
	F44/10	F125/10	F770/05	F510/08	F770/05 + F510/08	F1245/05
Number of bacterial strains tested	132		93			103
Bacterial strain's susceptibility	100%	100%	63.4%	68.8%	80.6%*	74.8%

*Percentage of *P. aeruginosa* isolates that were susceptible to at least one of the bacteriophages (only 51.6% of the isolates were susceptible to both)

Figure 2. Time-kill curves of the target bacteria during planktonic growth when challenged with their specific bacteriophages (alone or in combination). Bacterial strains were grown in TSB with constant agitation and with or without bacteriophages. Growth was monitored and quantified by calculating cfu/ml at 0, 1, 3, 5, and 24 hours. In the left panel (input multiplicity, IM 10/10), assays were performed on *S. aureus* 743/06, *P. aeruginosa* 433/07, and *A. baumannii* 1305/05 in which the specific bacteriophage suspensions were added to provide an IM of 10. In the right panel (IM 10/1), an additional assay was performed on *P. aeruginosa* 433/07 in which the bacteriophage F770/05 suspension was added to provide an IM of 1, alone or in combination with the bacteriophage F510/08 at an IM of 10.



Activity of bacteriophage combinations against established biofilms

We also studied the ability of the bacteriophages to eliminate cells in established biofilms by treating biofilms with species-specific bacteriophage combinations. AB, which quantitatively measures cell metabolic activity using an oxidation-reduction indicator that changes colour in the presence of metabolically active cells, was used to measure cell viability in biofilms with and without treatment. The viability of cells within a biofilm is one of the most important aspects when evaluating the effectiveness of antimicrobial agents; therefore, we used a quantification method based on metabolically active cells, as determined by AB. This assay is a reliable and reproducible method for evaluating biofilm suscept- ibility and is considered to be preferable over the viable plate-count method, as it is very difficult to recover all surviving adherent bacteria as single cells using the latter method (Pettit et al., 2005). The AB-based assay has been used to identify antimicrobials with enhanced efficacy against certain clinically important bacterial biofilms (Pettit et al., 2005 and 2009).

Figure 3 shows the percentage of AB reduction in control and treated biofilms at 4 and 24h using different IMs and frequencies of application. At 4h, the tested bacteriophage preparations strongly reduced the cell viability of all bacterial hosts, independently of the IM. There was only a statistically significant difference between IMs for *A. baumannii*; the higher IM resulted in a greater reduction in metabolic activity (71.9±5.8 vs 88.7±3.1%; $P<0.01$).

At 24h, after a one-time bacteriophage preparation application, the cell viability of all bacterial strains was less suppressed than at 4h but still significantly different from that of the control. At 24h, there were no statistically significant differences between IMs except for *S. aureus*, for which the higher IM resulted in a greater reduction in cell viability (34.8±8.5 vs 52.6±7.7%; $P<0.01$). In experiments using multiple bacteriophage treatments, a greater reduction in cell viability was observed compared with the reduction following one-time bacteriophage treatment. This trend was found for *S. aureus* and *P. aeruginosa* but not for *A. baumannii*.

Discussion

Effective bacteriophage preparations for therapeutic purposes require careful design through a multistep research process of bacteriophage characterization, cocktailing and dosing. This process includes in vitro studies, such as those presented here, and in vivo studies, which have been published previously (Mendes et al., 2013). Ideally, the characterization of bacteriophages for BT should be as thorough and complete as possible. However, in certain cases, it may be more practical to minimize this process and to focus the characterization on particular traits that are the most desirable for a specific application. Combining different bacteriophages in the same preparation (mixtures of two or more bacteriophages within a given formulation) frequently results in a broader spectrum of antibacterial activity and/or lytic efficacy and may allow targeting of bacteria under different conditions or in different environ-

ments (Chan & Abedon, 2012). Finally, in vitro experiments such as those described in this work are useful for evaluating the direct interaction between a drug and bacteria, which enables the selection of candidate bacteriophages. These studies also provide valuable information for the determination of optimal posology (Abedon & Thomas- Abedon, 2010).

Integration of the information emerging from the morphological and genomic analyses revealed that the bacteriophages used here were all tailed bacteriophages (order Caudovirales), with two belonging to the family Myoviridae and three to the family Podoviridae. Genome sequence analysis did not identify any known genes related to lysogeny or traits that might enhance the virulence of the target bacteria, which is an important observation regarding the safe use of bacteriophages. Another important selection criterion for bacteriophages for BT is their host range, which should be as broad as possible, particularly including clinically prevalent bacterial species (Gill & Hyman, 2010). In this study, members of family Myoviridae exhibited the broadest spectrum of lytic activity, whereas viruses of the family Podoviridae exhibited a narrower spectrum, particularly the pseudomonal bacteriophages. The spectrum of activity of the staphylococcal bacteriophages was relatively broad as expected, given their high relatedness to bacteriophages K and A5W, both of which have been described previously as polyvalent bacteriophages (O'Flaherty et al., 2005; GenBank accession no. EU418428). Nevertheless, the host ranges of the *Pseudomonas* and *Acinetobacter* bacteriophages were remarkable compared with those of other species-specific bacteriophages (Merabishvili et al., 2009; Popova, Zhilenkov, Myakinina, Krasilnikova & Volozhantsev, 2012). The overall morphology, genomic characterization and host-range results suggested that these bacteriophages are very good candidates for BT. However, care must be taken when generalizing these results, because the bacterial clinical isolates used for the bacteriophage host-range investigation reflect only the microbiological profile of diabetic foot ulcers in a particular geographical area, and these vary worldwide. Also the sensitivity of the spot test must be taken into account. Whilst the use of high bacteriophage titres (10^8 p.f.u. per spot) for host-range analysis is routine when considering bacteriophages for BT (Kutter, 2009), it should be noted that the use of lower titres may reduce host-range. In our host-range investigation, the use of bacteriophage stock dilutions up to 10^3 p.f.u. per spot yielded differences from the presented results by up to 23% (unpublished data).

Time-kill curves provide detailed information about antimicrobial efficacy against planktonic bacteria as a function of time. These curves are often used to study the antibacterial effect of single and combination drug compounds and dosing regimens before in vivo efficacy studies (NCCLS, 1999). In the current study, following bacteriophage exposure, all bacteria had an initial bacterial reduction to a nadir between 1 and 3h p.i., followed by regrowth that was noticeable after 5h and even more pronounced after 24h. The *Pseudomonas* bacteriophage combination resulted in a significantly greater reduction in bacteria compared with the reduction stimulated by most active single bacteriophage 24h after bacteriophage exposure.

However, the decrease was insufficient to be considered as a synergistic effect, defined as ≥2 log (c.f.u. ml^{-1})-fold decrease by a combination compared with the most active single agent (NCCLS, 1999). In the *Pseudomonas* combination study, when an IM of 1 was used for the *Pseudomonas* F770/05 bacteriophage instead of an IM of 10, we observed a greater initial bacterial reduction after 3h, but similar results were obtained at 24h. This interaction was not specifically analysed in our study, and there is no obvious explanation for this, but clearly further studies would be of interest.

The study presented here has certain limitations. First, only a single bacterial inoculum was used. This value was carefully selected based on several lines of evidence. A higher inoculum (10^7 c.f.u. ml^{-1}) was used than the normal 10^5 c.f.u. ml^{-1} inoculum used in previous time-kill studies testing antibiotics (NCCLS, 1999) because we wanted to mimic a worst-case scenario, similar to that found in wounds (Loc-Carrillo, Wu & Beck., 2012). In a previous epidemiological study (Mendes et al., 2012), microbiological products (aspirates, biopsies and swabs collected using the Levine method) of clinically infected foot ulcers in patients with diabetes were found to have a maximum bioburden of 10^7 c.f.u. $(g\ tissue)^{-1}$ (or cm^{-2} of ulcer area). Additionally, the most recent study using a previously optimized rodent model (Mendes et al., 2013) tested this bacteriophage cocktail on infected wounds with a known mean wound bioburden of 7.54±0.19 log (c.f.u.) per ulcer.

Secondly, the IM in nearly all experiments was 10 (fixed IM). The final IM chosen was selected based on the 'multiplicity of 10 rule', which states that if the goal is a significant reduction in bacterial density, then one should strive for in the order of 10 bacteriophages adsorbed to the average bacterium (Abedon, 2009; Kasman et al., 2002). Previous studies on infected animal and human burn tissue have concluded that low-titre bacteriophage administration (IMs lower than 10) is unlikely to be successful (Goode et al., 2003; Kumari, Harjai & Chhibber, 2010). Furthermore, increasing the IM increases the success of BT by reducing bacterial numbers.

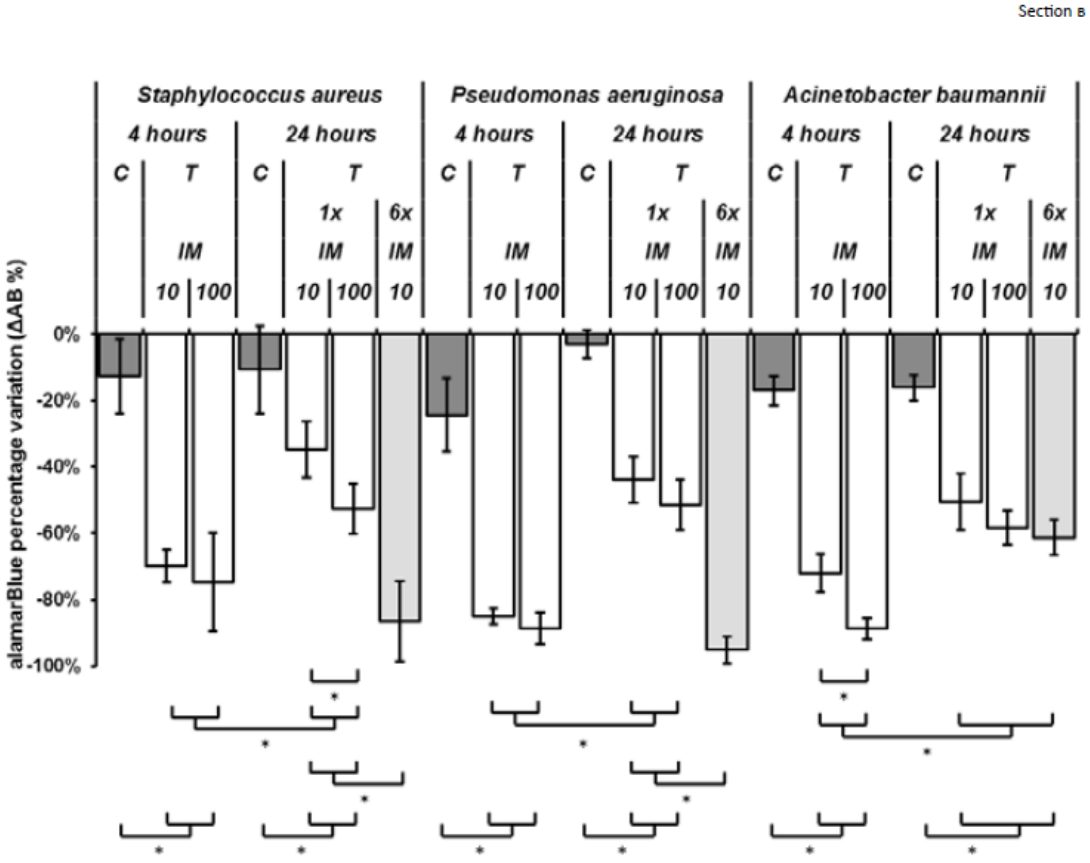
Thirdly, we observed regrowth in planktonic cells exposed to bacteriophages within 24h. This observation may be indicative of the development of resistance, as in vitro resistance is frequent in both BT and antibiotic therapy. For example, a study (O'Flynn, Ross, Fitzgerald & Coffey, 2004) previously found in vitro resistance frequencies of 102-1024 for single-phage treatments and 1026 for double-phage or triple-phage cocktails against Escherichia coli O157 : H7. Similarly, resistance to fusidic acid can readily be selected from an initial high inoculum, with a mean frequency of 1026-1028. This resistance has not limited the antibiotic's topical use and does not appear to be a clinical problem (Sahm, Deane, Pillar & Fernandes, 2013; Turnidge & Collignon, 1999). However, these observations do not imply in vivo resistance. According to certain studies, the rate of development of resistance to bacteriophages is approximately 10-fold lower than the rate of the development of antibiotic resistance (Carlton, 1999). Nonetheless, as observed here, in vitro studies show that bacteriophage resistance can evolve within hours, independently of the use of bacteriophage combinations. However, the

evolution of bacteriophage resistance in vitro does not seem relevant to in vivo scenarios, in which bacteria replicate more slowly and are challenged by more difficult environmental conditions. A previous study (Capparelli, Parlato, Borriello, Salvatore & Iannelli, 2007) found a mean resistance frequency of 1.2 x 10^8 for *S. aureus* treated with bacteriophages in vitro. However, the researchers were unable to isolate any bacteriophage-resistant *S. aureus* strains *in vivo*. Indeed, even though the resistance of bacteria to the bacteriophage cocktails used here was not specifically studied, we previously found that the presence of residual bacteria did not globally hinder planimetric or histological improvement (Mendes et al., 2013). In the current study, the greatest reduction in bacterial counts occurred at 3h, and regrowth was observed at 5 h, which enabled us to conclude that the best time to give a 'boost' application of bacteriophage would be between these two time points.

In a previous study (unpublished data), we found that the plaques of the bacteriophages F770/05, F510/08 and F1245/ 05 were surrounded by growing opaque halo zones, which could be related to the presence of a virion-associated exopolysaccharide depolymerase (Cornelissen et al., 2011). This and related enzymes have been found to enhance the biofilm-eradicating activity of bacteriophages compared with non-depolymerase-producing bacteriophages (Hughes et al., 1998). Based on genomic analysis, none of our bacteriophages seemed to produce any obvious extracellular polysaccharide or exopolysaccharide depolymerase. However, because bacteriophages that do not produce depolymerases have also been used in biofilm elimination (Chibeu et al., 2012), we sought to investigate the effect of bacteriophage combinations on the viability of target bacterial cells in pre- formed biofilms at 4 and 24h. Here, assays using an IM of 10 produced nearly identical results as assays using an IM of 100, with two exceptions. First, we observed different results between an IM of 10 and an IM of 100 after 4 h for *A. baumannii*; however, this discrepancy may have arisen because only one bacteriophage was used. When previous experiments used a combination of two bacteriophages, the IM doubled, producing synergistic results (Abedon & Thomas-Abedon, 2010). Secondly, differences between IM were observed after 24h for *S. aureus*. This result may have occurred because the receptor for the bacteriophage F44/10, which we speculate to be N-acetylglucosamine in the cell-wall teichoic acid, is very frequent (relative to other receptors) in both live cells and bacterial debris. This property means that active bacteriophages may adsorb to fragments of lysed cells (debris) instead of live cells, at a higher rate. This phenomenon may ultimately lead to injection of the genetic material in a suicidal manner, eliminating the bacteriophage from the system (Rabinovitch, Aviram & Zaritsky, 2003). Increasing the IM amplifies the probability of bacteriophage-bacterium interaction, resulting in a true cell infection. Moreover, in vivo, a bacteriophage dose sufficiently in excess of the target bacterium population (IM≥100) should be given to account for bacteriophage loss, dilution (associated with absorption and distribution), decay and/or

inefficiencies of bacteriophage adsorption to bacteria (e.g. inefficiencies in penetration into biofilms *in vivo*).

Figure 3. Analysis of the activity of the bacteriophages against bacterial biofilms. Bacteria were grown for 24h to establish biofilms and the bacteriophages were then added. The following bacteriophage suspensions were used for each bacterium: for *S. aureus*, a 1:1 combination of F44/10 and F125/10; for *P. aeruginosa*, a combination of F770/05 and F510/08 at a 1:10 ratio; and for *A. baumannii*, F1245/05 alone. Cell metabolism was quantified with AB and is reported as the percentage reduction relative to growth in untreated controls. C, negative control; T, treated with bacteriophage; 1x, one-time bacteriophage suspension application, 6x, bacteriophage suspension application every 4h for 24h. *P<0.01 (square brackets indicate the comparisons between different groups).



It is well known that bacterial regrowth occurs after biofilms have been exposed to antibiotics (Kussell, Kishony, Balaban & Leibler, 2005). One possible way to limit this regrowth is through multiple dose applications. Our results using multiple dose applications, as opposed to single-application dosing, are similar to the results observed in Georgia, where BT is the current standard of clinical care, and in Poland, where BT is used as an experimental treatment under a compassionate-use regulatory provision (Abedon et al., 2011; Kutter et al., 2010;

Międzybrodzki et al., 2012). These results were also corroborated experimentally in previously published animal studies (Capparelli et al., 2007). This implies that a significant proportion of the bacteria in biofilm do not have genotypic resistance but rather some form of phenotypic resistance, which is reversible by the modification of the causal environmental factors. Various equally valid and non-mutually excluding theories have been presented that could explain the possible coexistence dynamics of bacteriophages and susceptible bacteria: numerical refuge, physiological refuge and shielding by bacterial debris. The numerical refuge theory (Chao, Levin & Stewart, 1977) predicts that simple mass-action interactions between bacteriophages and sensitive and resistant bacteria determine the stability of the population. Thus, in our study, when new bacteriophages were added (creating a higher bacteriophage density), a decline in the number of sensitive cells resulted. The physiological refuge hypothesis (Lenski & Levin, 1985) postulates that during certain stages of bacterial life cycles sensitive bacteria may become transitorily resistant to bacteriophage infection. In the present study, fresh medium was then added. This altered the life cycle of the present sensitive bacteria (e.g. from stationary to logarithmic), thereby potentially converting them from a temporarily resistant state into a susceptible state. Finally, the shielding by bacterial debris theory (Rabinovitch et al., 2003) predicts that active bacteriophages adsorb onto fragments of lysed cells (debris) and inject their genetic material in a suicidal manner, thus discounting from the system as a bacteriophage. In the present study, as new bacteriophages were added, dead cells were removed, thus reducing non-productive binding as described. None of these observations was noted for *A. baumannii*, perhaps because this was the only case in which we used a single bacteriophage, limiting the importance of non-heritable mechanisms in the reduction in resistance induced by mutation.

Conclusions

In conclusion, we prepared, purified and characterized bacteriophage cocktails with a broad spectrum of activity against *S. aureus*, *P. aeruginosa* and *A. baumannii* strains that commonly cause DFI. The complementary studies on both planktonic cells and established biofilms allowed us to better evaluate the effects of a high IM (≥10) and a multiple-dose treatment protocol (every 4h for 24h). We believe that this work takes an important step towards the future future clinical application of BT.

CHAPTER VII

General Discussion, Conclusions and Future Perspectives

7.1. General Discussion

Diabetic foot ulcers are one of the most common complications of diabetes *mellitus*, affecting up to 10% of diabetic patients and representing the most common reason for non-traumatic limb amputations in developed countries (Katz et al., 2016). This chronic health problem represents a significant economic burden worldwide and causes a relevant impairment of life quality in affected patients (Sun, 2010). Foot sensorimotor neuropathy is responsible for the loss of protective sensation, rendering DFU prone to chronicity and infection (Richard, Lavigne & Sotto, 2012). In fact, the critical breach of the epithelial surface, coupled with neurological impairment, cardiovascular alterations and immune dysfunction, facilitates polymicrobial colonization and subsequent infection of the diabetic foot (Peters, Jabra-Rizk, O'May, Costerton & Shirtliff, 2012).

An early and precise diagnosis is important for the effective infection management, reducing the need of subsequent amputation, but often clinical and microbiological diagnostic may be difficult. In most cases, the antimicrobial management of DFI is empiric due to the lack of appropriate sample collection, which implies that superficial skin cultures only include the "causative pathogen" in less than 30% of the cases. Also, many patients are subjected to long-term antibiotherapy, which further decreases the culture yield (Lipsky et al., 2012b). Previous studies have claimed that sampling by deep tissue biopsy is the gold standard for culturing techniques, as swabs often contain high numbers of colonizers lacking the true pathogen; however, swabs can be collected from all wound types and by all healthcare personnel, being valuable in identifying pathogens in infected diabetic wounds of superficial and moderate depth with a high level of confidence (Slater et al., 2004). Therefore, swab sampling can be performed as long as standardized procedures strictly consistent with current clinical guidelines are used (Mendes et al., 2012).

Diabetic foot infections are mostly promoted by polymicrobial communities, frequently organized in biofilms that may be responsible for resistance to therapeutics and for infection chronicity.

The isolates used in this study belong to a collection of bacteria obtained in Lisbon hospitals, from patients whose DFU have been previously characterized both clinically and microbiologically (Mendes et al., 2012). An average of 3.0 ± 1.4 organisms per sample was obtained, mainly consisting of Gram-positive bacteria, especially belonging to the *Staphylococcus* genus, with the species *S. aureus* being present in 51% of the samples. *Corynebacterium* spp. and other uncommon Gram-positive bacteria were also identified but not in clinically significant concentrations; nonetheless, these genera deserve attention because they appear to be common players in chronic diabetic foot ulcer infections (Dowd et

al., 2008b). Gram-negative aerobes comprised 19.0% of the isolated organisms, with *P. aeruginosa*, the single most predominant species, being isolated from 12.2% of the samples. Anaerobic bacteria were found in about 30% of patients, especially in deep tissue samples (Mendes et al., 2012).

Others studies also have shown that DFI are polymicrobial in nature (Citron et al., 2007; Tascini et al., 2011), with *Staphylococcus* being the most frequent genus isolated, whereas Gram-negative aerobes isolation is frequently reported to be around 20% (Jones et al., 1985; Goldstein, Citron & Nesbit, 1996; Dang et al., 2003; Citron et al., 2007; Ramakant et al., 2011). Regarding anaerobic pathogens, they have been described as being present in deep tissues and related to diabetic foot osteomyelitis (van Asten et al., 2015). Although wounds are generally exposed to air, anaerobes may be able to survive if they co-aggregate with facultative anaerobes or aerobes, which would protect them from the harmful effects of oxygen and allow them to thrive (Smith et al., 2016).

One of the lifestyles adopted by bacteria, including by polymicrobial communities, is the formation of biofilm. The protection provided by these surface-associated communities is high, allowing bacteria to survive the action of several stressors, from antibiotics to host immune cells response, and facilitating the acquisition of nutrients and promoting exchange of genetic elements (Shrout, Tolker-Nielsen, Givskov & Parsek, 2012). It has been established that most biofilm growing bacteria can cause chronic infections (Costerton & Veeh, 2003), explaining their importance and persistence in DFU. Due to their importance, biofilm-related research has significantly increased in the past 10 years. However, studies on the microbiological diversity and biofilm formation by DFU isolates are scarce and, to our knowledge, this work first reported the time course biofilm formation by several species of diabetic foot bacteria (Mottola et al., 2015). As observed by previous studies (Swarna et al., 2012; Mirani et al., 2013), staphylococci presented a high ability to form biofilms after 24 hours, with the percentage of biofilm producer strains increasing significantly with incubation time. The same was observed for isolates belonging to the *Corynebacterium* genus, the second major biofilm producer, although the increase of biofilm production ability of these isolates with time was not statistically significant. *Pseudomonas* and *A. baumannii* showed the higher biofilm forming ability at 24 hours, which may explain high pathogenic effects caused by these bacteria. On the other hand, the lower biofilm production ability demonstrated by enterococci isolates can be explained by their dependence on environmental and genetic factors (Mohamed & Huang, 2007).

Variations of biofilm-forming ability by the different bacterial genera under study point out for the importance of time course evaluations to avoid false negatives results. Is also shows the importance of evaluating the ability of opportunistic human pathogens to form biofilms, since *Corynebacterium* isolates, usually considered skin commensals (Gontcharova et al., 2010), are able to produce biofilms, potentially contributing to wound chronicity. In fact, it is unclear if

the infection of diabetic foot wounds arises from specific combinations of pathogens or if a simple increase in the microbial load of opportunistic microbes can sustain infection (Gardner & Frantz, 2008). A better understanding of the DFU microbiome will help guide new strategies to effectively control the growth of polymicrobial biofilms and improve healing, directly benefiting patients suffering from these debilitating wounds.

Considering the high diversity in microbial composition and the true polymicrobial nature of diabetic foot wound diseases (Citron et al., 2007; James et al., 2007), the ability of dual-species communities to form biofilms was also tested. The bacterial combinations were chosen based on their major prevalence and were as follows: *Corynebacterium* + *Staphylococcus*; *Enterococcus faecalis* + *Staphylococcus*; *P. aeruginosa* + *Enterococcus*; *Acinetobacter* + *Staphylococcus* and *E. faecalis* + *Corynebacterium*. All these communities showed a higher biofilm forming ability when compared with respective individual cultures, but these differences were not statistically significant, with the exception for the combination that involved *Enterococcu*s and *Staphylococcus*. In fact, results demonstrated that biofilm production within communities may be influenced by the bacterial species or genus that is able to produce the higher biofilm values, resulting in a cumulative and not synergic effect. The cumulative biofilm formation in poly-colonized wounds may be responsible for their chronicity and further complications, representing an important barrier to effective treatment, as previously mentioned. The fact that the *Enterococcu*s + *Staphylococcus* combination produced a statistically significant higher biofilm formation when compared to individual isolates may be because although these two bacterial genera showed the lowest biofilm production when analysed individually or alternatively, their combination results in a synergistic effect. The fundamental question that remains to be answered is why some biofilms are 'healthy' and others are able to induce infection. Two hypotheses are currently under discussion (Richard et al., 2012): the 'specific bacterial hypothesis' suggests that only a few bacterial species within heterogeneous polymicrobial biofilms may be involved in the infectious process; conversely, the 'nonspecific bacterial hypothesis' (or 'community hypothesis') considers the whole bacterial composition of the biofilm as 'a functional unit' and does not take account of individual pathogenic bacteria. In 2008, Dowd et al. developed the concept of Functional Equivalent Groups (FEP), in which individual bacterial species considered as non-pathogenic can act synergistically to cause chronic infections when co-aggregated (Dowd et al., 2008b). Moreover, the possibility of transmission of multi-drug resistant determinants to other microorganisms sharing the same ecological niche must be considered in bacterial biofilms. DFI is a complex disease involving multispecies biofilms, frequently formed by bacteria traditionally considered low virulent. More knowledge regarding the microorganisms involved in DFU can direct specific therapeutic protocols to known causative organisms, improving significantly the treatment outcome and reducing infection-related morbidities.

Besides biofilm production, *Staphyloccoccus* pathogenicity is also related to the expression of several virulence factors that allow bacteria to adhere to surfaces, invade or avoid the host immune system, causing harmful effects (Holmes et al., 2005). Staphylococci virulence is multifactorial and caused by the combination of several virulence determinants (Bien et al., 2011). In study, virulence distribution results are in agreement with previous reports (Gillet et al., 2002; von Eiff, Friedrich, Peters & Becker, 2004; Holmes et al., 2005), since 70% of the isolates presented the *clfA* gene but only one *S. aureus* isolate was positive for the *tst* gene and none for the *pvl* gene. The *tst*-positive strains appear to be highly virulent and to cause a variety of illness, and their spread is of major concern (Durand, Bes & Meugnier, 2006).

The last two genes are associated to an increased pathogenic potential and to life-threatening invasive diseases (Holmes et al., 2005; Donate-Correa, Alcoba-Flórez & Méndez-Álvarez, 2011).

Genes related to one of the best-studied virulence regulation system of *S. aureus,* the accessory gene regulator - *agr*, were also detected in the analyzed isolates. As expected, *agrI* was the most frequently detected gene, followed by *agrII*, while no *agrIII* and *IV* were detected. The *agrI* class is considered common and linked to many types of disease, while *agrIII* and *IV* isolates are rare and linked to toxic shock and exfoliative toxin related syndromes, respectively (Thoendel, Kavanaugh, Flack & Horswill, 2011).

Since all staphylococci isolates in our study were able to form biofilm, the presence of the *icaA*, *icaD* and *atl* genes was expected. However, none of the isolates was positive for the *pls* gene, and considering that *pls* expression reduces these virulence features (Juuti, Sinha, Werbick, Peters & Kuusela, 2004), our results eventually suggest a higher potential of adhesion and cellular invasiveness of the isolates. In fact, Savolainen et al. demonstrated in 2011 that *pls* prevented adhesion of the clinical strain 1061 to immobilized fibronectin and immunoglobulin G (Savolainen et al., 2001), suggesting its inhibiting role at some stages during infection. Other studies successively demonstrated the reduced adherence and invasiveness of *pls*-positive *S. aureus* strains independently of MRSA/SCC*mec* background (Juuti et al., 2004; Hussain et al., 2009).

Clinical microbiology can provide useful information for clinical and research purposes in DFI, and cultural examination should be performed in any case of suspected DFI to avoid both the application of an ineffective systemic therapy and the emergence of resistant strains. The knowledge and integration of microbiological features from DFU bacteria, like virulence factors and antimicrobial patterns, should also lead to more accurate diagnosis and targeted antibiotic therapeutics, which can also be improved by clonal analysis of the bacteria involved in such

infections. Furthermore, the knowledge of the local predominant pathogens can help the selection of more adequate empirical antibiotic treatment for DFI.

Numerous *Staphylococcus* clones have emerged and disseminated worldwide (Deurenberg et al., 2007a) and techniques like PFGE and MLST are successfully used for epidemiological analysis of various bacterial species, namely *Staphylococcus*. MLST analysis performed in this study demonstrated a high diversity of clones in the DFU staphylococci isolates, detecting 18 *S. aureus* clones divided in five clusters by PFGE. *S. aureus* cluster I included only MRSA ST22 (Clonal Complex CC22) isolates, a major clone in Portugal since 2001, as it replaced the Brazilian clone in hospitals and communities (Aires-de-Sousa, Correia & De Lencastre, 2008). The most common CC detected in our study was CC5, including isolates from PFGE clusters III, IV and V. ST5 belonging to this clonal complex represented the second most frequent Sequence Type (ST), after ST22. A variant of this clone, ST105-II, had already been described as the second most predominant clone in Portuguese hospitals (Tavares et al., 2014). Besides ST5 and ST105, several ST belonging to CC5 and previously described in Portugal, were identified in this study, with the exception of ST1507 and ST2599, now identified for the first time in this country.

Patients with DFI are continuously attending health-care units for daily care and contrary to expectations, the virulence profile of the studied isolates was more similar to CA-MRSA than to HA-MRSA. These findings may suggest an increasing lack of barrier between health-care and community settings, and also that diabetic patients can be important vehicles for bacterial dissemination. The high prevalence of MRSA in patients with foot ulcers may reflect the increased prevalence of such strains in the community and must be taken into account when treatment with antibiotics is prescribed.

According to previous reports (Ge et al., 2002; Tentolouris et al., 2006; Cervantes-Garcia, Garcia-Gonzalez, Reyes-Torres, Resendiz-Albor & Salazar-Schettino, 2015), approximately half of the *S. aureus* isolates present in DFI are MRSA. Infection with these resistant strains is an increasing problem in both hospital and community. Due to frequent infections, diabetic patients are more exposed to antibacterial agents, which can lead to increased antibiotic resistance rates and justify the high prevalence of MRSA isolated from DFI (Boyanova & Mitov, 2013). Furthermore, the probability of a successful outcome in the treatment of chronic wounds like DFI, may be compromised by the presence of ischemic and necrotic tissue, which may impair antimicrobial distribution and therapeutic efficacy (Bowler et al., 2001). Since culture results are often not available when the decision to start systemic antibiotics is made, the choice is frequently empiric and it is important to consider local patterns of susceptibility and resistance (Hernandez, 2006). Our results suggest a good efficacy of linezolid, doxycycline, clindamycin and gentamicin in DFI treatment, and a very good efficacy of ceftaroline, an antimicrobial compound that was previously associated with high clinical

success in DFI, including patients with obesity, comorbidities, MRSA, mixed infections or requiring surgical intervention (Lipsky, 2015).

However, the high resistance rate of staphylococci (43%) to ciprofloxacin and erythromycin draws attention to the increasing use of these antibiotics in clinical practice (Gorbach, Bartlett & Blacklow, 2004; Reis et al., 2016). In addition, 36% of the isolates were considered to be MDR, which is in accordance with other reports (Malik et al., 2013; Sekhar et al., 2014), and represents a serious alarm regarding the control of this type of infection. These observations are of major importance, especially for patient management and the development of antibiotic treatment guidelines. Moreover, increasing prevalence of MDR organisms raises serious concerns since it limits the choice of antibiotic therapy protocols leading to poor prognosis. Therefore, proper MDR screening is also suggested, being essential for the selection of adequate antimicrobial treatment strategies for DFU management, contributing to the decrease of MDR strains incidence in DFU patients.

The antibiotic susceptibility patterns cannot be applied to biofilm-established infections.

Increased resistance towards antimicrobial agents is also observed in bacterial biofilms, as these communities can resist antibiotic concentrations 10 to 10.000 times higher than the ones required to kill planktonic cells (Kaplan, 2011). In this study, antibiotic concentrations required to inhibit or eradicate *S. aureus* biofilms were 10 to 1000 times higher than the respective MIC values, reaching toxic concentrations in human medicine, in other words, that render biofilms resistant to therapeutics. Furthermore, minimum biofilm eradication concentration (MBEC) values were often several times higher than minimum biofilm inhibitory concentration (MBIC) values.

Gentamicin and ceftaroline were the most potent agents against *S. aureus* biofilms, at concentrations that can be applied *in vivo* to inhibit and eradicate biofilm related infections, even the ones promoted by MRSA isolates. The efficacy of gentamicin against staphylococcal biofilm was already reported (Coraça-Huber et al., 2012) and more recently the ceftaroline efficacy was also demonstrated (Barber et al., 2015). It was observed that meropenem and linezolid were unable to eradicate *S. aureus* biofilms and cefoxitin was only able to inhibit and eradicate biofilms formed by MSSA isolates. Finally, clindamycin, doxycycline, linezolid, vancomycin, ciprofloxacin and erythromycin (Rose & Poppens, 2009), showed ability to inhibit biofilms but not to eliminate them. These results are in accordance with some previous reports (Pettit et al., 2005; LaPlante & Mermel, 2009; Smith et al., 2009; Cafiso et al., 2010) and may suggest that these antibiotics, although effective against planktonic bacteria, may not be the most appropriate for treating biofilm related infections, with the exception of gentamicin and

ceftaroline. These results once again alert us to the need for proper management of antibiotics to optimise patient care and improve clinical outcome in DFI.

Considering the high *S. aureus* biofilm phenotype resistance, an equally high presence of antibiotic resistance genes was not detected. In fact, only three isolates were positive for *bla*Z, which can be explained by the occurrence of multiple polymorphisms of this gene or by the expression of *mec*A in these isolates. Several genes are implicated in erythromycin resistance and *erm*A was the most prevalent *erm*-gene, followed by *erm*C, as previously described (Martineau et al., 2000; Strommenger et al., 2003). Similar to others reports (Smith et al., 2009; Andersen et al., 2015), *tet*K was the most frequent gene found among the tetracycline resistant *S. aureus* isolates (n=3), followed by *tet*M. It was not possible to detect *tet*L and *tet*O resistance determinants. The *tet*-positive isolates were susceptible to doxycycline, but this gene confers high resistance to tetracycline, oxytetracycline, chlortetracycline and low resistance to monocycline, 6-demethyl-6-deoxytetracycline and doxycycline (Andersen et al., 2015).

In the last years, fluoroquinolone resistance in *S. aureus* increased exponentially worldwide, involving mutations in genes that encode for subunits of DNA gyrase, DNA topoisomerase IV, and also in *norA* coding region (Takei, Fukuda, Kishii & Hosaka, 2001; Pourmand, Yousefi, Salami & Amini, 2014). Many isolates susceptible to ciprofloxacin were positive for *nor*A gene. From a clinical point of view, this discrepancy between genotype and phenotypic resistance expression can suggest that susceptible strains may be present but not express antibiotic resistance genes and should be regarded as potentially resistant to that antibiotic.

Besides staphylococci isolates, *Enterococcus* may play an important role in the establishment and persistence of DFI.

Traditionally, studies on wound microbiota have focused on the role of well-known pathogens such as *S. aureus* and *P. aeruginosa*, organisms that are easily cultured using traditional microbiological techniques. Nowadays, the medical and research communities are beginning to realize that the diversity of bacterial populations in wounds may be an important contributor to their chronicity, as observed in DFU (Dowd et al., 2008b).

Enterococci have emerged as increasingly important nosocomial and community-acquired pathogens, and although being generally considered as opportunistic pathogens, it is well recognized that these organisms can cause serious invasive infections, being also part of the complex diabetic foot microbiota (Zhang et al., 2010; Mendes et al., 2012). The high prevalence of *E. faecalis* among the DFU enterococci was expected, as this species is considered the most prevalent from this genus, being commonly isolated from clinical samples. Furthermore, *Enterococcus* was frequently isolated from DFI in previous studies, being present in 10% (Citron et al., 2007) to 35% of patients (Tascini et al., 2011). Two

enterococci isolates were identified as *E. gallinarum*, a species that has been implicated in a wide variety of human infections, especially in immunocompromised individuals (Reid, Cockerill & Patel, 2001).

Macrorestriction analysis of the enterococci isolates showed a high genomic diversity, allowing their distribution into nine genomic groups, sharing an elevated similarity that reveals their clonal relationships. All *E. faecalis* isolates were able to form biofilms and expressed haemolytic and gelatinolytic properties, and were also carriers of several virulence determinants considered relevant for enterococci pathogenicity, namely genes that encode for surface protein Esp (*esp*), cytolysin toxin (*cylA*) and aggregation substances (*agg*). Results from this study demonstrated the putative contribution of *Enterococcus* to the chronicity and pathogenicity of DFI due to their ability to form biofilm and express virulence traits. Moreover, although no enterococci isolates were resistant to vancomycin, but they were all resistant to different classes of antibiotics. In fact, the majority of the isolates were considered as MDR, a relevant feature for isolates present in chronic infections like DFI, often resulting in treatment failure.

Bacteriophage therapy represents a potential effective therapeutic approach for treatment of diabetic foot wounds infected with different pathogens.

In view of the increased resistance to antimicrobial treatments of infections promoted by bacterial biofilms, new strategies should be implemented aiming at their control. In spite of the increasing development of new promising approaches for biofilms prevention, like quorum sensing inhibitors and biofilm-dispersal agents (Rabin et al., 2015), the current therapeutic approaches for prevention of biofilms are essentially based on the application of antimicrobial agents (M. Chen, Yu & Sun, 2013). The use of bacteriophages to treat bacterial infections, known as phage therapy (BT), has a history substantially longer than that of antibiotics; this therapy has been used in Eastern Europe for over 60 years owing to its efficacy, low toxicity and low production costs (Burrowes et al., 2011). Recent animal and human clinical trials have confirmed phages to be safe and well tolerated, suggesting that phage therapy could be a major complement to antibiotic therapy (Rhoads et al., 2009; Vandersteegen et al., 2011; Mendes et al., 2013).

The development of effective bacteriophage suspensions for therapeutic use requires bacteriophage characterization, cocktailing and dosing, pre-clinical animal efficacy and toxicology studies, and finally regulated human clinical trials. Technophage, S.A., a Portuguese R&D biopharmaceutical company, previously isolated two *S. aureus* phages, two *P. aeruginosa* phages and one *A. baumannii* bacteriophage from environmental samples that were morphologically and genetically characterized. The objective was to develop a phage cocktail with a spectrum of activity directed to the most relevant DFI bacterial pathogens. To

assess their infectivity on target bacteria, these phages were tested against 44 DFI isolates (Mendes et al., 2014), including: *S. aureus*, the most common cause of DFI in our study population; *P. aeruginosa,* associated with multi-drug resistance and antibiotic failure; and *A. baumannii/calcoaceticus*, also related to high antibiotic resistance, being clinically relevant especially regarding diabetic patients (Boyanova & Mitov, 2013).

The ability of these bacteriophages to eliminate planktonic bacterial cells and established biofilms was evaluated using time-kill curves and a cell oxidation-reduction indicator, respectively. Time-kill curves provided detailed information about antimicrobial efficacy against planktonic bacteria as a function of time. After bacteriophage exposure with a input multiplicity of 10, all bacteria had an initial reduction to a nadir between 1 and 3 hours post-infection, but followed by regrowth that was noticeable after 5 hours post-contact and even more pronounced after 24 hours.

Regarding the use of BT in bacterial biofilms, the highest effect on biofilm cells occurred at a bacteriophage:bacterium input multiplicity of 10. The tested bacteriophage preparations strongly reduced the cell viability of all bacterial hosts at 4 hours after inoculation. This reduction was inferior at 24 hours after bacteriophage exposure, but still significantly different from the results obtained for planktonic cells.

The best inhibitory results on biofilms were obtained with multiple bacteriophage treatments, every 4 hours over a 24 hours incubation period, suggesting that a multiple dose treatment protocol would be more adequate, with the exception for *A. baumannii*. Phage cocktails are usually assembled in order to maximise the host range and to reduce the potential and emergence of resistance to phages (Fischer, Kittler, Klein & Glünder, 2013), and probably this did not happen regarding *A. baumannii* since it was the only case in which a single bacteriophage was used.

The tested bacteriophages represent a potentially effective therapeutic approach for the treatment of diabetic wounds infected with different pathogens, although further human clinical trials are mandatory in confirming the potential of bacteriophage therapy for the treatment of DFI and other chronic skin and soft tissue infections.

7.2 Conclusions and Future perspectives

Diabetic foot infections represent a significant burden for patients and the healthcare system. This study contributed to confirm that bacterial communities responsible for DFI are very complex and frequently organized in polymicrobial biofilms. For this reason, data regarding single-species cannot be extrapolated to multispecies biofilms.

To our knowledge, this is the first study on time-course of biofilm formation by diabetic foot isolates and one of the few reports on staphylococci isolated from DFI that include information about isolates origin, virulence factors and antimicrobial resistance profiles of planktonic and biofilm bacteria. The diabetic foot staphylococci studied presented high genomic diversity and antimicrobial resistance ability, and several virulence traits, of which biofilm may constitute one of the major obstacles to therapeutic success. *S. aureus* biofilms were resistant to antibiotic concentrations up to 10 to 1000 times higher than their planktonic counterparts and only gentamicin and ceftaroline were effective in eradicating biofilms. It is clear that antibiotic susceptibility values determined for planktonic populations are not necessarily applicable for an effective treatment of biofilm infections produced by the same organism. In fact, DFI therapeutics are empirically established in the beginning, considering infection severity, route of antimicrobial administration, co-morbidities and spectrum of microorganisms present. It is important to note that inappropriate empiric broad-spectrum antimicrobial therapeutic protocols can result in unfavourable outcomes for patients and contribute to the development and dissemination of antimicrobial resistant bacteria. More research is crucial concerning the effect of antibiotics on biofilms and could lead to the revision of antimicrobial guidelines in the clinical setting.

Other bacterial species involved in DFI and usually considered as minor pathogens may play an important role in these type of infections, including *Enterococcus* and *Corynebacterium.* Future attempts must be targeted at understanding the role of pathogen diversity in DFU. This will provide new insights to redirect therapy against all relevant species involved, improving clinical outcome.

The high level of resistance of biofilm-organized bacteria makes chronic infections, like DFI, extremely difficult to eradicate, rendering important the development of alternative therapeutic protocols such as bacteriophage therapy. The effect of previously developed bacteriophage cocktails, against biofilms formed by *S. aureus*, *P. aeruginosa* and *A. baumannii* strains isolated from DFI was evaluated, and the effects of a high input of multiplicity and a potential multiple-dose treatment protocol were established. This BT protocol represents a potentially effective therapeutic approach for treatment of diabetic wounds infected with different pathogens.

DFI studies are still scarce and studies about these complex bacterial communities are required. The next steps could include:

- Collection of more DFU isolates, especially targeting Gram-negative species that are less common in DFI but can be pathogenic and difficult to eradicate, like *P. aeruginosa* and *A. baumannii*. Virulence traits, with focus on biofilm formation and antimicrobial profile of the different species should be determined for such isolates;
- Analysis of polymicrobial biofilms with mass measurement using electronic microscopy for characterizing biofilm production by different bacterial species. This analysis would clarify the role and contribution of each species in the establishment, maintenance and dissemination of biofilms;
- Assessment of antimicrobial resistance patterns of the co-cultured microorganisms and comparison with the MIBC and MBEC of individual biofilms. The study of antimicrobial resistance of biofilm-enclosed mixed populations could elucidate on the probability of resistance traits dissemination among the most common bacterial species involved in DFI;
- Construction of a national database to link all available information obtained throughout this project and by other research groups on diabetic foot infections, including bacterial molecular typing and epidemiology, antimicrobial resistance and virulence traits and biofilm-forming ability, in order to identify clonal dispersion routes of virulent strains. The integration of all phenotypic and molecular data would contribute to unveil relationships among sets of isolates, clarifying epidemiological relationships and revealing the underlying population structure and spatial distribution.

The present study allowed to assess several microbiological properties of DFI isolates that include identification, virulence traits and antimicrobial resistance properties, encompassing many bacterial genera involved in diabetic foot infections. Furthermore, it also explored BT's potential in the treatment of DFI biofilms infections and other chronic skin and soft tissue infection diseases. Studies on DFI microbiology are scarce and represent a critical step to better understand and manage these infections.

CHAPTER VIII

References

8.1. References

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