The effect of NucB on the prevention and dispersal of biofilms of clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* from prosthetic joint infections

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Abstract

Total hip and knee replacements are one of the most common elective operations. Prosthetic joint infection (PJI) is a devastating and challenging complication. Often current antibiotic treatment is ineffective because PJI is commonly associated with biofilm formation. Prevention of biofilm attachment as well as disruption of established biofilms may therefore allow more effective treatment of such infections. NucB is a novel marine bacterial endonuclease, which degrades extracellular DNA, a structural component of biofilms and has shown promise in being able to degrade bacterial biofilms. The aim of this project was to investigate the effect of NucB on prevention of biofilm formation as well as dispersal of biofilms of clinical isolates of two important pathogens in PJI, *Staphylococcus aureus* and *Staphylococcus epidermidis*. In addition, enzyme activity against biofilms attached to surgically relevant surfaces such as titanium, polyethylene and cobalt chrome was quantified in order to understand how this enzyme would work on biofilms grown on surgically relevant surfaces. Biofilms were grown in microtiter plates and on metal and polyethylene discs and quantified using crystal violet staining as well as confocal microscopy. High purity NucB (>95%) was used. In the presence of low concentrations of NucB (1µg/ml), we observed between 34 and 76% inhibition of biofilm formation. NucB could also effectively disperse between 38 to 96% of biofilm attached to cobalt chrome, polyethylene, stainless steel and titanium surfaces. We also observed an increase in the ability of antibiotics to kill bacterial cells in the presence of NucB compared to controls. NucB can therefore successfully prevent the formation, and can disperse biofilms of clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis*. These results demonstrate a new approach to biofilm prevention and dispersal, and provide the foundation for the further development of NucB into a therapeutic product which could improve the treatment of PJI in the future.

To my parents, Loly and Jesus and my wonderful husband Matei; for their unconditional love and support.

Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time.
Thomas A. Edison

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Table of Contents

List of abbreviations

UHMWPE- Ultra-high molecular weight polyethylene

MoP- Metal on Polyethylene

MoM- Metal on metal

CoC- Ceramic on ceramic

CoP-Ceramic on polyethylene

RCT- Randomized controlled trial

PJI- Periprosthetic joint infection

UK- United Kingdom

ESR- Erythrocyte sedimentation rate

CRP- C-reactive protein

WBC- White blood cell

LE- Leukocyte esterase

PMN- Polymorphonuclear neutrophil

PTC- Procalcitonin

IL-6- Interleukin 6

AUC - Area under the curve

α-defensin- Human α-defensin

ELA-2- Neutrophil elastase 2

BPI - Bactericidal/permeability-increasing protein

NGAL- Neutrophil gelatinase-associated lipocalin

SF- Synovial fluid

G-CSF- Granulocyte colony-stimulating factor

VEGF- Vascular endothelial growth factor

EANM- European Association of Nuclear Medicine

EBJIS- European Bone and Joint Infection Society

MSSA- Methicillin sensitive *Staphylococcus aureus*

MRSA- Methicillin resistant *Staphylococcus aureus*

P. acnes- Propionibacterium acnes

CN- Culture negative

DAIR- Debridement and implant retention

CLSM- Confocal laser scanning microscopy

ECM- Extracellular matrix

eDNA- Extracellular DNA

CWA- Cell-wall anchored proteins

Aap- Accumulation-associated protein

PIA- Polysaccharide intracellular adhesin

PNAG- Poly-N-acetyl glucosamine

Ica- Intercellular adhesin

PSMs- Phenol-soluble modulins

MF- Major facilitator

ATP- Adenosine triphosphate

RND- Resistance-Nodulation-Division

SMR- Small multidrug resistance

MATE- Multidrug and toxic compound extrusion

NAC- N-acetyle-L-cysteine

MIC- Minimum inhibitory concentration

MBC- Minimum bactericidal concentration

ppGpp- Guanosine 5′-diphosphate 3′-diphosphate

pppGpp- Guanosine 5′-triphosphate 3′-diphosphate

FnBP- Fibronectin binding protein

rhDNase- Recombinant human DNase

NHS- National health service

MALDI-TOF MS- Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

TSB- Tryptic soy broth

TSA- Tryptic soy agar

PBS- Phosphate buffer saline

CV- Crystal violet

NB- Nutrient broth

LB- Luria-Bertani broth

CT- Calf thymus

EDTA- Ethylenediaminetetraacetic acid

UHMWPE- Ultra-high molecular weight polyethylene

RA- Roughness average

ETS- Exeter trauma stem

XTT- 2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide

Chapter 1. Introduction

1.1 History of arthroplasty.

1.1.1 The beginning of a surgical specialty

Over the last twenty years in the UK and in many parts of the developed world, increased life expectancy and an ageing population have contributed to the ubiquity of joint disease and the requirement for increased surgical intervention. Arthroplasty surgery, the surgical reconstruction or replacement of a joint (Oxford English Dictionary) has undergone rapid development over the past 60 years but has its beginnings in the 19th century when the first attempt at implant surgery was successfully made by the german surgeon Themistocles Glück (1853-1942). On the 20th May 1880, Glück performed the first hinge knee replacement made of ivory (Figure 1-1). He also performed the first hip replacement by attaching an ivory ball to the neck of the femur with a nickel plate and screws in 1881(Rang, 1966). His results were successful in the short term but all 5 cases developed complications due to infection (Wessinghage, 1991; Eynon-Lewis *et al.*, 1992). Glück was also the first surgeon to use bone cement, experimenting with different materials including plaster of Paris, copper amalgam and stone putty (Eynon-Lewis *et al.*, 1992; Gomez and Morcuende, 2005).

Very early on, it was apparent that infection was associated with a negative surgical outcome. Joseph Lister, a British surgeon, was aware of the importance of post-operative

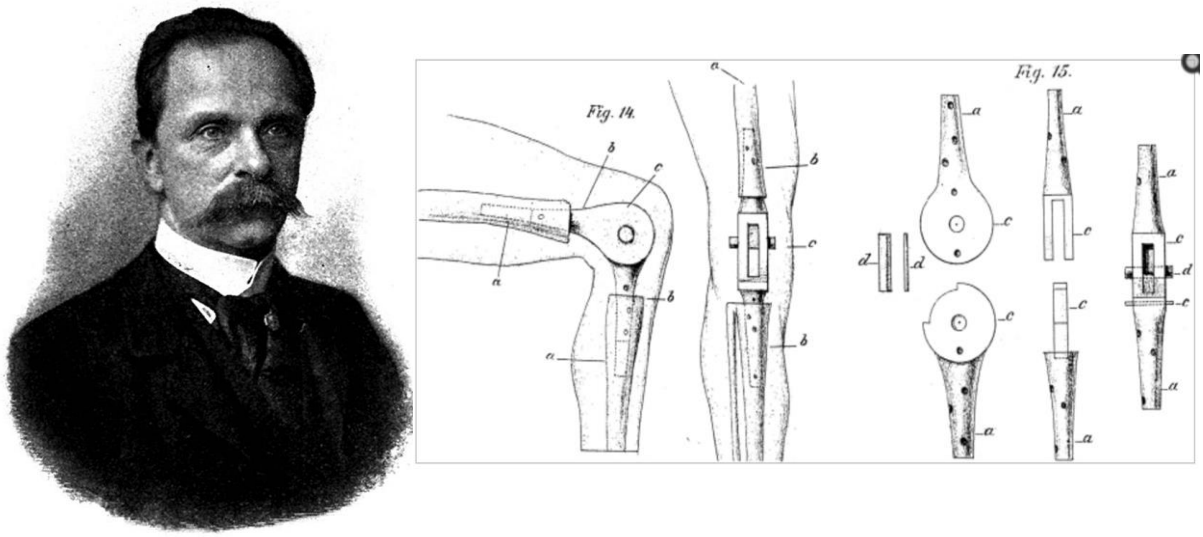

Figure 1-1. (A) Themistocles Gluck, a pioneer orthopaedic surgeon. (B) Illustration of Gluck's ivory knee replacement (Reprinted from Gluck T. ***Arch klin chir***. 1891;41:187–239).

infection and its role in high mortality rates (Lidwell *et al.*, 1984; Pitt and Aubin, 2012). Towards the second half of the 19h century, he developed the antiseptic technique using carbolic acid in the wound, which reduced infection (Lidwell *et al.*, 1984). The high failure rate due to infection in Gluck's work as well as the Lister's studies underlines that infection has been a significant problem from the beginnings of arthroplasty. Today, 150 years later, despite numerous improvements in surgical technique, implant design and medical treatment, there remains an urgent need for novel approaches to prevent and treat post-operative infection.

The 20th century was a revolutionary era for the development of hip and knee arthroplasty. In the 1960s, Sir John Charnley, an orthopaedic surgeon working in Manchester, developed several new concepts that completely changed surgical practice. Charnley was aware that infection continued to be a problem in orthopaedics. He developed a clean air operating system and a body exhaust system in an attempt to reduce surgical infection (Charnley, 1974). He introduced the use of bone cement; he studied the properties of acrylic dental cements and along with the dental industry developed bone cement in an attempt to improve implant fixation (Charnley, 1965). Another important development was the description of the low friction concept. Charnley showed that the friction coefficient of normal articular cartilage is extremely low. He developed a low friction prosthesis using a small socket femoral head (the smallest possible for the expected load) and a High Molecular Weight Polyethylene cup with a large outside diameter (to minimize cup displacement) (Hammond and Charnley, 1967; Charnley, 1968; Charnley and Eftekhar, 1969). This design and concept of a small head and a socket with a larger outside diameter remains today, almost 50 years later. Charnley not only revolutionized the design of the hip prosthesis but he was constantly trying to improve surgical technique to reduce complications. Another development in surgical technique included the design of a cylindrical surgical gown cuff to avoid contamination during glove exchanging in a surgical procedure (Charnley, 1976). Since Charnley's discoveries and designs, the principles of joint replacement surgery have evolved very little. Advances have focused on minor improvements to implant design and surgical techniques to reduce complication rates. In addition, there is a growing focus on methods to try to reduce infection.

1.1.2 Benefits and outcomes of prosthetic joint replacement

Joint replacement is now one of the most common elective operations in the UK with 259,859 operations performed in 2018 (National Joint Registry, 2019). It radically improves quality of life by restoring mobility and providing pain relief (Osmon *et al.*, 2013).

Modern surgical techniques and implants, have improved the outcomes of total hip and knee replacements with a current survival rate of 95% at 10 years (National Joint Registry, 2019) and 58% at 25 years (Evans *et al.*, 2019). Cemented implants have the lowest revision rate at 10 years in both hip and knee replacement (National Joint Registry, 2015). In an era where evidence-based medicine is the gold standard of clinical practice (Charles *et al.*, 2011), outcomes published by the registries have a significant impact on clinical practice and drive changes to improve treatment and clinical standards.

1.1.3 Infection as an emerging problem

Infection has been a problem since the beginning of arthroplasty and if left uncontrolled can lead to surgical failure and death (Charnley and Eftekhar, 1969). The causes of infection following arthroplasty are multifactorial (Solarino *et al.*, 2015) and involve both the patient and the environment (Shahi and Parvizi, 2015). Patient related factors include comorbidities such as congestive heart failure, pulmonary disease, preoperative anaemia, diabetes mellitus, renal disease, obesity, rheumatoid arthritis, hypercholesterolemia, metastatic tumours, venous thromboembolism and certain mental health disorders such as depression and psychoses (Pulido *et al.*, 2008; Bozic *et al.*, 2012). A large study of over 9000 joints published in 2016 demonstrated that patients over 100kg had twice the incidence rate of prosthetic joint infection than that of patients with lesser weight (Lübbeke *et al.*, 2016). A possible reason for this elevated risk is the increase in subcutaneous tissue with poor perfusion as well as increased wound tension predisposing to wound breakdown leading to increasing the risk of infection (Fujii *et al.*, 2010). Sarcopenia, or loss of skeletal muscle, is known to be an independent predictor of morbidity and mortality, but a recent study published in 2019 by Cohen et al. demonstrates a correlation between increased prosthetic infection rate and reduced central skeletal muscle mass (Babu *et al.*, 2019). Both of these factors are modifiable with simple measures such as a healthy weight loss regime and increased protein intake to slow muscle mass loss. Similarly, malnutrition, poorly controlled diabetes and pre-operative anaemia should be identified and corrected when possible to optimise the patient prior to surgery and reduce the risk of post-operative infection (Eka and Chen, 2015; Maempel *et al.*, 2016). Another modifiable patient related factor is the burden of

skin commensals often involved in subsequent PJI. Full body wash with an antiseptic solution such as chlorhexidine prior to surgery can be effective in reducing the risk of post-operative infection (Rao *et al.*, 2008; Rao *et al.*, 2011). There is emerging evidence showing the effectiveness of de-colonisation programs prior to elective arthroplasty in reducing post-operative infection rates. A double blind placebo controlled multi-centre trial showed a reduction in staphylococcal infection rate in the treatment group (3.4% vs 7.7%) (Moroski *et al.*, 2015) and a more recent large study of 12,911 hip and knee joint replacements supported this evidence showing a significant reduction rate in prosthetic joint infection while being cost-effective (Jeans *et al.*, 2018).

Environmental factors include the design of the operating room, draping and traffic of theatre staff. These factors can be modified to minimize the risk of bacterial contamination during a surgical procedure. In the late 1960s, the infection rate following arthroplasty was above 9%. (Evans, 2011). Charnley believed that the air in the theatre environment had an impact on wound contamination at the time of surgery. He developed the first clean air theatre ventilation system and after its implementation infection rate was reduced 20 fold (Charnley, 1972). The use of a clean air operating theatre when performing joint replacement surgery is still common practice today. Orthopaedic theatres have a laminar flow system installed although there are some controversies regarding its efficacy in reducing infection (James *et al.*, 2015). Lidwell et al. back in 1982 published a randomised controlled trial of over 8000 joints and demonstrated that the use of laminar air flow systems in combination with prophylactic antibiotics reduced the infection rate from 3.4% to 0.3% (Lidwell *et al.*, 1982). Further evidence published in subsequent years supported this evidence, ultimately leading to the routine use of laminar air flow systems in arthroplasty surgery (Lidwell *et al.*, 1987; Ahl *et al.*, 1995; Kakwani *et al.*, 2007). More recently, new evidence is emerging disputing the effectiveness of such air systems. A meta-analysis of 12 studies with a total of 464,514 joints published by Birchoff et al. in 2017 suggested that there was no difference in risk of prosthetic joint infection when using laminar airflow compared with conventional ventilation (Bischoff *et al.*, 2017) with some studies even showing an increased infection rate associated with the use of laminar flow systems (Brandt *et al.*, 2008; Hooper *et al.*, 2011). The laminar flow system works by pushing air and debris vertically from the ceiling to the floor, but the presence of theatre personnel, as well as the theatre lights and equipment can alter the vertical flow and produce turbulences that moves the air particles into the surgical field and could increase the exposure of the surgical wound to contaminated air and debris (Andersson *et al.*, 2012; Jain and Reed, 2019). Traditionally, reusable cloth drapes were used

in theatre to prepare and isolate the surgical field but it has been shown that these are less effective than disposable plastic drapes in reducing bacterial penetration and wound contamination (French *et al.*, 1976; Markatos *et al.*, 2015). Theatre personnel traffic can also increase the load of airborne microorganisms (Shahi and Parvizi, 2015). Pre-surgical planning to prepare instruments and materials needed during surgery can reduce traffic and unnecessary personnel and thereby further reduce the risk of infection.

1.1.4 Current materials used in arthroplasty

Over the years different material combinations have been tested in total hip arthroplasty. Ivory was one of the first materials used (Eynon-Lewis *et al.*, 1992) but, over time, new materials and designs emerged which were more biocompatible and had better wear resistance (Gomez and Morcuende, 2005). Modern prostheses are made of stainless steel, cobalt chrome, titanium, ceramic and ultra-high molecular weight polyethylene (UHMWPE). These materials can be combined in several ways:

Metal on polyethylene (MoP)- This is the most common type of prosthesis currently implanted in the UK (National Joint Registry, 2019) due to its reliability and cost effectiveness (Knight *et al.*, 2011). It consists of a stainless steel, cobalt chrome or titanium stem with a cobalt chrome or stainless steel femoral head and an UHMWPE acetabular component.

Metal on metal (MoM)- This prosthesis consists of a metal femoral stem and head with a metal acetabular cup. MoM prostheses have been used since the 1960s. There are some controversies surrounding the use of MOM designs due to the release of metal particles produced by surface wear. Metal particles can have an adverse effect on patients (Hartmann *et al.*, 2013) including higher mortality and revision rates (Pijls *et al.*, 2016). Cobalt chrome ions have been found to be 3 to 5 times higher in the blood of patients with MOM prosthesis than those with metal on polyethylene (Cuckler, 2005). MOM designs have evolved and improved over the years but despite this, the use of MOM implants in England, Wales and Northern Ireland remains very limited with <0.1% and 2.7% of all cemented and uncemented total hip replacements registered in the National Joint Registry being MOM (National Joint Registry, 2019).

Ceramic on ceramic (CoC)- This type of prosthesis was developed by Pierre Boutin in 1970 (Boutin, 2014) in an attempt to address the problems arising with particles released due to wear in MOM or metal on polyethylene designs (Knight *et al.*, 2011). The benefit of CoC implants is their excellent wear resistance. They are mainly made of alumina or zirconia

based materials which are extremely hard wearing and biocompatible (Sentuerk *et al.*, 2016). Lower infection rates with CoC implants have been reported (Pitto and Sedel, 2016). The downside of using CoC implants is their higher fracture rate and a squeaking noise during movement (Macdonald and Bankes, 2014; Dong *et al.*, 2015). This has been associated with lower patient satisfaction (Gillespie *et al.*, 2016). The number of CoC total hip replacements performed has been gradually falling over the past eight years(National Joint Registry, 2019).

Ceramic on polyethylene (CoP)- This combination of materials has the potential to maintain the advantages of softer and less rigid polyethylene surfaces and the smooth but hard ceramic head (Cash and Khanduja, 2014). Although it has been demonstrated to have similar outcomes to CoC implants, CoP prostheses have shown lower implant fracture rates, reduced component-related noise (Amanatullah *et al.*, 2011) and lower wear rates (Urban *et al.*, 2001). The overall revision rates for CoP are lower at 13 years when compared to other material combinations (National Joint Registry, 2019). Thanks to this emerging data indicating good survival rates and reduced complications, the number of CoP based total hip replacements has been steadily gaining popularity in recent years.

There has been some discussion in the literature suggesting that the bearing surface of the joint replacement may influence the risk of prosthetic joint infection. Pitto el al. published work based on the New Zealand joint registry with over 97,000 joint replacements and concluded that Ceramic on Ceramic arthroplasty had a lower revision rate for infection than the other bearing combinations (MoM, MoP, CoP) (Pitto and Sedel, 2016). Further evidence supported this claim with a large series of 177,237 primary total hip replacements, suggesting a higher revision rate for infection in the MoP and CoP combinations when compared to CoC (Madanat *et al.*, 2018). Perhaps the reason for this association of lower infection risk with ceramic on ceramic bearings is related to the lower surface roughness and higher hydrophylicity compared to metal bearings (Kurtz and Ong, 2009) as these properties have been associated with lower bacterial adhesion to surfaces (Zmantar *et al.*, 2011; Koseki *et al.*, 2014) Interestingly, a recent meta-analysis of 17 articles including 11 randomised controlled trials and 6 observational studies concluded that the bearing surface does not influence the risk of prosthetic joint infection (Hexter *et al.*, 2018). This meta-analysis included 158,430 MoP, 17,459 CoC and 17,489 CoP hip replacements and the overall infection rate for each bearing combination was MoP 0.85%; CoC 0.53%; and CoP 0.38% but these results were not statistically different. The studies included in this meta-analysis did not have a standardized definition of PJI and the randomized controlled trials (RTC) included were underpowered for evaluation of prosthetic joint infection as they did not have this as a primary outcome.

Nevertheless, pooling the results of the RCT together should overcome this flaw. The controversies and lack of strong evidence in the current literature warrant further studies comparing post-operative infection rates in matched patients with different bearing combinations (Hexter *et al.*, 2018).

1.2 Periprosthetic joint infection (PJI)

1.2.1 Definition and classification

Periprosthetic joint infection (PJI) remains one of the most serious complications of arthroplasty surgery with devastating effects for patients and major socio-economic consequences (Cooper and Della Valle, 2013; Osmon *et al.*, 2013). Patients with PJI have recurrent hospital admissions, long term antibiotic therapy, repeated surgical procedures and an extended rehabilitation process (Kapadia *et al.*, 2013; Shanmugasundaram *et al.*, 2013). Despite the improvement of prevention techniques such as clean air theatres, full body suits and prophylactic antibiotics, the rate of PJI remains in UK at 0.6 to 2.5% following primary total hip or knee arthroplasty, and 2.1 to 5.8 % in revision surgery (Vanhegan *et al.*, 2012b).

There is no universally accepted definition of PJI and therefore its diagnosis is challenging (Alijanipour *et al.*, 2013). The Musculoskeletal Infection Society described a new definition for PJI in an attempt to create a "gold standard" to improve current practice (Workgroup Convened by the Musculoskeletal Infection, 2011). This definition includes the presence of a sinus tract communication with the prosthesis or isolation by culture of a pathogen from ≥ two separate tissue or fluid samples from the affected joint, or the presence of four of the following six criteria:

- Erythrocyte sedimentation rate (ESR) greater than 30 mm/h
- C-reactive protein (CRP) concentration greater than 10 mg/L
- Elevated synovial leukocyte count, Elevated synovial neutrophil percentage
- Purulence in the affected joint
- Isolation of a microorganism in one culture of periprosthetic tissue or fluid
- More than five neutrophils per high-power field in five high-power fields observed from histologic analysis of periprosthetic tissue at x 400 magnification

This definition has been criticized for several reasons: the low threshold for considering positive CRP and ESR which improves the sensitivity but reduces the specificity and the acceptance of 2 positive tissue cultures instead of 3 which has been shown to have greater specificity (Oussedik *et al.*, 2012). PJI is possible even if the above criteria are not

met and the clinician should use clinical judgment to determine if this is the case (Parvizi *et al.*, 2013). The Infectious Diseases Society of America in 2012 produced guidelines on the diagnosis of PJI, again in an attempt to provide consensus across the medical community. Their definition of PJI also included the presence of a sinus tract communication with the prosthesis, presence of acute inflammation on histopathological examination of tissue at the time of the surgical debridement, the presence of purulence around the prosthesis without another known aetiology, two or more intraoperative cultures of combination of pre-operative aspiration and intra-operative cultures that yielded the same organism (Osmon *et al.*, 2013). This definition does not include any inflammatory markers and suggests clinical judgment should be used for cases that do not fulfill the criteria. More recently, Parvizi et al proposed combining use of history, physical examination, imaging, ESR and CRP levels as diagnostic criteria, adding that, if the diagnosis is still unclear, joint aspiration with analysis of synovial leukocyte count, polymorphonuclear cell percentage and leukocyte esterase levels in addition to the routine pathogen culture should be obtained. He also advised the use of more novel technologies in those cases of indolent infection including alpha-defensin or interleukin 6 (Parvizi *et al.*, 2016).

In 2013 the International Consensus Group on Peri-prosthetic Joint Infections made some modifications to the 2011 Musculoskeletal Infection Society definition (Parvizi *et al.*, 2013) advising two positive periprosthetic cultures with phenotypically identical organisms, or a sinus tract communicating with the joint, or having 3 of the following minor criteria:-

- Elevated serum; C-reactive protein (CRP) AND erythrocyte sedimentation rate (ESR)
- Elevated synovial fluid white blood cell (WBC) count; OR ++ change on leukocyte esterase (LE) test strip
- Elevated synovial fluid polymorphonuclear neutrophil (PMN) percentage
- Positive histological analysis of periprosthetic tissue
- A single positive culture

In 2018, the same consensus modified the definition once again, including novel diagnostic tests and developing a new scoring system to more accurately define PJI (Table 1-1) (Parvizi *et al.*, 2018).

Because there is no absolute test available that accurately diagnoses PJI, clinicians have to use their clinical judgment along with a combination of tests. There is significant evidence demonstrating a primitive but specific immune response to pathogens (Manger and Relman, 2000; Fessler *et al.*, 2002; Deirmengian *et al.*, 2005; Matussek *et al.*, 2005; Kim *et al.*, 2008). The innate immune response triggers a cascade of protective pathways in the host

when a pathogen is identified (Deirmengian *et al.*, 2005). This specific response is also observed at the level of the proteome, revealing a number of biomarkers that have the potential to be developed into a diagnostic test for prosthetic joint infection (Jacovides *et al.*, 2011) (Deirmengian *et al.*, 2010).

Table 1-1- New diagnostic scoring system for PJI (Parvizi *et al.*, 2018).

Major criteria (at least one of the following)	**Decision**
Two positive cultures of the same organism	Infected
Sinus tract with evidence of communication to the joint or visualisation of the prosthesis	

	Minor criteria		**Score**	**Decision**
Pre-operative Diagnosis	Seru	Elevated CRP or D-Dimer	2	≥ 6 Infected 2-5 Possibly infected 0-1 No infected
		Elevated ESR	1	
	Synovial	Elevated synovial WBC or LE	3	
		Positive alpha-defensin	3	
		Elevated synovial PMN(%)	2	
		Elevated synovial CRP	1	

	Inconclusive preoperative score or dry tap	**Score**	**Decision**
Intra-op. Diagnosis	Preoperative score	-	≥ 6 Infected 4-5 Possibly infected ≤ 3 No infected
	Positive histology	3	
	Positive purulence	3	
	Single positive culture	2	

Serum biomarkers are more favourable than synovial biomarkers due to the low risk nature of a blood test compared to synovial fluid aspiration (Shahi and Parvizi, 2016). The most commonly used serum markers in the diagnosis of PJI are CRP and ESR. Both tests have a reported relatively low specificity. A meta-analysis published in 2010 described a pooled sensitivity for CRP of 88% and for ESR 75% with a pooled specificity of 70% and 74% respectively (Berbari *et al.*, 2010). Despite being part of the routine workout to diagnose PJI, both tests can produce results that are often unreliable as these biomarkers can also be raised when the patients suffer from other inflammatory disorders or comorbidities like

obesity (Lee and Pratley, 2005; Liu *et al.*, 2014). Both markers are also raised post-operatively. CRP can take two weeks to normalise after surgery while ESR can take up to 6 weeks and therefore these markers are not diagnostically useful for the first few weeks post-surgery (Parvizi and Della Valle, 2010).

Another potential diagnostic serum biomarker is procalcitonin (PTC). PTC is elevated in the presence of bacteria and can be useful to differentiate between bacterial joint infections and other causes of inflammation (Shahi and Parvizi, 2016). The results of PTC value as a diagnostic tool in PJI remain controversial (Drago *et al.*, 2011). In some studies PTC was useful in the diagnosis of PJI with a sensitivity of 93% and specificity of 75% when the threshold was 0.25ng/mL had (Hugle *et al.*, 2008). More recent studies had opposite results showing PTC had no value in diagnostic PJI (Worthington *et al.*, 2010; Drago *et al.*, 2011) and a meta-analysis performed in 2013 showed a pooled sensitivity of 67% and a specificity of 90% ; although the studies included in this meta-analysis included only patients with septic arthritis and osteomyelitis, and no prosthetic joint infections (Shen *et al.*, 2013).

Interleukin 6 (IL-6) is secreted by non-lymphoid cells, and it is a major regulator of the acute phase response (Song and Kellum, 2005). It stimulates the secretion of CRP and therefore IL-6 raises quicker than CRP in response to infection or trauma (Heinrich *et al.*, 1990) and the serum level returns to normal much faster than CRP or ESR, 48-72 hours after surgery (Wirtz *et al.*, 2000) and therefore is believed to be useful for early post-operative diagnosis of PJI (Shah *et al.*, 2009). Serum IL-6 has been shown to be elevated in PJI cases (Berbari *et al.*, 2010; Worthington *et al.*, 2010) and has been demonstrated to have a sensitivity of 72% and a specificity of 89% (Xie *et al.*, 2017). Synovial IL-6 has a higher diagnostic value for PJI and has been shown to have a sensitivity between 89-91% and a specificity between 90-97% (Deirmengian *et al.*, 2014; Xie *et al.*, 2017).

Another synovial biomarker which has shown promising results is α-defensin. Alpha defensin is a microbicidal peptide produced by neutrophil, macrophages and Paneth cells and in response to microbial products or pro-inflammatory cytokines (Shahi and Parvizi, 2016). A recent meta-analysis has shown a pooled sensitivity and specificity 98% and 97% respectively (Li *et al.*, 2017a). It is the most successfully marketed biomarker that has been commercialised as a diagnostic test for PJI, has been proven to have consistent results regardless of the infecting organism (Deirmengian *et al.*, 2015) and it is not affected by systemic antibiotics (Shahi *et al.*, 2016). Although available, it is not widely used due to its high cost (£500 per test) (Wyatt *et al.*, 2016; Wouthuyzen-Bakker *et al.*, 2017).

A large number of synovial biomarkers have been tested showing promising results

(Table 1-2) but further studies with higher numbers of patients and different sub-groups are needed to further assess the diagnostic value of such biomarkers (Deirmengian *et al.*, 2014).

More recently calprotectin, a protein present in the cytoplasm of neutrophils and released upon their activation, has been shown to have good potential as a diagnostic test for PJI with 89% sensitivity and 92% specificity. It has a 94.4% negative predictive value, making it a good tool to rule out infection in the emergency setting and revision surgery (Wouthuyzen-Bakker *et al.*, 2017). Each test had an estimated cost in 2018 of £17 which makes it more attractive and easier to implement in routine practice (Wouthuyzen-Bakker *et al.*, 2018). But these studies have relatively low numbers of patients and therefore further studies will be needed to fully assess the diagnostic value of this test.

Table 1-2 Diagnostic characteristics of synovial fluid biomarkers (Deirmengian *et al.*, 2014).

Biomarker	**AUC**	**Cut-off**	**Specificity (%)**	**95% CI (%)**	**Sensitivity (%)**	**95% CI (%)**
α-defensin	1.000	4.8μg/mL	100	95-100	100	88-100
ELA-2	1.000	2.0μg/mL	100	95-100	100	88-100
BPI	1.000	2.2μg/mL	100	95-100	100	88-100
NGAL	1.000	2.2μg/mL	100	95-100	100	88-100
Lactoferrin	1.000	7.5μg/mL	100	95-100	100	88-100
IL-8	0.992	6.5μg/mL	95	87-99	100	88-100
SF CRP	0.987	12.2μg/mL	97	90-100	90	73-98
Resistin	0.983	340μg/mL	100	95-100	97	82-99
Thrombospondin	0.974	1061μg/mL	97	90-100	90	73-98
IL-1β	0.966	3.1μg/mL	95	87-99	96	82-100
IL-6	0.950	2.3μg/mL	97	89-100	89	71-98
IL-10	0.930	32.0μg/mL	89	79-96	89	72-98
IL-1α	0.922	4.0μg/mL	91	81-97	82	63-94
IL-17	0.892	3.1μg/mL	99	92-100	82	63-94
G-CSF	0.859	15.4μg/mL	92	82-97	82	62-94
VEGF	0.850	2.3μg/mL	77	65-87	75	55-89

AUC = area under the curve; α-defensin = human α-defensin; ELA-2 =neutrophil elastase 2; BPI = bactericidal/permeability-increasing protein; NGAL = neutrophil gelatinase-associated lipocalin; SF = synovial fluid; CPR = C-reactive protein; G-CSF = granulocyte colony-stimulating factor; VEGF= vascular endothelial growth factor

Despite the identification of numerous promising biomarkers, no single highly accurate, cost effective and feasible test has yet been identified (Alvand *et al.*, 2017).

Another method of diagnosis is by polymerase chain reaction (PCR). This method targets bacterial deoxyribonucleic acid (DNA) or ribonucleic acid (RNA), which involves using universal primers; genetic templates from most bacterial strains can be amplified (Kuo

et al., 2018). This method is complex and has been criticized for its high false-positive incidence although some studies have demonstrated a sensitivity of 100% and a specificity of 99.5% (Kuo *et al.*, 2018).

The European Association of Nuclear Medicine (EANM) along with the European Radiology Society (ERS) and the European Bone and Joint Infection Society (EBJIS) have recently published guidelines on the use of advanced imaging tests as an aid to diagnose PJI including the use of WBC scans in those suspected infections in prostheses implanted within 2 years and 3-phase bone scans for prostheses implanted for more than 2 years (Signore *et al.*, 2019).

Classifications often guide the management plan. Currently there is no consensus on a universal classification for PJI (Kuiper *et al.*, 2014). It is often divided into early and late infection, but the difficulty is to clearly establish the time that defines early or late infection. Albotins et al. propose a classification where infection is classified as early if presentation is within the first 3 months after the operation, delayed if it is between 3 months and 2 years and late if it is beyond 2 years (Table 1-3) (Aboltins *et al.*, 2014). Medical bodies such as the Infectious Disease Society of America and the American Academy of Orthopaedic Surgeons define early infection as an infection that occurs within 3 weeks of implantation of the prosthesis or within 3 weeks of the initial symptoms of a haematogenous infection (Della Valle *et al.*, 2011; Osmon *et al.*, 2013). In their definition any infection happening beyond three weeks is considered late (Gehrke *et al.*, 2015). Later on Shahi et al. described a four stage classification system based on the interval between surgery and the onset of symptoms: Stage one or early infection is defined when symptoms start within the first 4 to 8 weeks post operatively, stage two or delayed infection when symptoms present between 3 to 24 months after surgery, stage 3 or late onset infection when symptoms manifest after 2 years postoperatively and stage four or silent PJI is defined when a positive culture is obtained at the time of revision surgery in an asymptomatic patient (Shahi and Parvizi, 2015). This variation in the literature highlights the difficulty in adequately defining and classifying the different stages of prosthetic joint infection. Appropriate classification of PJI is essential as it dictates surgical treatment and possible outcomes (Petretta *et al.*, 2016).

Table 1-3. PJI classification (Aboltins *et al.*, 2014).

Classification	Time from arthroplasty to symptoms	Common clinical features	Pathogenesis	Typical organisms
Early	<3 months	Pain , erythema, prolonged post-operative wound discharge, fever	Organisms introduced through the surgical wound during arthroplasty of the post-operative period	*Staphylococcus aureus*, gram negative bacilli, enterococci
Delayed	3-24 months	Indolent onset of pain, implant loosening, sinus	Organisms introduced through the surgical wound with delayed manifestation	Coagulase negative staphylococci, *Propionibacterium acnes*
Late (haematogenous)	>24 months	Acute onset of pain, fever, erythema, bacteremia	Organisms seed to prosthesis haematogenously	*Staphylococcus aureus*, gram negative bacilli, streptococci

1.2.2 Microorganisms associated with PJI

Mirza et al. demonstrated that the number of *Staphylococcus aureus* cells required to establish an infection is reduced 10,000 fold when a prosthetic implant is present (Mirza *et al.*, 2016). The immune system also has difficulty accessing the infection site due to the avascular nature of the implant (Campoccia *et al.*, 2006). Because of these and other human and environmental factors, a multitude of microorganisms can infect artificial joints. It has been shown that in up to 36% of cases studied, the infection can be polymicrobial in origin (Moran *et al.*, 2007). In a large proportion of cases staphylococci, including methicillin-sensitive *Staphylococcus aureus* (MSSA), as well as coagulase-negative Staphylococci, are responsible for PJI (Blom *et al.*, 2004; Pulido *et al.*, 2008; Geipel, 2009; Mortazavi *et al.*, 2010; Peel *et al.*, 2012; Sukeik *et al.*, 2012; Aggarwal *et al.*, 2014; Tande and Patel, 2014; Hickson *et al.*, 2015; Lewis *et al.*, 2015; Benito *et al.*, 2016; Mirza *et al.*, 2016; Sambri *et al.*, 2017), with *Staphylococcus epidermidis* being one of the most common amongst the coagulase-negative staphylococci (Bogut *et al.*, 2014). Some studies have found as many as 57 % of the cases of PJI are thought to be caused by *Staphylococcus aureus* (Peel *et al.*,

2012) and 27% by Coagulase-negative Staphylococci. Trends are continuously changing and there is an increase in Gram negative bacteria such as *Enterobacteriaceae* being associated with PJI (Benito *et al.*, 2016).

Staphylococcus aureus is an opportunistic pathogen that lives harmlessly on the skin but is able to cause disease under certain circumstances. It is responsible for PJI as well as osteomyelitis, endocarditis, and it is the second leading causative organism of nosocomial infection and community acquired blood stream infections (Laupland and Church, 2014; Mirza *et al.*, 2016). *Staphylococcus aureus* is an aggressive organism, causing high morbidity and mortality, despite treatment (Lewis *et al.*, 2015; Makki *et al.*, 2017). *Staphylococcus aureus* is most commonly isolated in early infection (Moran *et al.*, 2007). Poor prognosis is often associated with methicillin-resistant *Staphylococcus aureus* (MRSA) infection (Salgado *et al.*, 2007; Parvizi *et al.*, 2009; Cunningham *et al.*, 2017), due to the limited effective antibiotic therapy (Ferry *et al.*, 2010; Vaudaux *et al.*, 2012) although the addition of rifampicin as part of the treatment of MRSA prosthetic joint infection has shown increased success in implant retention (55-90%) which is comparable to the success rate of those with other staphylococcal infections (Aboltins *et al.*, 2007; Senneville *et al.*, 2011; Lora-Tamayo *et al.*, 2013).

Coagulase-negative *Staphylococcus* species are often associated with delayed prosthetic joint infection (Moran *et al.*, 2007; Tornero *et al.*, 2012; Lewis *et al.*, 2015). Of all the Coagulase-negative staphylococcus species, *Staphylococcus epidermidis*, a human skin flora commensal, has been shown to be one of the most prevalent pathogens associated with PJI (Zimmerli *et al.*, 2004). The prevalence of Coagulase-negative *Staphylococcus* species is increasing, and often associated with methicillin and fluoroquinolone resistance, making the antibiotic options limited (Tornero *et al.*, 2012). Its infection is often associated with biofilm formation (Otto, 2008). Although is thought to be less virulent than *Staphylococcus aureus*, the treatment should remain equally aggressive with these infections (Murgier *et al.*, 2016).

Between 4 and 12% of prosthetic joint infections are associated with *Streptococcus* species (Peel *et al.*, 2012; Benito *et al.*, 2016). These infections often present as a haematogenous infection (Lora-Tamayo *et al.*, 2017) and the success rate of their treatment has been shown to be 79-94% (Everts *et al.*, 2004; Marculescu *et al.*, 2006), although these results come from smaller series and a more recent and large multicentre study published by Lora-Tamayo et al. showed a success rate of 57% and it is likely to demonstrate a more accurate representation of the success rate with the study being multicentre and significantly larger (462 cases vs 99) (Lora-Tamayo *et al.*, 2017). The use of β-lactam antibiotics as part of

the medical management of *Streptococcus* PJI is recommended (Osmon *et al.*, 2013) although their activity against biofilms is poor due to their high minimum biofilm eradication concentration (Olson *et al.*, 2002).

Polymicrobial infection accounts for 19 to 37% of all PJI (Wimmer *et al.*, 2016). Older patients, over 65 and patients who suffer wound dehiscence are at higher risk of developing a polymicrobial PJI (Marculescu and Cantey, 2008). Polymicrobial infection is often associated with more virulent organisms including *Enterococcus* species, anaerobic bacteria and methicillin resistant *Staphylococcus aureus* (Duijf *et al.*, 2015; Tan *et al.*, 2016a; Kheir *et al.*, 2017). Treatment includes the use of multiple broad spectrum antibiotics which is associated with higher costs (Peel *et al.*, 2013), mortality and failure following surgical treatment than monomicrobial infection (Bozhkova *et al.*, 2016; Tan *et al.*, 2016a). Mardulescu et al. found no difference in surgical outcomes between monomicrobial and polymicrobial PJI (Marculescu and Cantey, 2008). This study included a small number of 34 patients with polymicrobial infections and therefore it may lack the power to detect differences in the patient sub-groups.

A small percentage of PJI is caused by *Enterococcus* species (Tornero *et al.*, 2014; Kheir *et al.*, 2017). Patients with *Enterococcus* infection often present with pain and loosening of the prosthesis but minimal systemic features (El Helou *et al.*, 2008). This species is often resistant to several antibiotics, making the treatment of PJI challenging and resulting in poor outcomes (Tornero *et al.*, 2014). The medical treatment of polymicrobial PJI remains controversial: some authors report no difference in outcome between patients treated with monotherapy or a combination of antibiotics (El Helou *et al.*, 2008) while others advocate combination therapy (Tornero *et al.*, 2014; Kheir *et al.*, 2017).

Anaerobic bacteria can also be the cause of PJI. The most common anaerobic pathogen associated with PJI is *Propionibacterium acnes* (*P. acnes*) (Kierzkowska *et al.*, 2017). *P. acnes* is a Gram-positive anaerobic rod found in the skin, usually non-pathogenic, but in certain cases can cause severe infections including PJI (Zappe *et al.*, 2008). It is estimated that *P. acnes* is involved in 4-6% of all prosthetic infections (Figa *et al.*, 2017) although it is believed the actual number of cases is higher because it is often misdiagnosed (Zappe *et al.*, 2008). Because it is part of the normal skin flora, when grown on cultures *P. acnes* is commonly labeled as a contaminant (Zeller *et al.*, 2007). *P. acnes* infection has few clinical symptoms and often produces normal test results due to its low virulence, making its diagnosis extremely challenging (Figa *et al.*, 2017).

Fungal infection associated with PJI is very rare, representing around 1% of prosthetic

infections and mainly occurs in immunocompromised patients (Yilmaz *et al.*, 2011). The majority of the fungal prosthetic joint infections are caused by *Candida* species, particularly by *Candida albicans* (Yilmaz *et al.*, 2011; Cobo *et al.*, 2017) but a few cases of *Aspergillus* have also been reported (Yilmaz *et al.*, 2011). Symptoms are usually mild and the diagnosis can often be delayed as *Candida* infection is not considered in the differential diagnosis scheme due to the scarcity of cases (Cobo *et al.*, 2017). The outcome of these infections has been shown to be poor compared to other organisms perhaps due to other variables including the immunocompromised state from which patients with fungal infections often suffer (Cunningham *et al.*, 2017).

On certain occasions, it is not possible to identify a causative organism. Culture negative (CN) prosthetic joint infection is particularly challenging due to the subsequent difficulty in selecting the appropriate antibiotic regime. Previous antibiotic therapy and the use of post-operative wound drainage are risk factors for developing CN infection which has an incidence of 7-42% (Malekzadeh *et al.*, 2010; Yoon *et al.*, 2017). Although there is no consensus on the appropriate treatment of CN PJI, glycopeptides and cephalosporins are the two most common antibiotics of choice for the medical treatment of PJI and a two stage revision the most effective surgical treatment, with success rates varying between 70 and 100% (Yoon *et al.*, 2017).

Despite staphylococcal species often being the causative organism of prosthetic joint infections, there are geographical variations in the prevalence of such pathogens (Hickson *et al.*, 2015). This variation contributes to the difficulty of a national or international standardisation of the management and treatment of PJI. It is essential to understand and follow local policies and guidelines when prescribing antibiotic treatment and prophylaxis to ensure the more likely organisms are covered.

Following the identification of the most prevalent pathogens associated with prosthetic joint infection it was decided to focus the work of this thesis on examination of the two main bacterial species associated with PJI: *Staphylococcus aureus* and *Staphylococcus epidermidis.* Both strains are aerobic Gram positive bacteria, but as mentioned above, other organisms including Gram negative species and anaerobic pathogens as well as fungi can cause PJI. Therefore further investigations with such strains will be necessary to fully assess the effect of NucB on PJI.

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1.2.3 Current surgical treatment

The goal for the treatment of PJI is the complete eradication of the infection and restoration of mobility (Sukeik *et al.*, 2012; Gehrke *et al.*, 2013). PJI is often associated with biofilm formation (Geipel, 2009; Patenge *et al.*, 2012) whereby the causative microorganisms are embedded in a thick and viscous protective extracellular matrix (Vanhegan *et al.*, 2012b). Biofilms are known to reduce the effectiveness of antibiotics (Mah and O'Toole, 2001; Donlan and Costerton, 2002; Chiang *et al.*, 2013). The resulting poor response to antibiotic therapy increases the need for higher doses of antibiotics (Welliver *et al.*, 2014) and aggressive surgical treatments. Currently in the UK, prosthetic joint infections are voluntarily reported to Public Health England (Dryden, 2014). These reports produce national figures that help us to understand the patterns and epidemiology of infection (Dryden, 2014; Lamagni, 2014).

A multidisciplinary approach to the treatment of PJI is essential, as it has been shown that patients treated by a multidisciplinary team have better outcome and prognosis (Ibrahim *et al.*, 2014). The multidisciplinary team includes orthopaedic surgeons, plastic surgeons, musculoskeletal radiologists, microbiologists and infectious disease specialists who can offer advice regarding the best antimicrobial for each individual case as well as monitor antibiotic use and response (Cooper and Della Valle, 2013; Osmon *et al.*, 2013; Perez-Jorge *et al.*, 2016). One of the main challenges is to identify whether the infection is superficial or deep and whether the implanted prosthesis is involved (Dryden, 2014). The lack of consensus on the definition and classification of infection, as well as the individuality of many cases makes it difficult to identify and establish a standardised effective treatment. Each case has to be tailored to the individual needs depending on the comorbidities, clinical presentation, causative organism and patient's wishes, and can include a wide variety of treatment options such as individual antibiotic regime treatment, surgery with or without removal of prosthesis and long term suppression treatment (Dryden, 2014).

There are several different strategies used to treat PJI. The current gold standard remains two-stage revision surgery (Della Valle *et al.*, 2011; Cooper and Della Valle, 2013). The first stage is to remove the infected prosthesis and insert a temporary spacer often loaded with antibiotics. The second procedure, performed weeks later, is to remove the spacer and re-implant a new prosthesis. In between operations the patient will receive targeted intravenous antibiotic therapy for a period of weeks (Cooper and Della Valle, 2013; Zmistowski *et al.*, 2016). The antibiotic regime is not standardised and varies depending on hospital policy. In the US a common regime is 6 weeks of antibiotic therapy followed by 2

weeks antibiotic free before considering re-implantation of prosthesis (Muhlhofer *et al.*, 2018). Other regimes include two weeks of intravenous therapy followed by 4 weeks of oral antibiotic therapy and with regular inflammatory marker tests every two weeks until re-implantation (Wang *et al.*, 2018). The two-stage revision is associated with increased morbidity and it is poorly tolerated by patients due to long combined hospital stays, antibiotic treatments and the need for removal of the prosthesis with the insertion of a temporary spacer (Leonard *et al.*, 2014). Less aggressive approaches are becoming more popular as they have less morbidity, shorter hospital stays and lower costs (Kim *et al.*, 2014).

One stage revision is appropriate when the causative organism is isolated prior to surgery so that targeted antibiotic treatment can be given in the intra and postoperative period (Gehrke and Kendoff, 2012; Gehrke *et al.*, 2013). In 2014 an international group of arthroplasty surgeons concluded that single stage revision surgery was a reasonable surgical treatment when appropriate antibiotics were available as long as none of the following exclusion criteria were present: a presence of sinus; generalised sepsis; infection caused by antibiotic-resistant bacteria; infection where bacteria cannot be identified and the presence of severe soft tissue deficiency over the joint (Lichstein *et al.*, 2014). Although is difficult to establish the true cost difference between one and two stage revision surgery, it appears that single stage revision is likely to save costs related to length of patient stay, morbidity, surgical costs and duration of antibiotic administration (Klouche *et al.*, 2010; Gulhane *et al.*, 2012; Kurtz *et al.*, 2012). Success rates for single stage revision surgery are variable in the literature and range between 65% and 100 % (von Foerster *et al.*, 1991; Goksan and Freeman, 1992; Sofer *et al.*, 2005; Parkinson *et al.*, 2011; Gulhane *et al.*, 2012). The cases where a 100% success rate was obtained the studies were small series in very selected patients adhering to a very strict criteria (Parkinson *et al.*, 2011; Gulhane *et al.*, 2012). These results also highlight that single stage can be a successful treatment option when chosen appropriately with the help of strict protocols. A more recent systematic review showed similar re-infection rates between single and two stage revision, but single stage revision had better functional outcomes, probably due to early mobilization (Leonard *et al.*, 2014). Single stage revision surgery is gaining popularity and more evidence is coming out in favour of such a treatment for PJI. An almost 95% successful eradication of infection after a single stage revision hip surgery with cementless implants has been reported (Bori *et al.*, 2014; Bori *et al.*, 2018) including when the causative organism is fungi (Ji *et al.*, 2017). These are small case series and further larger studies are required to validate the results. There is currently no randomised controlled trial comparing single vs two stage revision surgery and therefore

there is no high quality evidence to demonstrate the superiority of either surgical approach to the treatment of PJI (Masters *et al.*, 2013). Single stage revision surgery for PJI is an established approach in selected patients and this method has been shown to be cost effective and preferred by patients due to reduced morbidity and improved patient experience.

Aggressive early debridement is reserved for cases where surgical treatment occurs within days or weeks of the onset of infection (Romano *et al.*, 2012; Sukeik *et al.*, 2012; Alijanipour and Parvizi, 2014). The rationale behind debridement and implant retention (DAIR) is the attempt to remove biofilm with aggressive surgical debridement and exchange of modular components, followed by eradication of the causative organism with antibiotic treatment. It has been shown that the shorter the period of infection the higher the chance of DAIR being successful (Kuiper *et al.*, 2013) with evidence suggesting that if performed within 2-4 weeks of implantation the success of DAIR is higher (Tsukayama *et al.*, 1996; Crockarell *et al.*, 1998; Fink *et al.*, 2017; Narayanan *et al.*, 2018). Some studies have shown no relationship between the timing of DAIR and success rates (Byren *et al.*, 2009; Fehring *et al.*, 2013). A consensus meeting in 2014 strongly agreed that surgical debridement was an option for infections within 3 months of initial implantation or 3 weeks from onset of symptoms (Haasper *et al.*, 2014). A more recent consensus strongly agreed with a moderate level of evidence that symptoms should not be present for longer than 4 weeks (Argenson *et al.*, 2019). A possible explanation for the conflicting evidence is that biofilm formation is extremely variable, depending on the infective organisms (some organisms are more aggressive, some have a lower virulence), the inoculum size that contaminates the wound also plays a role as well as the host (Lovati *et al.*, 2017; Lowik *et al.*, 2019). It is thought that it may not be possible to completely eradicate fully mature biofilms without explanting all the prosthetic components and therefore DAIR should not be an option for those patients whose infective symptoms have been present for longer than 4 weeks (Argenson *et al.*, 2019). Multiple studies have demonstrated that symptoms present for less than 7 days have been associated with a higher success rate (Marculescu *et al.*, 2006; Qasim *et al.*, 2017; Tsang *et al.*, 2017; Urish *et al.*, 2018) but establishing the exact length of symptoms can be challenging (Kim *et al.*, 2019).

The results of debridement and implant retention surgery are very variable. The failure of DAIR is likely due to the inability to successfully disperse the biofilm and a substantial amount remains on the implanted prosthesis (Urish *et al.*, 2014). Between 20 and 87% success has been reported in the literature (Sukeik *et al.*, 2012; Alijanipour and Parvizi, 2014; Fink *et al.*, 2017). The variable success of this method of treatment may again be

explained by the combination of complex and multifactorial process of biofilm formation and multiple host related factors.

A failed DAIR compromises the successful outcome of further surgical treatment and limits the ability to control the infection (Urish *et al.*, 2017; Rajgopal *et al.*, 2018), such findings have been disputed (Brimmo *et al.*, 2016; Kim *et al.*, 2019). DAIR is less invasive with a faster recovery, it is often used as a first step in an attempt to save the prosthesis and prevent more aggressive surgical procedures as well as obtaining histological samples for laboratory analysis and bacterial identification. DAIR relies on the patients' immune system to fight the infection, so in patients where the immune system is compromised this procedure is not suitable. It may have a role for patients deemed too high risk due to comorbidities for multistage revisions (Qasim *et al.*, 2017).

1.3 The role of biofilms in PJI

1.3.1 Definition

Biofilms are communities of microorganisms which adhere to a surface and become embedded in a thick and protective self-produced extracellular matrix (Costerton *et al.*, 1999; O'Toole *et al.*, 2000; Donlan and Costerton, 2002). These bacterial communities were first observed by Van Leeuwenhoek, inventor of the microscope, in the seventeenth century (Costerton *et al.*, 1999; Donlan and Costerton, 2002) but it was Henrici who described biofilm in 1933, "The deposit of bacteria becomes apparent in a few days and increases progressively, eventually becoming so thick that individual cells may be distinguished with difficulty" (Henrici, 1933). It was not until the 1980s with new technological advances such as Confocal Scanning Laser Microscopy (CSLM) that biofilms were examined and described in more detail (Costerton *et al.*, 1994).

1.3.2 Structure and life cycle

Biofilms are complex living structures (Fletcher, 1994). The study of the biofilm structure has evolved during the past twenty years. The development of CLSM allowed researchers to observe the biofilm in a hydrated state (Costerton *et al.*, 1995; O'Toole *et al.*, 2000).

The image of a biofilm as a homogenous structure with cells piled on top of each other (Costerton *et al.*, 1994; O'Toole *et al.*, 2000) was discarded and a complex heterogeneous structure with different density microcolonies, sometimes in mushroom-shaped structures, intersected by water channels and held together by extracellular polymers

was established (Costerton *et al.*, 1994; Fletcher, 1994; Costerton *et al.*, 1995; Branda *et al.*, 2005).

Biofilm formation can occur at any solid-liquid interface (Costerton *et al.*, 1994; Allegrucci *et al.*, 2006). The first step for the formation of the biofilm is the attachment of the cell to the surface (Figure 1-2). This attachment is mediated by physical forces and by bacterial appendages such as pili or flagella (Maric and Vranes, 2007). During this attachment an initial reversible phase occurs where the bacteria can detach from the surface (Garrett *et al.*, 2008). This reversible attachment phase will progress to an irreversible attachment due to the presence of extracellular polymers (Stoodley *et al.*, 2002) and may be mediated by type IV pili or other adhesin proteins (O'Toole *et al.*, 2000). Type IV pili are displayed on the surface of Gram-negative and Gram-positive bacteria (Melville and Craig, 2013). They are involved in bacterial motility, attachment to surfaces and biofilm formation (Mikkelsen *et al.*, 2011). During the attachment phase a number of genes are activated to initiate the production of the extracellular matrix (ECM) (Costerton *et al.*, 1999). With the production of ECM the biofilm moves to a maturation phase where cell-to-cell communication (also called quorum sensing) plays an important role (Stoodley *et al.*, 2002; Yarwood *et al.*, 2004). To complete the life cycle and to colonise new surfaces, some bacteria have to be released from the biofilm and revert to a planktonic state (Costerton *et al.*, 1999; Stoodley *et al.*, 2002). This event might be due to the release of the bacteria by matrix

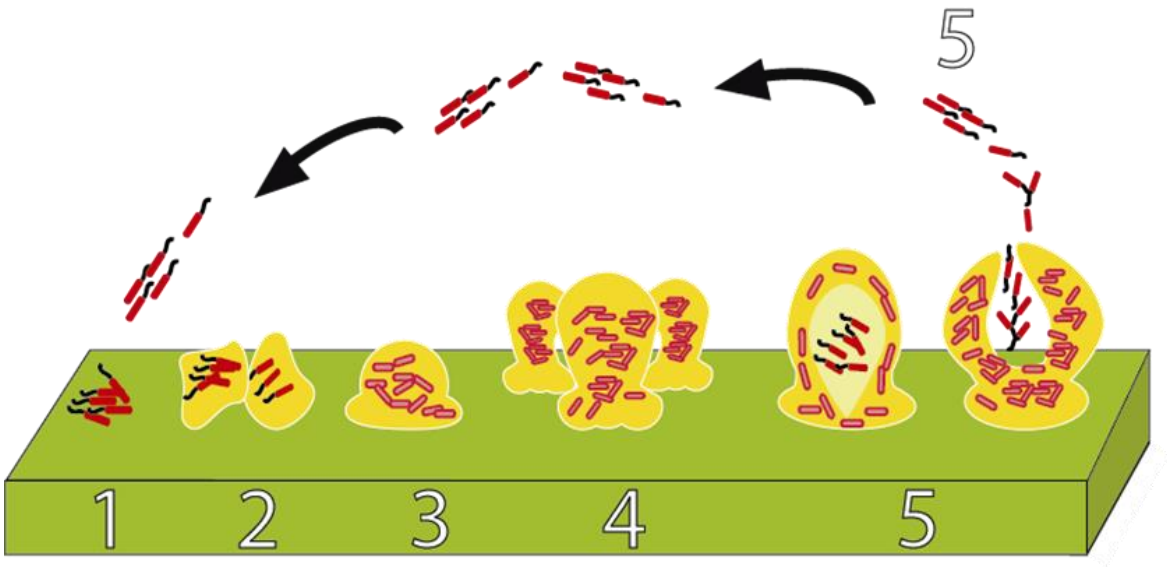


Figure 1-2 Biofilm life cycle: 1-individual cells attach to the surface, 2- an extracellular matrix is produced, attachment becomes irreversible, 3-biofilm matures, 4- biofilm develops, 5- finally single cells are released from the biofilm (modified from Stoodley *et al.*, 2002)).

degrading enzymes, which break down the ECM releasing the bacteria into the medium (Stoodley *et al.*, 2002). The detached bacteria can be in the form of a single cell or clusters of cells of different sizes (Stoodley *et al.*, 2001). Large clusters of cells, although less commonly detached from the biofilms, have a disproportionately higher number of cells and therefore that can potentially have a higher infective activity (Stoodley *et al.*, 2001).

The extracellular matrix is a shared feature in biofilms. In the majority of cases the extracellular matrix accounts for 90% of the biofilm mass, leaving the remaining 10% to the microorganism (Flemming and Wingender, 2010). The matrix provides structure to the biofilm, adhesion to surfaces (Flemming and Wingender, 2010) and protects the bacteria against the attack of external particles such as the human innate response, antibiotics or disinfectants (Mah and O'Toole, 2001; Donlan and Costerton, 2002; Chiang *et al.*, 2013). The composition and structure of the ECM can vary between species and even between strains of the same species (Branda *et al.*, 2005; Das *et al.*, 2014). Within a biofilm, the ECM is heterogeneous, suggesting a physical structure that segregates different extracellular activities (Lawrence *et al.*, 2007). Commonly the ECM is a combination of polysaccharides, proteins, water and and also extracellular DNA (eDNA) (Nijland *et al.*, 2010; Boles and Horswill, 2011).

It is possible to study biofilm formation practically in real time (Moormeier and Bayles, 2017). This technology has helped to identify and describe the biofilm formation of *Staphylococcus aureus* as a 5 stage process: 1) attachment, 2) multiplication, 3) exodus, 4) maturation, and 5) dispersal (Figure 1-3) (Moormeier *et al.*, 2014).

When introducing a new material into the human body, this material is rapidly coated by a multitude of host-related matrix proteins (O'Gara, 2007). The process of biofilm formation is comprised of a wide range of functional activities including molecule mediating binding to surfaces (Otto, 2009). The initial attachment phase of *Staphylococcus aureus* to material surfaces is mediated by different cell-wall anchored proteins (CWA) that vary depending on the host matrix component that the bacteria is attaching to (Moormeier and Bayles, 2017) and can be altered by growth conditions (Speziale *et al.*, 2014). These proteins are covalently attached to peptidoglycans and are not exclusively expressed by *Staphylococcus aureus* (Foster *et al.*, 2014). *Staphylococcus epidermidis* also produce CWA although to a much lesser degree than *Staphylococcus aureus* (Bowden *et al.*, 2005).

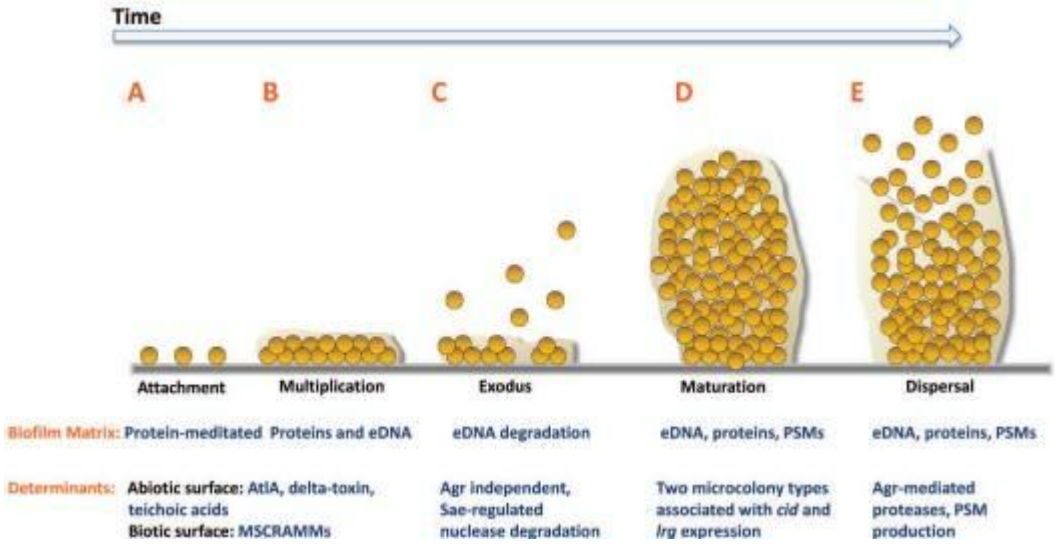


Figure 1-3 *Staphylococcus aureus* 5-step biofilm formation process (Moormeier and Bayles, 2017)

Some of the most common CWA are the microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) which includes Protein A (Nguyen *et al.*, 2000), SasG (Roche *et al.*, 2003) or fibronectin binding proteins (FnBPA and FnBPB) (Greene *et al.*, 1995; O'Neill *et al.*, 2008) amongst others. In *Staphylococcus epidermidis* biofilm, the surface proteins SSP-1 and SSP-2 have been shown to play a role in the initial attachment stage (Ammendolia *et al.*, 1999). Similarly, accumulation-associated protein (Aap) also contributes to the initial attachment of *Staphylococcus epidermidis* to surfaces (Speziale *et al.*, 2014). All these proteins play a crucial role when attaching to host surfaces but when it comes to abiotic material surfaces, it is the electrostatic and hydrophobic interactions involving Autolysin A and teichoic acid which play a major role in *Staphylococcus aureus* and *Staphylococcus epidermidis* initial attachment (Gross *et al.*, 2001; O'Gara, 2007; Houston *et al.*, 2011; Moormeier and Bayles, 2017).

Following the initial attachment to a surface, the cells begin to multiply and divide, the newly divided cells are at risk of detachment due to shear forces; *Staphylococcus aureus* is capable of producing an extracellular matrix that facilitates intercellular binding to prevent cellular detachment. This is what Moormeier determined as the multiplication stage (Moormeier and Bayles, 2017). Part of the extracellular matrix is the polysaccharide intracellular adhesin (PIA) (Cramton *et al.*, 2001; O'Gara, 2007) also called poly-N-acetyl glucosamine (PNAG) (Mack *et al.*, 1996) which promotes cell to cell adhesion by binding to the negatively charged surfaces of bacterial cells (Heilmann *et al.*, 1996; Speziale *et al.*, 2014). Its expression has been shown to be variable depending on the bacterial strain or

environmental conditions- anaerobic conditions have been shown to increase PIA expression (Cramton *et al.*, 2001). In *Staphylococcus aureus* and *Staphylococcus epidermidis*, the adhesion phase is associated with the presence and expression of the *ica* (intercellular adhesin) operon and the consequent production of the polysaccharide intercellular adhesin (Cramton *et al.*, 1999; Cafiso *et al.*, 2004). PIA is not the only component with a major role in cell adhesion, others include Protein A and SasC (Merino *et al.*, 2009; Schroeder *et al.*, 2009). The production of an early extracellular matrix that helps binding the cells and preventing its detachment may be mediated by a "regulated autolysis" (Bayles, 2014) that produces the secretion of extracellular DNA and cytoplasmic proteins such as cytoplasmic nucleoid-associated proteins (NAPs) (Goodman *et al.*, 2011; Foulston *et al.*, 2014). These cytoplasmic proteins bind to eDNA (Goodman *et al.*, 2011) and may have a critical importance during the multiplication stage of biofilm formation before the matrix components have had a chance to accumulate (Moormeier and Bayles, 2017).

The third stage in *Staphylococcus aureus* biofilm formation is called "exodus". This next stage takes place 6 hours after the multiplication phase starts and is mediated by nuclease-dependent degradation of eDNA (Moormeier and Bayles, 2017). This phenomenon induces a coordinated biofilm cell dispersion and produces a re-structure of the biofilm (Moormeier and Bayles, 2017). The exact role of this third stage is not fully understood but it is believed to be essential for the complete development of biofilm as has been shown that mutant *Staphylococcus aureus* cells that do not produce the necessary nucleases to initiate the exodus phase do not produce microcolonies (next phase in *Staphylococcus aureus* biofilm formation) (Moormeier *et al.*, 2014).

After the exodus stage come the maturation and dispersal stages (Moormeier and Bayles, 2017). During these phases, microcolonies: conglomerates of cells, proteins, polymers including exopolysaccharide, teichoic acids and eDNA, are formed from rapid cell division of the remaining cells after the exodus phase (Otto, 2008; Mann *et al.*, 2009; Moormeier and Bayles, 2017). In these microcolonies, the cells are organised and surrounded by channels that ensure nutrition to all cells including deeply embedded cells (Otto, 2008). The mechanism that leads to the formations of these channels is not fully understood. It is believed that cell to cell signaling plays an important role (Otto, 2008). The expression of Phenol-soluble modulins (PSMs) beta peptides leads to the detachment of cell clusters and likely to the formation of channels in both, *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilm (Otto, 2008; Periasamy *et al.*, 2012). The dispersal stage has been shown

to be mediated by Agr quorum sensing in *Staphylococcus* biofilms by regulating the formation of PSMs (Yarwood *et al.*, 2004; Wang *et al.*, 2007; Periasamy *et al.*, 2012).

The dispersion of cells is essential for biofilm development and it enables the biofilm to spread (Otto, 2008; Wang *et al.*, 2011). This phase is not only dependent on cell expression and other factors may influence the dispersal of biofilm cells such as mechanical forces such as flow in a vein or artery or the synovial fluid in a hip or knee joint (Otto, 2008).

By understanding the biochemical nature of the ECM in greater detail, it may be possible to develop antibiofilm strategies which attack or degrade certain biofilm components. One ECM component which is attracting such attention is extracellular DNA.

1.3.3 Extracellular DNA.

Extracellular DNA has been demonstrated to be a significant component of the ECM (Whitchurch *et al.*, 2002; Flemming and Wingender, 2010). The amount of eDNA within the ECM is variable (Izano *et al.*, 2008) but eDNA has been shown to be present in biofilms from different species including *Pseudomonas aeruginosa*, *Staphylococcus aureus* and *Staphylococcus epidermidis* (Whitchurch *et al.*, 2002; Qin *et al.*, 2007; Izano *et al.*, 2008). There are two main theories explaining the secretion of eDNA into the extracellular matrix: cell lysis (Gunn *et al.*, 2016) and active secretion. Cell autolysis mechanisms such as fratricide increase eDNA release and biofilm production (Jakubovics *et al.*, 2013). There is evidence that cell lysis is mediated by the AtlE protein, an autolysin that when inactivated in *Staphylococcus epidermidis*, significantly reduced DNA release (Qin *et al.*, 2007). Active secretion of eDNA via membrane vesicles that carry eDNA on the surface or in the lumen has been described in *Pseudomonas aeruginosa* (Schooling *et al.*, 2009).

Further investigation to determine the role of eDNA has been performed. Das et al demonstrated that extracellular DNA plays a crucial role on the initial attachment phase of biofilm formation. It was demonstrated that the presence of eDNA on the bacterial cell surface enhanced bacterial adhesion and aggregation (Das *et al.*, 2010). eDNA also provides important structural support for mature biofilms (Izano *et al.*, 2008; Harmsen *et al.*, 2010). These discoveries have opened a new research line for the treatment of biofilm related infections as by degrading the extracellular DNA using nucleases, biofilms can potentially be disrupted (Nijland *et al.*, 2010). Breaking up protective biofilms may also allow more effective use of antibiotics (Kaplan, 2009).

1.3.4 Antibiotic resistance of cells within biofilms

Antibiotics are an essential part of the treatment of prosthetic joint infections but the efficacy of the antibiotics appears to be significantly reduced when they are against bacteria within a biofilm as opposed to the free planktonic form (Stewart, 2002). This resistance is due to several mechanisms and they appear to differ from those resistant mechanisms observed in the planktonic bacterial form (Sharma *et al.*, 2019).

Antimicrobial penetration: A possible mechanism of antibiotic resistance is the inability of the antibiotic to penetrate through the extracellular matrix particularly into the deeper layer and therefore not reaching the bacterial cells to inactivate and kill (Singh *et al.*, 2016). As it has been demonstrated the limited ability of antibiotics such as cefotaxime and vancomycin to penetrate *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms (Jefferson *et al.*, 2005; Singh *et al.*, 2010). But the literature on this is not unanimous as authors have been able to demonstrate good penetration of antibiotics in biofilms including those of *Pseudomonas aeruginosa*, *Escherichia coli* and even *Staphylococcus* (Stone *et al.*, 2002; Mathur *et al.*, 2005; Rodríguez-Martínez *et al.*, 2007). A possible explanation for this discrepancy is that it is not only the physical barrier of the extracellular matrix which provides resistance to antibiotics but the interaction of different molecules within the extracellular matrix with the antibiotics reducing their capacity to act against bacterial cells (Mulcahy *et al.*, 2008). This may explain why some antibiotics have shown good biofilm penetration but no effect on the bacterial viability. This phenomenon was observed for several antibiotics such as ciprofloxacin, rifampicin and daptomycin; antibiotics were able to penetrate staphylococcal biofilms but did not have a bactericidal or bacteriostatic effect (Dunne *et al.*, 1993; Zheng and Stewart, 2002; Stewart *et al.*, 2009).

Nutritional limitation and hypoxia: There is a gradient of oxygen and nutrients present within some species biofilms with the deeper layer being more deprived of oxygen (Stewart *et al.*, 2016). Bacterial cells present in the deeper, more hypoxic layer present lower metabolic activity (Ciofu *et al.*, 2017). This stationary phase provides them with resistance to antibiotics since antimicrobials are more effective in actively growing bacteria (Borriello *et al.*, 2004; Zheng and Stewart, 2004; Ciofu *et al.*, 2017).

Efflux pumps: Bacterial cells have membrane-associated proteins that act by moving antimicrobial agents from the bacterial cell into the extracellular space providing resistance (Poole, 2007; Routh *et al.*, 2011). These proteins can be divided into different families: the major facilitator (MF) superfamily, the ATP (adenosine triphosphate)-binding cassette (ABC) family, the resistance-nodulation-division (RND) family most commonly seen in gram

negative bacteria, the small multidrug resistance (SMR) family, and the multidrug and toxic compound extrusion (MATE) family. These proteins provide resistance to specific antibiotics depending on the bacterial cell, for example *Staphylococcus* species biofilms provide resistance against fluoroquinolones and tetracyclines via efflux pump of the MF family, the ABC family provides resistance against streptogramins (Poole, 2007).

Horizontal gene transfers: It is believed that horizontal gene transfer may occur through the transfer of plasmids between cells in a biofilm via conjugation (Hall and Mah, 2017). Savage et al. demonstrated that the horizontal transfer of an antibiotic resistance plasmid was 10 000 times greater in *Staphylococcus aureus* biofilms than in their planktonic cultures (Savage *et al.*, 2013). Similar observation was demonstrated with *Enterococcus faecalis* biofilms. The cells in the biofilm had up to 2-fold increase in plasmid copy number compared to planktonic cells, and this correlated with increased transcription of plasmid-borne antibiotic resistance genes (Cook and Dunny, 2013).

Antibiotic modifying enzymes: β-lactamases, are enzymes present in the biofilm matrix of some bacterial species, that can degrade antimicrobials preventing them from reaching their targets (Hall and Mah, 2017). Several authors have demonstrated the presence of β-lactamases on the extracellular matrix increases antibiotic resistance to certain bacterial biofilms. Bowler et al. suggests that mature *Pseudomonas aeruginosa* biofilms are more resistant to ceftazidime and meropenem than younger biofilms due to an increased amount of β-lactamase in the matrix (Bowler *et al.*, 2012). Anderl et al. demonstrated that β-lactamases secreted by *Klebsiela pneumoniae* biofilms were capable to degrade ampicillin and prevent it from reaching cells within the biofilm and therefore reducing antibiotic activity (Anderl *et al.*, 2000).

The mechanisms of antibiotic resistance in biofilms is intricate and multifactorial which makes the fight against biofilm related infections challenging. The need to develop new approaches to enhance antibiotic activity within the biofilm and reduce resistance are essential to increase the chances to win this complex battle.

1.4 Surface materials and bacterial interaction

Bacterial cells in a planktonic phase are attracted to different surfaces by different forces such as Brownian motion, van der Waals or gravitational forces (Katsikogianni and Missirlis, 2004). Once the bacteria have adhered to the surface, they form stronger adhesion using pili. Subsequently these bacteria can reproduce and secrete extracellular matrix

ultimately forming a biofilm that will protect them against the immune system and other external insults such as antimicrobial agents (Orapiriyakul *et al.*, 2018).

The bacterial adhesion to a surface is influenced by multiple factors some of them related to the bacteria itself, some to the surface and some to the environment:

Bacterial properties: Bacterial species behave differently when in contact with different biomaterials surfaces due to their physicochemical characteristics and preferred environment (Katsikogianni and Missirlis, 2004). Multiple bacterial characteristics play a role in the adherence to surfaces:

Bacterial hydrophobicity/hydrophilicity: Bacterial species with hydrophobic properties adhere better to hydrophobic surfaces and vice versa (Orapiriyakul *et al.*, 2018). In vitro studies have shown that *Staphylococcus epidermidis* strains with higher surface hydrophobicity are more adherent to polyethylene surfaces (An and Friedman, 1998; Vacheethasanee *et al.*, 1998). Bacterial hydrophobicity can be altered by multiple factors such as the age of the bacteria, the medium growth and surface structure adding to the complexity of bacteria-surface interaction (Orapiriyakul *et al.*, 2018).

Bacterial surface charges: Bacteria in aqueous solution is almost always negatively charged (Katsikogianni and Missirlis, 2004) but can be affected and modified by multiple factors, similar to those affecting the bacterial hydrophobicity, including the growth medium, the environmental PH as well as the age of the bacteria (Katsikogianni and Missirlis, 2004; Orapiriyakul *et al.*, 2018). Due to its complexity and multiple variations, the relationship contribution of the bacterial surface charge to bacterial adhesion to biomaterials has not been yet fully understood (An and Friedman, 1998; Katsikogianni and Missirlis, 2004).

Biomaterial properties: The initial adhesion phase is significantly influenced by the topography of the biomaterial (Crawford *et al.*, 2012) but the surface chemistry and functional groups of the surface also have an effect of bacterial adhesion (Lorenzetti *et al.*, 2015). Bacterial adhesion to surfaces is very complex and multifactorial. In titanium implants bacterial adhesion is influenced by the crystalline phase of the titanium oxide present on the surface (Lorenzetti *et al.*, 2015). The surface roughness plays a role in bacterial attachment. Increased surface roughness promotes bacterial attachment due to the increase in contact area between the material surface and bacterial cells as well as protection from shear forces (Teughels *et al.*, 2006; Anselme *et al.*, 2010; Lorenzetti *et al.*, 2015). Smooth surfaces might help prevent the development of biofilm (Ionescu *et al.*, 2012) but there is no optimal surface roughness to prevent all bacterial biofilm development because the effect of the surface roughness on bacterial adhesion alters with the bacterial type, size and shape as well as other

environmental factors making extremely difficult to identify the universal optimal surface roughness (Renner and Weibel, 2011).

Environment: Multiple environmental factors can affect the bacteria-surface interaction including the bacterial concentration, pH, temperature, time of exposure and flow conditions (An and Friedman, 1998; Katsikogianni and Missirlis, 2004). Temperature for example can change the morphology of the bacteria, increasing or reducing the number of flagella and therefore altering their capacity to adhere to surfaces (Orapiriyakul *et al.*, 2018).

The presence of certain serum proteins has been shown to affect bacterial adhesion. There are some controversies regarding the influence of fibronectin on *Staphylococcus epidermidis* attachment to surfaces (Herrmann *et al.*, 1988) but studies have shown that in the case of *Staphylococcus aureus,* the adherence to surfaces is significantly increased in the presence of fibronectin (Vaudaux *et al.*, 1984; Herrmann *et al.*, 1988). Albumin and fibrinogen have also shown an effect on bacterial adhesion. Albumin appears to have an inhibitory effect on bacterial adhesion, likely due to binding to the bacterial cell wall or by modifying the substratum hydrophilicity (Fletcher and Marshall, 1982).

It is clear that the relationship between bacterial adhesion and surface and surface materials is highly complex, multifactorial and overall poorly understood. This work is focused on the development of biofilm of two bacterial species: *Staphylococcus aureus* and *Staphylococcus epidermidis* on four different material surfaces: cobalt chrome, titanium, stainless steel and high molecular weight polyethylene of particular relevance to arthroplasty. Surface roughness, wettability and surface energy has been shown to play a vital role in the adhesion properties of *Staphylococcus aureus* (Alam and Balani, 2017) and *Staphylococcus epidermidis* has been shown to have lower adhesion to cobalt chrome than titanium or stainless steel (Koseki *et al.*, 2014; Malhotra *et al.*, 2019) but still low adhesion when compared to highly cross-linked polyethylene (Malhotra *et al.*, 2019). It is not the aim of this work to study the underlying biochemical mechanism of bacterial adhesion to surface materials, but rather to investigate the growth of clinically relevant bacteria on clinically relevant surfaces as this may help to shed some light on this highly complex and variable phenomenon. There is growing awareness that biofilm prevention can have important clinical advantages.

1.5 Strategies for biofilm prevention

Prosthetic joint biofilm related infections are a huge economic burden (Tande and Patel, 2014) and carry morbidity and mortality (Shahi and Parvizi, 2015). Preventing infection in the first place has been an on-going concern for the orthopaedic community. Multiple efforts to prevent infection have been developed: optimising the patient pre-operatively, reducing theatre traffic and the use of laminar flow.

One of the most well established steps currently used to prevent infection is the use of prophylactic antibiotics (AlBuhairan *et al.*, 2008; Prokuski, 2008). Ultraclean air theatres, body-exhaust suits and prophylactic antibiotics led to large reduction in infection (Lidwell *et al.*, 1984). Antibiotics are given shortly before skin incision (Prokuski, 2008). In England there is a large variation regarding the precise nature of the antibiotic prophylaxis regime (Hickson *et al.*, 2015) which depends on local guidelines. Second generation cephalosporins are adequate for the majority of patients undergoing elective arthroplasty. In some circumstances, administration of vancomycin or a teicoplanin is also indicated in methicillin resistant *Staphylococcus aureus* carriers, in patients with penicillin allergy, patients on dialysis and health care professionals (Shahi and Parvizi, 2015). Currently the three most common antibiotic prophylaxis regimes in England are Flucloxacillin in combination with gentamicin, cefuroxime alone and teicoplanin with gentamicin (Hickson *et al.*, 2015).

The use of antibiotic loaded cement was developed in 1970 by Buchholz for the prevention and treatment of infected join arthroplasties (Buchholz and Engelbrecht, 1970). It delivers high concentration of antibiotics locally and reduces the risk of toxicity (Bistolfi *et al.*, 2011; Anagnostakos, 2017). Low and high dose antibiotic cement preparations depending on whether he intended use is prophylactic or therapeutic (Randelli *et al.*, 2010). The use of antibiotic loaded cement in the treatment of prosthetic joint infection is well established (Chen and Parvizi, 2014). Antibiotic loaded cement is used routinely in Europe/UK, Australia (Schiavone Panni *et al.*, 2016) less so in the USA (Hansen *et al.*, 2014). The increase in cost, risk of hypersensitivity, bacterial resistance and the possible reduction of mechanical properties of the cement are some of the reasons why routine use in primary elective arthroplasty remains controversial (Randelli *et al.*; Schiavone Panni *et al.*, 2016).

Research has focused on developing strategies to prevent the establishment of biofilm on the surface of implanted prosthesis. Biofilm formation has several stages and therefore each stage is a possible target to prevent the complete development of biofilm. Targeting the initial attachment of the cells to the surface material seems logical, but this initial attachment is complex and variable depending on the bacteria and the surface (Bhattacharya *et al.*, 2015).

This includes the development of antibacterial and anti-adhesion coatings for the artificial surfaces (Zhao *et al.*, 2009; Bhattacharya *et al.*, 2015). The surface may be modified to stop growth of the bacterium or cause its death, to inhibit the initial adhesion without bactericidal or bacteriostatic effect or to prevent the expression of molecules essential for the development of biofilm formation such a PIA (Oliveira *et al.*, 2018).

Several particles and materials coated with antibacterial properties have been investigated developed and tested in an attempt to prevent colonisation of *Staphylococcus aureus* and *Staphylococcus epidermidis* (Table 1-4) (Bhattacharya *et al.*, 2015; Norambuena *et al.*, 2017; Pilz *et al.*, 2019). Pel and Psl polysaccharides produced by *Pseudomonas aeruginosa*, hydrophobin proteins from fungal species and organic compounds can reduce *Staphylococcus epidermidis* biofilm formation by modifying the abiotic surface properties, changing the wettability of the surface, modifying its charge and preventing bacterial attachment although the precise mechanism is yet to be fully understood (Qin *et al.*, 2009; Artini *et al.*, 2017). Polysaccharides also are capable of modifying the bacterial surface adhesin proteins that have a role in the initial attachment (Rendueles *et al.*, 2013), similar mechanism of action to aryl-rhodanine molecules (Opperman *et al.*, 2009). Esculetin and fisetin, two biological compounds derived from natural plants have been shown to reduce *Staphylococcus aureus* biofilm formation up to 90% in concentrations of 128 and 16µg/ml, although the mechanism of action was not described (Dürig *et al.*, 2010).

Antibiotic and antiseptic coatings have been shown to be effective in biofilm prevention in animal models. Gentamicin and vancomycin coatings on stainless steel and titanium surfaces are effective in reducing *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilm formation (Antoci *et al.*, 2008; Kruszewski *et al.*, 2013; Jennings *et al.*, 2015); Chlorhexidine coated devices have also been shown to reduce infection up to 7 fold (Darouiche *et al.*, 1998).

Anti-adhesion coatings can also be combined with antibiotic treatment. The anti-adhesion surface prevents bacterial attachment, maintaining the bacteria in a planktonic phase for longer, increasing effectiveness of antibiotic therapy. These anti-adhesion properties can be achieved by modifying the surface charge or roughness but care has to be taken not to alter the properties of the implant (Bhattacharya *et al.*, 2015). The initial attachment of bacteria to surfaces is partly mediated by surface proteins, which can be used as a target to prevent biofilm formation. Proteins such as Sortase A, a surface protein of *Staphylococcus aureus*, have been the focus of research and have been demonstrated to effectively reduce virulence and reduce infection (Zhang *et al.*, 2014; Cascioferro *et al.*, 2015).

Table 1-4 Coatings which have been used to prevent *Staphylococcus aureus* surface attachment (Bhattacharya *et al.*, 2015).

Anti- *Staphylococcus aureus* coating	Mechanism of action
Aryl rhodanines	Small molecule. Prevents attachment of bacteria to surfaces but is not antibacterial. Effective against multiple Gram-positive
Quaternary ammonium silane	Quaternary ammonium groups have antimicrobial activity. These were tested in a singular ATCC *Staphylococcus aureus* strain
Calcium chelators	Deprive bacteria of essential Ca^{2+}
Polymer brushes	Repulsive osmotic forces, discourages bacterial adhesion to the surface
Organoselenium	Catalyzes the formation of superoxides, reducing possibility of bacterial survival on surface
PLL-g-PEG	PLL-g-PEG reduces adsorption of host matrix proteins onto the surface, preventing bacterial attachment
Silver nanoparticles	Ag+ ions enter cells to interact with protein and DNA, disrupting cell division and respiration, leading to cell death
Chitosan	Osteoconductive, antimicrobial coating. Biocompatible with host tissue

N-Acetyl-L-cysteine (NAC) is widely used in the treatment of chronic bronchitis (Olofsson *et al.*, 2003) and also as an antidote to acetaminophen overdose (Chiew *et al.*, 2018). A recent large retrospective cohort study has shown that it can reduce the risk of prosthetic joint infection within 5 years of primary surgery (Chang *et al.*, 2018). Its mechanism of action is by affecting the production of extracellular polysaccharide (Olofsson *et al.*, 2003). The dose of NAC given was not uniform across the patients included in the study, therefore more studies including other ethnicities with a uniform protocol of treatment may be necessary to support this evidence. This is the first study to demonstrate the efficacy

of NAC in vivo in prosthetic join infection. The efficacy of NAC against biofilms until then, had only been demonstrated in vitro (Olofsson *et al.*, 2003; Drago *et al.*, 2013).

Extracellular DNA, plays a role in initial bacterial attachment to a surface and therefore the use of DNA degrading enzymes is a potential tool for biofilm prevention. DNAse I has been proven to be effective in the prevention of *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilm formation (Eckhart *et al.*, 2007; Qin *et al.*, 2007; Izano *et al.*, 2008). Coating surfaces of polymethylmetacrylate with DNase I effectively prevented biofilm formation by disrupting the initial cell attachment of *Pseudomonas aeruginosa* and *Staphylococcus aureus* to the surface (Swartjes *et al.*, 2013). In addition, newly discovered marine endonucleases such as the secreted enzyme NucB may be able to degrade eDNA and thereby prevent biofilm formation.

1.6 Strategies for biofilm eradication

Biofilm related infections are difficult to eradicate once they have formed, with routine antibiotic treatment (Edmiston *et al.*, 2015). Bacteria growing in biofilms exhibit increased tolerance against antibiotics, as well as host immune mechanisms (Hoiby *et al.*, 2010; Hoiby *et al.*, 2011). When polymorphonuclear phagocytes are involved in immune response with non-degradable implants, the bactericidal and phagocytic response become defective and therefore allowing bacterial growth and development on infection (Zimmerli *et al.*, 1984). The bacteria embedded in the biofilm are more resistant to antibiotics as the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) have been shown to be up to 100–1000-fold higher than compared with planktonic bacteria (Anwar and Costerton, 1990; Bjarnsholt *et al.*, 2007). Cells in a biofilm are in a dormant state in a reduced metabolic activity, rendering them less susceptible to antimicrobials (Stewart, 2015). This dormant state is related to the deprivation of nutrients, cells in the periphery acquire most of the nutrients leaving those cells in the deepest, dense areas deprived (Stewart and Franklin, 2008). The antibacterial agents induce their target to produce toxic products but as the cells are in a dormant state, they will produce less protein mistranslation and will not trigger cell lysis (Nguyen *et al.*, 2011). The presence of secreted β-lactamases in the matrix of the some biofilms can degrade antimicrobials, therefore preventing these agents from reaching their cellular targets (Anderl *et al.*, 2000). It also has been speculated that the presence of eDNA in the matrix increases antibiotic tolerance by altering the extracellular environment and by enabling the transfer of antibiotic resistance genes between biofilm cells (Hall and Mah, 2017).

Due to the difficulty of treating biofilm related infections with traditional methods the development of new approaches for management of established infections is needed. A number of different strategies have been explored in an attempt to improve biofilm eradication, but given promising results with a number of enzymes, this work focussed on those that are capable of dispersing biofilms with the most relevance to PJI.

The use of nanoparticles has been developed in recent years particularly as a method of drug delivery (Suresh *et al.*, 2019) but has also been proven successful in the treatment of biofilm related infection. Thanks to their small size (less than 1μm) they are able to penetrate deeper into the biofilm and enhance the interaction with their target (Mu *et al.*, 2016). Chaudry et al. and Applerot et al. were able to demonstrate a synergistic effect of Silver and Zinc oxide nanoparticles with numerous antibiotics against *Staphylococcus aureus* biofilms (Applerot *et al.*, 2012; Chaudhari *et al.*, 2012). Silver oxynitrate has promising results in vitro against dual species bacterial biofilms including Staphylococcal species as is able to reduce the minimal bactericidal concentration up to 10 fold (Lemire *et al.*, 2017).

Peptide 1018, which works by targeting RelA and SpoT enzymes, mediate the synthesis of guanosine 5′-diphosphate 3′-diphosphate (ppGpp) and guanosine 5′-triphosphate 3′-diphosphate (pppGpp). Both nucleotides are often referred to as (p)ppGpp and are important molecules in biofilm development (de la Fuente-Nunez *et al.*, 2014). Peptide 1018 is effective in dispersing multiple Gram negative and Gram positive bacterial species including methicillin resistant *Staphylococcus aureus* although the exact mode of action has not yet been identified (de la Fuente-Nunez *et al.*, 2014; Reffuveille *et al.*, 2014).

The natural immune response or targeted immunization can disrupt pre-formed biofilms by attacking particular proteins within the extracellular matrix producing a structural collapse of the biofilm (Goodman *et al.*, 2011). The family of proteins denominated DNABII are extracellular proteins that bind DNA. They are known to have a structural role in the biofilms of *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Salmonella enterica* (Goodman *et al.*, 2011). These proteins exist in two subtypes, histone-like protein from *Escherichia coli* strain U93 (also known as HU) and integration host factor (IHF). When this family of proteins is attacked by anti-IHF, the eDNA within the biofilm is weakened and breaks down (Goodman *et al.*, 2011). Animal studies have also shown a disruption of *Haemophilus influenzae* biofilms by targeting the outer membrane protein P5 and type IV pilus with a chimeric immunogen (Novotny *et al.*, 2011). More specifically related to our work are the antimicrobial peptides (AMPs). These peptides are part of the natural immune system and act by disrupting the bacterial membrane (Li *et al.*, 2017b) but

also have biofilm dispersion properties (Suresh *et al.*, 2019). LL-37 and RNAIII-inhibiting peptide showed promising results in vitro but they were unstable and had very little effect in vivo so derivatives were created and found to have a higher dispersal activity against *Staphylococcus aureus* biofilms (Dean *et al.*, 2011; Zhou *et al.*, 2016).

The accessory gene regulator system or agr; is one of the quorum sensing systems of staphylococci (Kavanaugh and Horswill, 2016). This system is activated by the presence of an auto-inducing peptide (AIP) that is released by the *agr* operon (Lister and Horswill, 2014). After the activation of the *agr* system, cells involved within the biofilm can detach themselves (Yarwood *et al.*, 2004) and by adding AIP to the existing biofilm these can be dispersed (Boles and Horswill, 2008; Lauderdale *et al.*, 2010).

The use of natural viruses (bacteriophage) has been studied as an alternative treatment against bacterial infection to overcome the problem of muli-drug resistance (Lin *et al.*, 2017). Some of these viruses include Bacteriophage K and DRA88. These two phages were shown to be able to reduce staphylococcal biomass by 50% in 4 hours (Alves *et al.*, 2014). This novel approach requires further extensive research. There has been some recent promising animal studies that demonstrate a 22.5-fold reduction in S*taphylococcus aureus* burden in the joint tissue of animals that were treated with phage and vancomycin combination (Morris *et al.*, 2019). The mechanisms of phage-antibiotic synergy are not yet fully understood and remains an area of current active research (Schmidt, 2019).

Despite extensive research attempting to identify an effective way of biofilm eradication, no single strategy has been encountered which is 100% effective. Some methods attack the physical structure of the biofilm to disperse the bacterial cells, some enhance antibiotic activity, some target the bacterial cell-cell communication and some directly attack the bacteria embedded in the biofilm. The complexity, variability and evolving nature of biofilms makes their eradication extremely difficult. Targeting biofilm through different strategies simultaneously in combination with antimicrobial agents may be a way to enhance eradication and successfully treat biofilm related infections. Increasingly, matrix degrading enzymes are being evaluated as an important part of these strategies.

1.7 Matrix degrading enzymes

The extracellular matrix (ECM) is a structural component of the biofilm which makes it an excellent target for developing biofilm dispersion strategies. Identifying molecules that target each of these structural components of the ECM should help to disrupt the mature biofilm. This theory opens numerous research lines for the development of novel treatment

options. Several bacteria including staphylococcal species produce an extracellular matrix polysaccharide called poly-N-acetylglucosamine (PNAG) which contains a chain of *N*-acetyl-D-glucosamine residues in β(1,6)-linkages (Ramasubbu *et al.*, 2005). Dispersin B is a polysacharidase produced by *Aggregatibacter actinomycetemcomitans*, specifically a β-*N*-acetylglucosaminidase (Kaplan *et al.*, 2003) capable of dispersing preformed biofilms of *Staphylococcus epidermidis* (Manuel *et al.*, 2007; Gokcen *et al.*, 2013) as well as enhancing antibiotic activity against certain bacterial strains (Donelli *et al.*, 2007; O'Neill *et al.*, 2008). Hyaluronidase is also capable of disrupting *Staphylococcus aureus* biofilms by breaking down the glycosidic bridges of hyaluronic acid of the extracellular matrix (Ibberson *et al.*, 2016).

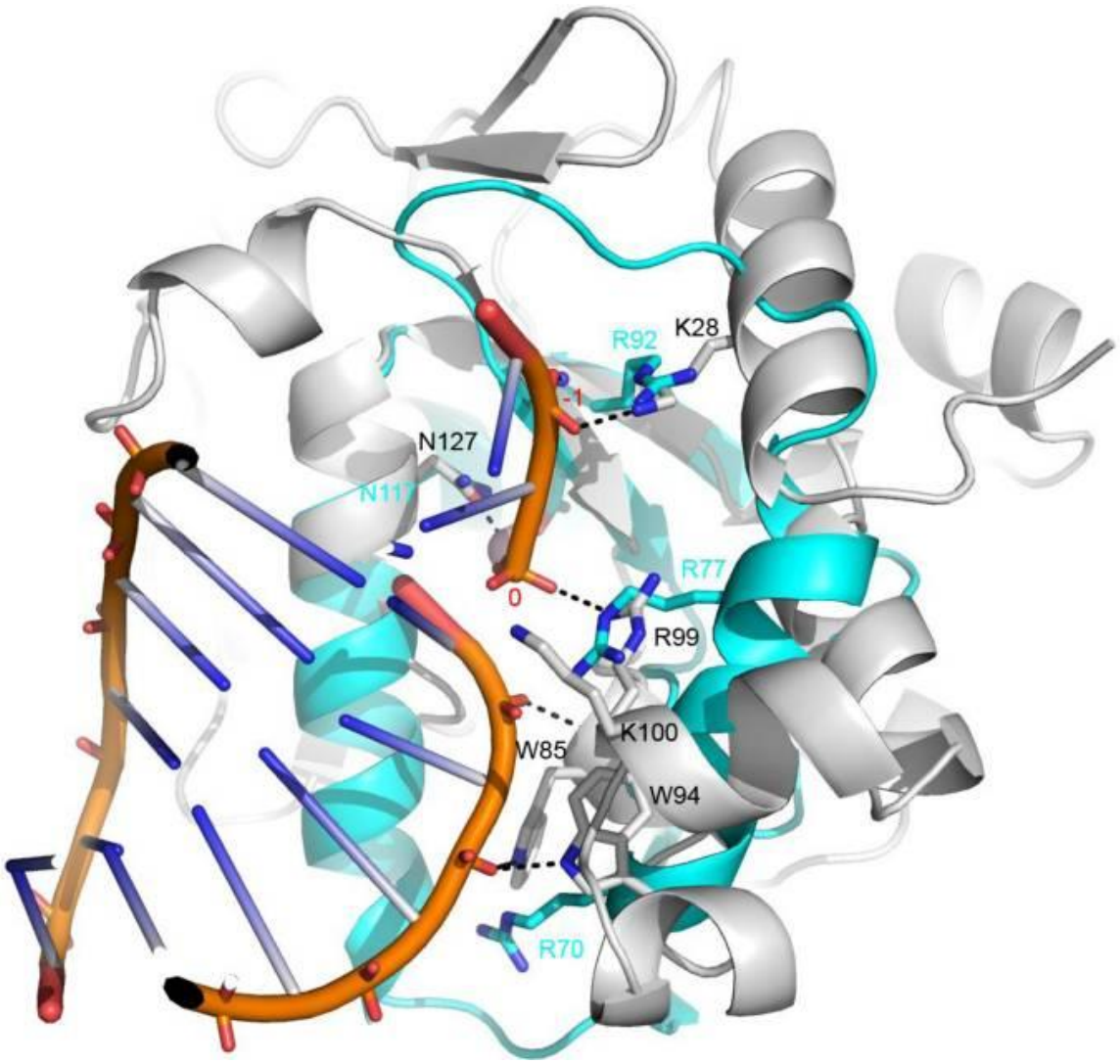
Numerous proteases have been studied as a possible method to combat biofilm related infection (Sugimoto *et al.*, 2013). Proteases with biofilm dispersal activities include proteinase K, staphopain proteases and V8 protease. Proteinase K are effective in the removal of staphylococcal biofilms (Lauderdale *et al.*, 2010; Kumar Shukla and Rao, 2013). Dispersin B has a synergistic effect with certain antibiotics (Lauderdale *et al.*, 2010). Extracellular cysteine proteases SspB and ScpA also called Staphopains disperse preformed biofilms and prevent biofilm formation (Mootz *et al.*, 2013). Protease V8 is effective in preventing biofilm formation and dispersal in staphylococcal species. V8 protease works by degrading the cell surface fibronectin-binding protein (FnBP) (McGavin *et al.*, 1997; O'Neill *et al.*, 2008).

Nucleases are enzymes capable of digesting nucleic acid into nucleotides (Miller-Keane, 2012). Deoxyribonuclease is a type of nuclease that specifically hydrolyses DNA (Collins, 2009). With the discovery of extracellular DNA it was hypothesised that the use of nucleases may be a method of biofilm dispersal. One of the first attempts of disrupting biofilm by attacking the extracellular DNA was successfully done half a century ago (Catlin, 1956). Studies have been performed to investigate the effect of DNases such as bovine DNase I or recombinant human DNase (rhDNase) on biofilm formation and dispersal (Tetz *et al.*, 2009; Tang *et al.*, 2011; Nur *et al.*, 2013). rhDNase is capable of inhibiting *Staphylococcus aureus* biofilm formation by up to 90% (Kaplan *et al.*, 2012). Experiments performed with bovine DNase I in vitro and in vivo also showed a decreased formation of *Gardnerella vaginalis* biofilm and also demonstrated a 50% disruption of pre-existing biofilms when a concentration of 100µg/mL DNaseI (Hymes *et al.*, 2013). This evidence strongly supports the concept that targeting extracellular DNA can be an effective tool in the treatment of biofilm related infection.

1.8 NucB

The marine bacterium *Bacillus licheniformis* EI-34-6 was observed to have bacterial biofilm disruptive properties (Nijland *et al.*, 2010). Nijland et al. were able to isolate and purify the enzyme NucB. It is a non-specific endonuclease capable of hydrolysing single and double stranded DNA, it belongs to the divergent His-Me finger family of endonucleases, although it is unique in terms of sequencing as it only shares 12% of DNA with its closest structural neighbour, an endonuclease produced by *Serratia marcescens* (Basle *et al.*, 2018). NucB interacts with its double stranded substrates in the DNA minor groove (Figure 1-4). NucB can be produced in quantities of up to 50mg per batch for experimental studies (Rajarajan *et al.*, 2013). The nucleotide sequence of the *nucB* gene is available in GenBank with accession number 323145044.

Figure 1-4 DNA and NucB nuclease interaction (Basle et al. 2018).



NucB has also been shown to be thermally robust and regains its function after the Heat-Cool cycle by re-folding its structure which is something that human DNAse is not capable of (Basle *et al.*, 2018). This thermal property is particularly important when considering the potential use of NucB in the orthopaedic setting as the use of cement during routine arthroplasty produces an exothermic reaction rapidly increasing the local temperature. This small nuclease of 12kDa can effectively disperse biofilms from Gram-positive and Gram-negative bacteria (Nijland *et al.*, 2010; Rajarajan *et al.*, 2013). NucB has attracted attention from the medical community as it may be a potential tool for the treatment of biofilm related medical infections. Subsequent experiments have demonstrated the efficacy of the enzyme against mixed species biofilms on medical devices such as tracheoesophageal speech valves (Shakir *et al.*, 2012) and samples from patients with chronic rhinosinusitis (Shields *et al.*, 2013). It has been shown to be more effective at reducing biofilm than bovine DNaseI (Nijland *et al.*, 2010).

Completely removing all biofilm when perfoming a DAIR, is extremely difficult. The use of NucB during the procedure could promote biofilm dispersal and aid in the eradication of the infection. Another possible use of NucB is as part of a coating of the metal implant that will help to prevent biofilm formation inside the body. This could be used in primary procedures to prevent initial attachment of bacteria to the artificial surfaces; as well as during a single stage revision surgery to prevent recurrence of the disease. The potential of NucB in the prevention of biofilm formation on orthopaedic materials as well as a treatment for established biofilms will therefore be evaluated in this work.

1.9 Aims and Objectives

The management of biofilm related infections remains a challenge for orthopaedic surgeons (Petretta *et al.*, 2016). DNase enzymes such as NucB have not yet been tested against bacterial strains involved in orthopaedic prosthetic join infection. The aim of this research was therefore to evaluate the efficacy of NucB in preventing the formation of biofilms as well as breaking down established biofilms from infected joint replacement prostheses. The combined effect of NucB with standard antibiotic treatment was also investigated to understand if nucleases might allow greater antibiotic efficacy against biofilm covered bacteria.

Objectives:

1. Set up a model to investigate the effect of NucB on clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis*.
2. Investigate the effect of NucB on the formation of biofilms from clinical PJI isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* on prosthetic joint surfaces.
3. Investigate the effect of NucB on established biofilms of clinical PJI isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* on prosthetic joint surfaces as well as to investigate the effect of NucB and antibiotic treatment (gentamicin, vancomycin and teicoplanin) on established *Staphylococcus aureus* and *Staphylococcus epidermidis* clinical biofilms and planktonic bacterial cells.

1.10 List of scientific presentations made

- 24th Annual Open Scientific Meeting, Musculoskeletal Infection Society, 9th August 2014 Charleston, South Carolina, USA. Podium presentation.
- Oxford Bone Infection Conference, 27th March 2015, Oxford, UK. Podium presentation and Poster presentation.
- British Orthopaedic Association Annual Convention, 15th September 2015 Liverpool, UK. Podium presentation.
- Furlong Christmas Lecture ORUK, 3rd December 2015 London, UK. Poster presentation.
- Society of Academic and Research Surgery Annual Meeting, 7th January 2016 London, UK. Podium presentation- Invited.
- American Academy of Orthopaedic Surgeons, 4th March 2016 Orlando, USA. Podium presentation - Invited.
- North East Surgical Society Annual Meeting, Registrar Prize Session, 7th April 2016 Northumbria, UK. Podium presentation.
- 3rd Annual Quality Improvement for Surgical Teams Conference, 19th October 2016, Newcastle upon Tyne, UK. Podium presentation- Invited.

1.10.1 Research Grants Secured

Dispersal of clinical biofilms from titanium and cobalt chrome surfaces using a novel marine nuclease. 2nd October 2014. Orthopaedic Research UK (ORUK) awarded a £58,696.90 grant. Project 513.

http://www.oruk.org/funding-research/research-projects/- Impact report 2015, p109

1.10.2 Awards

British Orthopaedic Association Young Investigator of the year 2015. 17th September 2015, Liverpool, UK.

Chapter 2. Clinical strains and optimisation of in vitro model

2.1 Introduction

To be able to assess the efficacy of NucB in biofilm prevention and biofilm dispersal, an in-vitro model was designed. Clinical samples were sourced from prosthetic joint infections. The bacteria chosen for this model were *Staphylococcus aureus* and *Staphylococcus epidermidis.* A number of clinical isolates were sourced from two different hospitals from patients undergoing treatment from prosthetic joint infections. Often reference strains are also used alongside clinical strains. Reference strains have been shown to evolve over time due to multiple subcultures. The resulting changes in properties often make reference strains less virulent and can diminish their ability to produce biofilm (Head and Yu, 2004). Fux et al. suggested that genes controlling the spatial and metabolic interactions within biofilms could be lost after numerous sequential passages of planktonic sub-culturing (Fux *et al.*, 2005). Our group had previously worked with reference strains (*Staphylococcus aureus* 6571 and *Staphylococcus epidermidis* 11047) and assessed the efficacy of NucB against such biofilms (unpublished data). It was therefore deemed unnecessary to duplicate these experiments but to focus specifically on clinically relevant pathogens going forward. These pathogens have clearly been capable of colonizing in vivo artificial joint surfaces and had the virulence to develop infections that required medical and surgical treatment. These known pathogenic properties made them the ideal subjects with which to test the efficacy of NucB.

Once the isolates were obtained from two local hospitals, it was necessary to establish whether these isolates were capable of forming biofilm on surfaces in-vitro. Strains were incubated in polystyrene microtitre plates and biofilm was stained with crystal violet, a well-established technique for biofilm quantification (Xu *et al.*, 2016). Crystal violet stains charged surface molecules and the polysaccharide components of the extracellular matrix. It stains all live and dead cells hence can quantify biofilm biomass. Crystal violet staining will not be able to distinguish the living and dead cells within biofilm (Pitts *et al.*, 2003). At the early stage of the in-vitro model development, it was deemed not necessary to discriminate functional from non-functional biofilm. The main objective was to assess the capability of the clinical strains to grow as a biofilm in the clinically relevant model system. The next step was to decide on an effective nutrient media to allow biofilm formation in-vitro. Environment and nutrients strongly influence biofilm formation, with biofilm structure developing differently as a response to changes in nutrient conditions (Tolker-Nielsen, 2015). Identifying the

optimum growth medium to robustly reproduce biofilm under in vitro conditions was essential to allow any NucB activity to be studied. The optimal time for biofilm growth was also determined. The enzymatic activity of NucB was assessed using gel electrophoresis to demonstrate that DNA degradation was reproducible and constant over time. NucB is non-toxic to cells, but we needed to ensure NucB remained active in the presence of live cells, and their presence did not impair the activity of live cells. This was assessed by gel electrophoresis of NucB degradation of calf thymus DNA in the presence of different concentrations of bacteria.

The objectives of this chapter were:

1. To obtain clinical isolates of bacteria from NHS patients affected by prosthetic joint infections.
2. To evaluate the ability of such isolates to form biofilm in an in-vitro model and optimise their growth conditions in order to allow reliable growth of a static biofilm.
3. To evaluate the activity of NucB in digesting DNA and the effectiveness of NucB in digesting DNA in the presence of live bacterial cells, which had not been clearly demonstrated before.

2.2 Materials and methods

2.2.1 Bacterial strains

Clinical isolates were obtained from the microbiology department at Northumbria Healthcare NHS Foundation Trust (North Shields, UK) and the Freeman Hospital (Newcastle upon Tyne, UK). Strains were isolated from patients suffering from implant associated infections. Strains were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) by Dr Michael Ford, Freeman Hospital, according to previously described methods (Shields *et al.*, 2013). This technique has been shown to be a quick and reliable way to identify microorganisms (Harris *et al.*, 2010; Drevinek *et al.*, 2012). The clinical isolates were also identified using 16S rRNA gene sequencing as previously described (Shields *et al.*, 2013).

For ethical reasons we obtained no clinical patient information regarding diagnosis or treatment. The project was focussed on assessing the potential of a nuclease to control biofilms from clinically relevant staphylococci, and at this stage, there was no benefit in linking data back to patient information.

2.2.2 Growth and maintenance of bacterial strains

Tested strains were maintained at -80°C in tryptic soy broth (TSB) (Sigma-Aldrich, Dorset, UK) containing 20% glycerol and recovered onto tryptic soy agar (TSA) plates. The plates were incubated for 24 hours at 37°C and stored at 4°C for a maximum of 24 hours, after which plates were discarded. An isolated colony of each strain was inoculated into TSB and incubated overnight at 37°C, 200 rpm prior to each biofilm experiment. Regular Gram stain and microscopy visualisation was performed to ensure no contamination.

2.2.3 Assessment of biofilm formation by clinical strains

An adaptation of the method described by Christensen et al. was used (Christensen *et al.*, 1985). An isolated colony of each strain was inoculated into Tryptic Soy Broth (TSB) (Melford Biolaboratories Ltd, Suffolk, UK) and incubated overnight at 37°C, 200 rpm prior to the biofilm experiment. A Corning® flat bottom polystyrene 96 well plate (Sigma-Aldrich, Dorset, UK) was inoculated with 200µl/well OD_{600} = 0.1 of the overnight broth. The plates were covered and incubated statically at 37°C for 24 hours. Negative controls, which had no cells, were set up for each of the experimental preparations. Following growth, liquid medium was aspirated, and the plates were gently washed with sterile isotonic phosphate buffer (KH_2PO_4 20mM, Na_2HPO_4 20mM, NaCl 0.15 mM, pH 7.3) (PBS). The remaining

biofilms were stained with 200µl of 0.1% crystal violet (CV) (Sigma-Aldrich, Dorset, UK), and incubated at room temperature for 15 minutes. Excessive stain was removed by washing with sterile distilled water three times. Wells were dried at room temperature for 30 minutes. The amount of biofilm biomass was quantitated by solubilizing the crystal violet stain for 15 minutes with 200µl of 33% (v/v) acetic acid (Merritt *et al.*, 2005). The absorbance of the CV solution at 595nm was measured with a Fluostar Optima plate reader (BMG Labtech, Bucks, UK), using the MARS software package (BMG Labtech).

2.2.4 Optimization of an in vitro model of biofilm formation

Four clinical strains were chosen to optimise the in vitro model: *Staphylococcus aureus* 76933, *Staphylococcus aureus* 518F, *Staphylococcus epidermidis* 76933 and *Staphylococcus epidermidis* 096R (Table 2-1). An isolated colony of each strain was inoculated into Nutrient Broth (NB) (Sigma-Aldrich, Dorset, UK), Luria Bertani (LB) (Sigma-Aldrich, Dorset, UK) and Tryptic Soy Broth (TSB) (Melford Biolaboratories Ltd, Suffolk, UK) and incubated overnight at 37°C, 200 rpm prior to the biofilm experiment. A Corning® flat bottom polystyrene 96 well plate (Sigma-Aldrich, Dorset, UK) was inoculated with 200µl/well OD_{600} = 0.1 of the overnight broth. The plates were covered and incubated statically at 37°C for 24 hours. Appropriate controls of each media were included in the experimental preparation. Following growth, liquid medium was aspirated, and the plates were gently washed with sterile isotonic phosphate buffer (KH_2PO_4 20mM, Na_2HPO_4 20mM, NaCl 0.15 mM, pH 7.3) (PBS). The remaining biofilms were stained with 200µl of 0.1% crystal violet (CV) (Sigma-Aldrich, Dorset, UK), and incubated at room temperature for 15 minutes. Excessive stain was removed by washing with sterile distilled water three times. Wells were then dried at room temperature for 30 minutes. The amount of biofilm biomass was quantified by solubilizing the crystal violet stain for 15 minutes with 200µl of 33% (v/v) acetic acid (Merritt *et al.*, 2005). The absorbance of the CV solution at 595nm was measured with a Fluostar Optima plate reader (BMG Labtech, Bucks, UK), using the MARS software package (BMG Labtech). Experiments were performed using different times of biofilm growth: 6, 12, 18 and 24 hours. Experiments were done in triplicate, three independent times.

2.2.5 Assessment of NucB activity by gel electrophoresis and spectrophotometry measurement

To evaluate NucB enzyme activity and stability, a series of agarose gel electrophoresis experiments were conducted to compare the variation of activity between enzyme batch and effect of storage over time. A reaction mixture was created using 125µg of calf thymus (CT) DNA (stock concentration of 2mg/ml) added to Tris-HCl buffer 50mM (pH8.0) mixed with 5mM $MnSO_4$ (stock 100mM) and sterile distilled water to make a final reaction volume of 250µl. The reaction mixture was pre-equilibrated at 37^{o}C for 10 minutes. The reaction was started by the addition and mixing of NucB (5ng/ml) followed by further incubation at 37^{o}C. The incubation time intervals were 5 minutes, 15 minutes and 30 minutes. Controls were made without enzyme and the volume was made up with buffer solution. For analysis by agarose gel (0.8% w/v) electrophoresis, 50µl of the reaction was halted by the addition and mixing of 50µl of phenol/chloroform/isoamyl alcohol mix to both enzyme and control samples. The mixture was shaken forming an emulsion and was centrifuged at 13,000 rpm at 4^{o}C for 3 minutes in a benchtop centrifuge (MiniSpin Eppendorf) forming two layers. For electrophoresis the DNA was taken from the upper layer and stored in the fridge at 8 ^{o}C until all time intervals were processed. DNA was separated and visualised on 0.8 % molecular biology grade agarose (Melford). 100mls 1 x TAE buffer (40mM Tris, 20mM glacial acetic acid (Fisher Scientific), 1mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, pH 8.0) was transferred to a 250ml conical flask 0.8g agarose was added to buffer and dissolved by gently heating in a microwave. After the solution had cooled but remained liquid 5µl of Gel Red was added and swirled to mix. The agarose solution was then poured into gel trays, with gel combs, and allowed to solidify. Set gels were placed into a gel tank and immersed in 1x TAE buffer. 5µl of Hyperladder 1kb Plus (Bioline) to first well. Samples containing DNA were mixed with 5x DNA loading dye (1:5) (Bioline). 5µl of each interval DNA was mixed with DNA loading buffer (Bioline) and loaded onto an agarose gel. Gels were run at 100V for up to 90 minutes. DNA bands were visualised with an ultraviolet source (G:Box, Syngene).

The second part of the experiment was the quantification of NucB activity by spectrophotometry. Independent reaction volume samples were stored for different time periods (0 days, 15 days and 30 days). Samples were prepared as previously described and incubated for 60 minutes at 37^{o}C. The reaction was halted by the addition of 250µl of cold (4 ^{o}C) 4% (v/v) perchloric acid. The reaction mixture was incubated for 60 minutes on ice to precipitate high molecular weight DNA and then centrifuged at 13k rpm at 4 ^{o}C in a benchtop

centrifuge (MiniSpin Eppendorf). 250µl of the supernatant was diluted to 1ml with Tris-HCl buffer (pH8.0) and the amount of low molecular weight DNA present was measured by the absorbance at 260nm using a NanoDrop (ND-1000 NanoDrop) spectrophotometer. For each sample, 2µl volume was loaded onto the device and values were recorded.

A similar approach was taken to monitor NucB activity in the presence of *Staphylococcus aureus* with minor modifications. An isolated colony of *Staphylococcus aureus* 518F (Table 2-1) was inoculated into Tryptic Soy Broth (TSB) (Melford Biolaboratories Ltd, Suffolk, UK) and incubated overnight at 37°C, with shaking at 200 rpm. The OD_{600} was adjusted to 0.1. Individual Eppendorfs with reaction combinations were made. The total volume of 300ml of reaction solution per combination included: 125µg of calf thymus (CT) DNA (stock concentration of 2mg/ml), Tris buffer 50mM (pH8.0) mixed with 5mM $MnSO_4$ (stock 100mM), 10µl, 50µl or 100µl of bacterial broth at OD_{600} of 0.1 was added to the wells as per Figure 2.6 and sterile distilled water to make up the final volume. The reaction was started by the addition of NucB at a concentration of 1µg/ml. All samples were incubated statically at 37^{o}C for 15 minutes. Cells were harvested by centrifuging 1ml of overnight bacterial culture for 3 min at 13k rpm at 4^{o}C in a benchtop centrifuge (MiniSpin Eppendorf), the supernatant was removed and stored in a sterile eppendorf. Wash 1 cells were obtained by centrifuging 1 ml of overnight bacterial culture as described above, supernatant discarded, and cells were washed by re-suspension in TSB, further centrifugation and re-suspension again in TSB. Wash 2 cells were obtained similarly by repeating the washing step once more. Unwashed cells were obtained by taking 10µl and 100µl of overnight culture at OD_{600} of 0.1 and added to the reaction solution. Samples were added to different wells as per table in Figure 2-6. The gel was run at 100V for 90 minutes. The DNA bands were visualised with an ultraviolet source (G:Box, Syngene).

2.2.6 Statistical analysis

Statistical analysis was performed with SPSS (IMB SPSS Statistics-version 22) by using a one-way ANOVA. To calculate significant differences between control and experimental samples, p values below 0.05 were considered statistically significant.

2.3 Results

2.3.1 Bacterial strains

Staphylococcus aureus and *Staphylococcus epidermidis* were chosen as they are the two most common organisms associated with PJI (Pulido *et al.*, 2008; Geipel, 2009; Hickson *et al.*, 2015; Sambri *et al.*, 2017).

Clinical isolates were collected from patients undergoing treatment from prosthetic joint infections from two local hospitals (Freeman Hospital and Northumbria Healthcare NHS Foundation Trust). After identification by MADI-TOF MS, samples were transferred into agar slopes to allow transportation to Newcastle University. Confirmation of the bacterial strain was perform using 16S rRNA gene sequencing (see annex). Each individual sample was then were inoculated into TSB and incubated at 37°C overnight. The overnight broth was then centrifuged to obtain a pellet before re-suspension on TSA and preparation of a glycerol stock for storage at -80°C. Prior to this, the purity of each strain was checked by repeatedly streaking out to single colonies on TSB agar, and all isolates were found to be pure. A summary of the bacterial strains used is listed in Table 2-1.

Table 2-1. Bacterial strains.

Strain	Source
Staphylococcus aureus 559C	Freeman Hospital
Staphylococcus aureus 722P	Freeman Hospital
Staphylococcus aureus 76901	Northumbria Healthcare NHS Foundation Trust
Staphylococcus aureus 717T	Freeman Hospital
Staphylococcus aureus 089G	Freeman Hospital
Staphylococcus aureus 476A	Freeman Hospital
Staphylococcus aureus 518F	Freeman Hospital
Staphylococcus aureus 171F	Freeman Hospital
Staphylococcus aureus 649D	Freeman Hospital
Staphylococcus aureus 378S	Freeman Hospital
Staphylococcus aureus 107H	Freeman Hospital
Staphylococcus epidermidis 286G	Freeman Hospital
Staphylococcus epidermidis S76933	Northumbria Healthcare NHS Foundation Trust

Staphylococcus epidermidis 033G	Freeman Hospital
Staphylococcus epidermidis 684X	Freeman Hospital
Staphylococcus epidermidis 150T	Freeman Hospital
Staphylococcus epidermidis 096R	Freeman Hospital
Staphylococcus epidermidis 248X	Freeman Hospital
Staphylococcus epidermidis 414W	Freeman Hospital

Bacterial strains were confirmed to be *Staphylococcus aureus* and *Staphylococcus epidermidis* by 16S rRNA gene sequencing (see Annex).

2.3.2 Quantitative determination of biofilm formation

The ability of the clinical strains to form biofilms was assessed using a 96-well microtitre plate biofilm model and stained with crystal violet. The results are summarised below. Eight *Staphylococcus aureus* and eleven *Staphylococcus epidermidis* strains were tested. All nineteen strains grew biofilm after 24 hours (Figure 2-1). We observed a large variability of biofilm growth in all species. This can be observed by the large error bars seen across the graph in Figure 2-1. Tests were performed three independent times in triplicate.

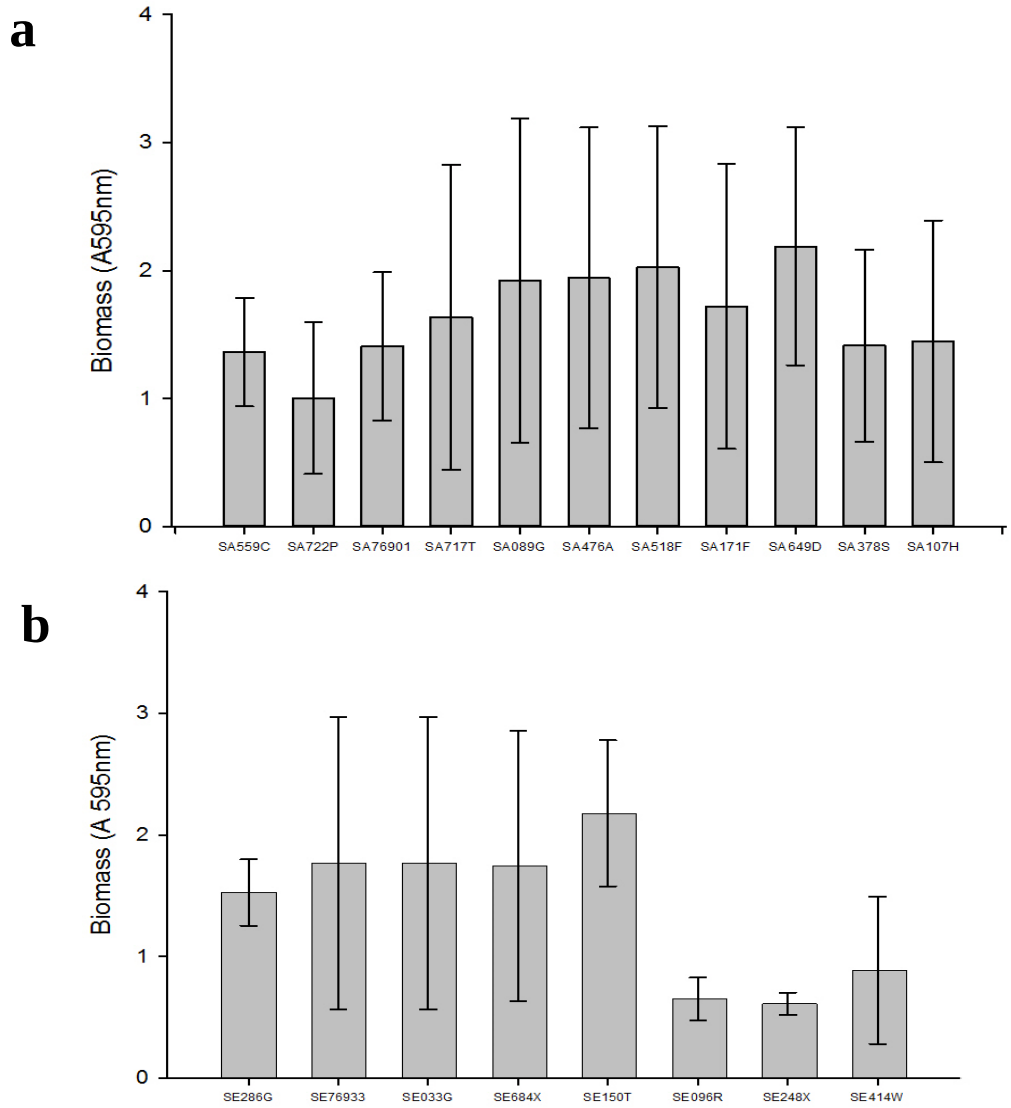


Figure 2-1 Biofilm formation of a) *Staphylococcus aureus* and b) *Staphylococcus epidermidis* clinical strains grown for 24 hours in a 96 well plate and stained with crystal violet. Absorbance measured at 595nm. Mean and standard deviations are shown. Experiments were done in triplicate, three independent times.

2.3.3 Optimisation of in-vitro biofilm formation model

Once it was demonstrated that all clinical strains were capable of producing biofilm in in vitro, a series of experiments were set up to identify the optimum growth media and incubation time for production of a biofilm. Ideally all 19 strains would have been tested but time and funding constraints prevented this. Two *Staphylococcus aureus* and two *Staphylococcus epidermidis* strains were selected for the optimisation experiments. We included the two strains obtained from Northumbria Healthcare NHS Foundation Trust

(SA76901 and SA76933) and two from Freeman Hospital (SA518F and SE096R). These strains are capable of average biofilm formation; we also included a strain that forms weaker biofilm as we ultimately wanted to assess the efficacy of NucB with a wide range of biofilm forming bacteria. Three different generic media for bacterial growth were tested: Nutrient Broth (NB), Luria Bertani (LB) and Tryptic Soy Broth (TSB). All three are reliable media for bacterial growth for *Staphylococcus aureus* and *Staphylococcus epidermidis* species (Missiakas and Schneewind, 2013; Shida *et al.*, 2013; Koseki *et al.*, 2014; Hiltunen *et al.*, 2019; Jenkins and Bean, 2019; Wijesinghe *et al.*, 2019). All four strains grew in all three media (Figure 2-2).

Table 2-2 describes the amount of biofilm growth of each strain in different media tested: LB, TSB and NB. As all four strains grew in TSB significantly stronger than in LB or NB, particularly SA76901 and SE76933, it was decided to continue using TSB as our medium of choice for future experiments.

	LB	TSB	NB	p value
SA518F	0.254	0.354	0.103	0.012*
SE096R	0.475	0.483	0.248	0.001*
SA76901	0.599	1.981	0.126	<0.001*
SE76933	0.588	2.811	0.318	<0.001*

Biofilms of all four clinical strains were grown for 6, 12, 18, and 24hours to establish the optimal incubation time. Table 2-3 presents the biomass of each strain at each specific time point. We observed most biofilm formation at 24h in strains SE096R, SA76901 and SE76933 (Figure 2-3). Although SA518 demonstrated higher biofilm formation at 18hour (Figure 2-3), to ensure consistency in the methodology, 24hour biofilm growth time was chosen for future experiments as it was the optimal incubation time for three of the four tested strains

	LB	TSB	NB	p value

SA518F	0.254	0.354	0.103	0.012*
SE096R	0.475	0.483	0.248	0.001*
SA76901	0.599	1.981	0.126	<0.001*
SE76933	0.588	2.811	0.318	<0.001*

Table 2-2 Clinical strains biofilm growth on different media. Columns represent the amount of biomass growth after 24 hours in different media. Means are represented in the table. *The significant statistical difference between groups was calculated using one-way Anova.

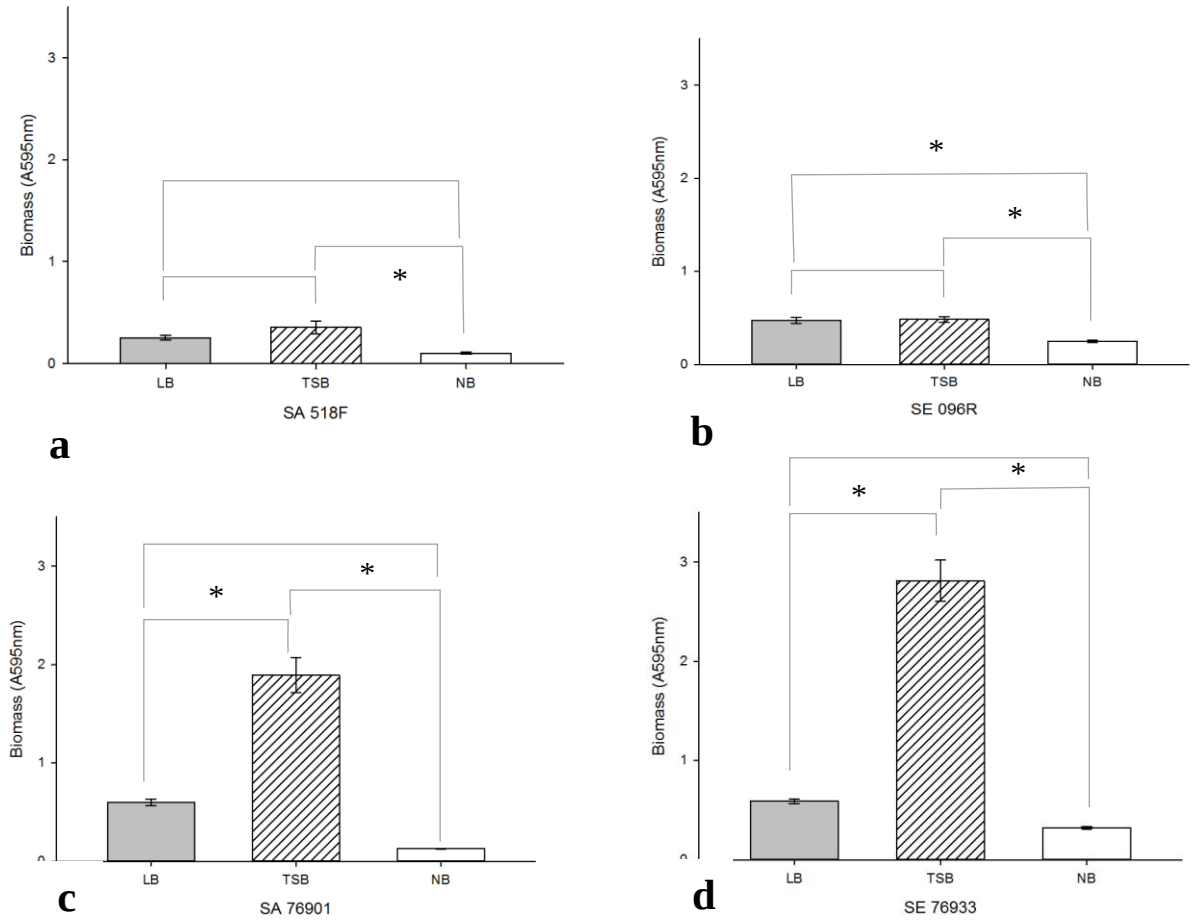


Figure 2-2 Effect of different media on the growth of different clinical strains a)SA 518F, b)SE 096R, c)SA 76901, d)SE 76933 grown for 24 hours in a 96 well plate in Luria Bertrani (LB), Tryptic Soy Broth (TSB) or Nutrient Broth (NB) and stained with crystal violet. Absorbance measured at 595nm. Mean and standard deviation are shown in the graphs. *Statistical analysis was done using a one-way Anova and a post-hoc Tukey HSD test to show the multiple comparison between media. Experiments were done in triplicate, three independent times

Table 2-3 Biofilm growth over time of four clinical strains. Columns represent the amount of biomass growth at each time point. Means are represented in the table. *The significant statistical difference between groups was calculated using one-way Anova.

	6h	12h	18h	24h	p value
SA518F	0.435	0.477	0.571	0.413	0.019*
SE096R	0.492	0.585	0.711	1.405	<0.001*
SA76901	0.689	0.586	1.345	1.426	<0.001*
SE76933	1.440	0.951	1.208	2.055	<0.001*

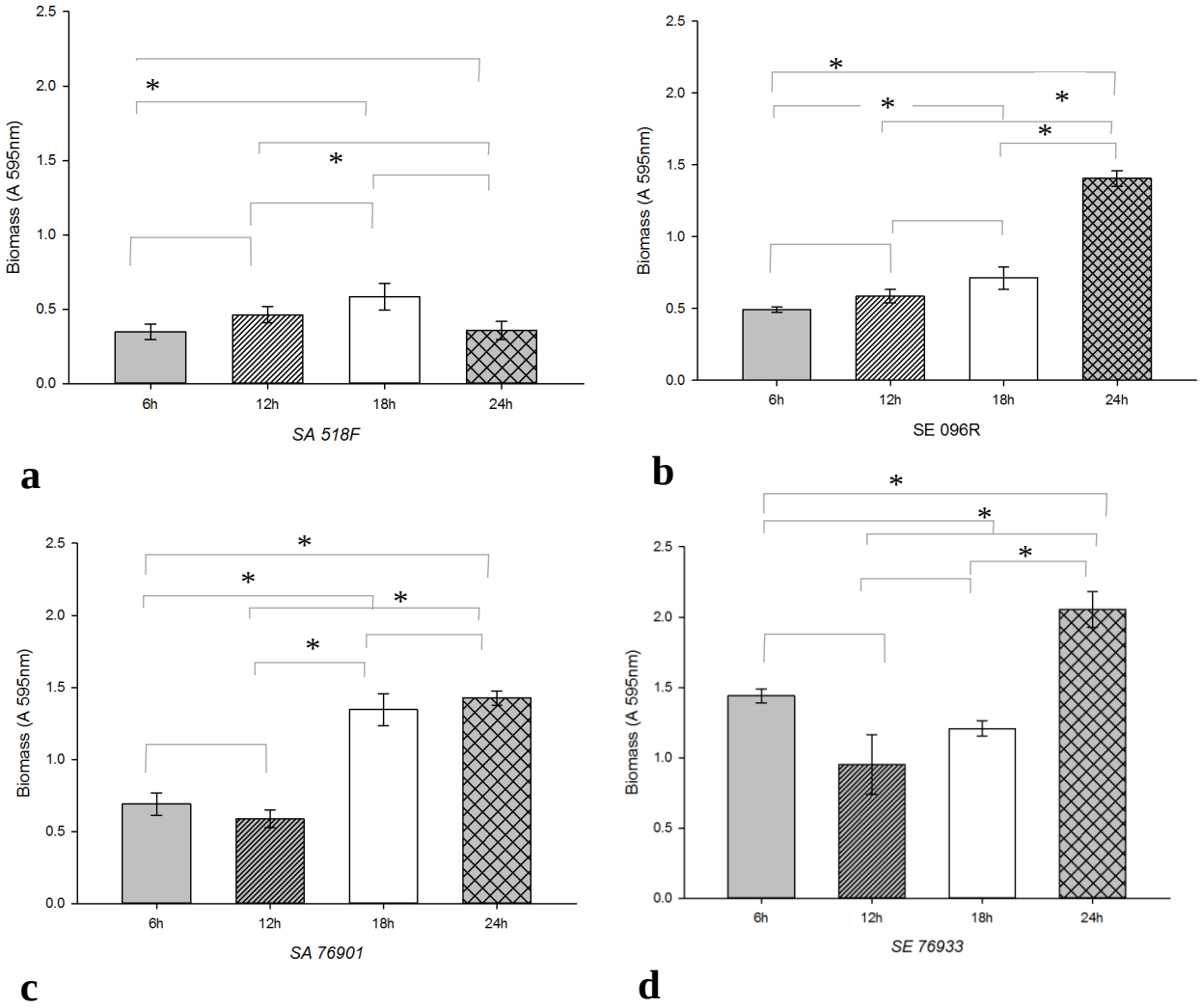


Figure 2-3 Effect of different incubation times on biofilm formation of strains a)SA 518F, b)SE 096R, c)SA 76901, d)SE 76933 grown in a 96 well plate and stained with crystal violet. Absorbance measured at 595nm. Mean and standard deviation are shown in the graphs. *The significant statistical difference between groups was calculated using one-way Anova and a post-hoc Tukey HSD test to demonstrate the multiple comparison between times Experiments were done in triplicate, three independent times

2.3.4 Quantification of NucB activity by agarose gel electrophoresis and spectrophotometry.

NucB was stored at -80 ^{o}C. A series of experiments were devised to evaluate the stability, reproducibility and efficacy of NucB over time. The enzyme preparation was stored and assessed at 3 time points: 0 days, 15 days and 30 days by gel electrophoresis and quantified by spectrophotometry. Gel electrophoresis is a reliable and effective method to differentiate DNA fragments based on size (Lee *et al.*, 2012) therefore it is a good method to assess the nuclease activity of NucB. Figure 2-4 demonstrates that NucB was capable of digesting 125µg/mL Calf thymus DNA within 5 minutes compared to control samples. Rows 1, 3 and 5 contain the enzymatic mixture of NucB and DNA. Digested DNA can be seen at the bottom of the gel in these rows while undigested DNA remains at the top of the gel in the control rows 2, 4 and 6 where no NucB was added. The efficacy of NucB to digest DNA was also measured using a NanoDrop spectrophotometer. Figure 2-5 demonstrates that NucB remained stable after 30 days in storage at -80 ^{o}C with no significant difference in its enzymatic activity over time.

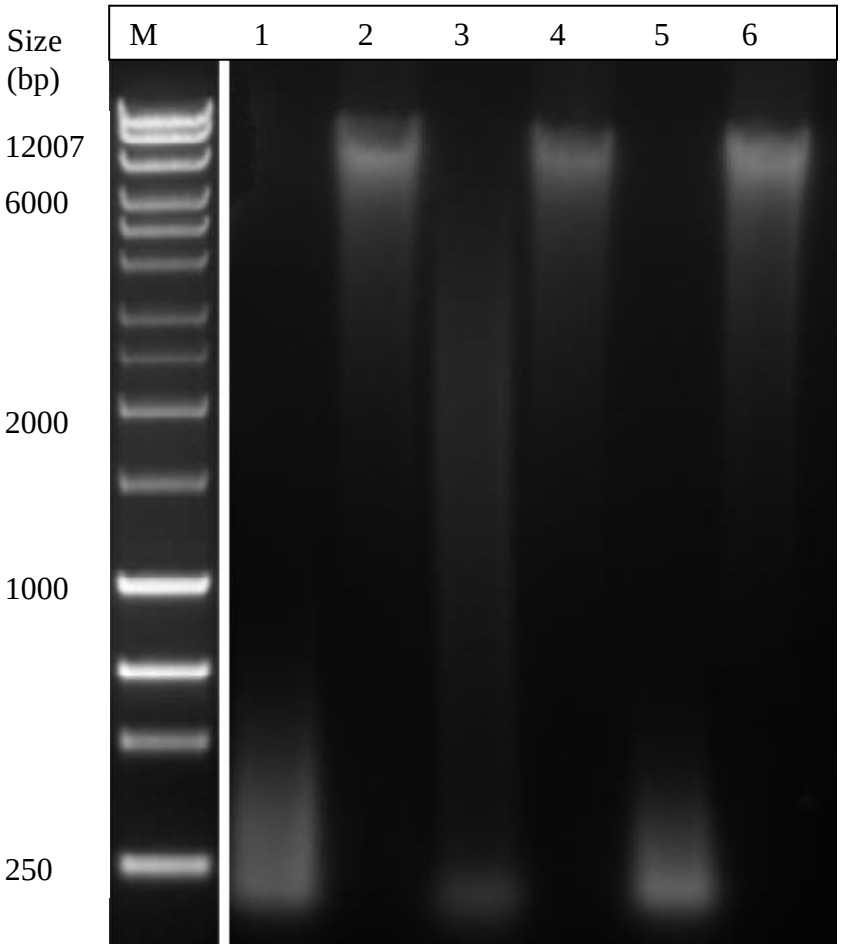


Figure 2-4 Enzymatic digestion of CT DNA. Row 1 represents DNA digested by NucB for 5 minutes, row 2 represents control DNA that was incubated for 5 minutes, row 3 represents DNA digested by NucB for 15 minutes, row 4 represents control DNA incubated for 15 minutes, row 5 represents DNA digested by NucB for 30 minutes and row 6 represents control DNA incubated for 30 minutes. Ladder is represented in row M with weight values

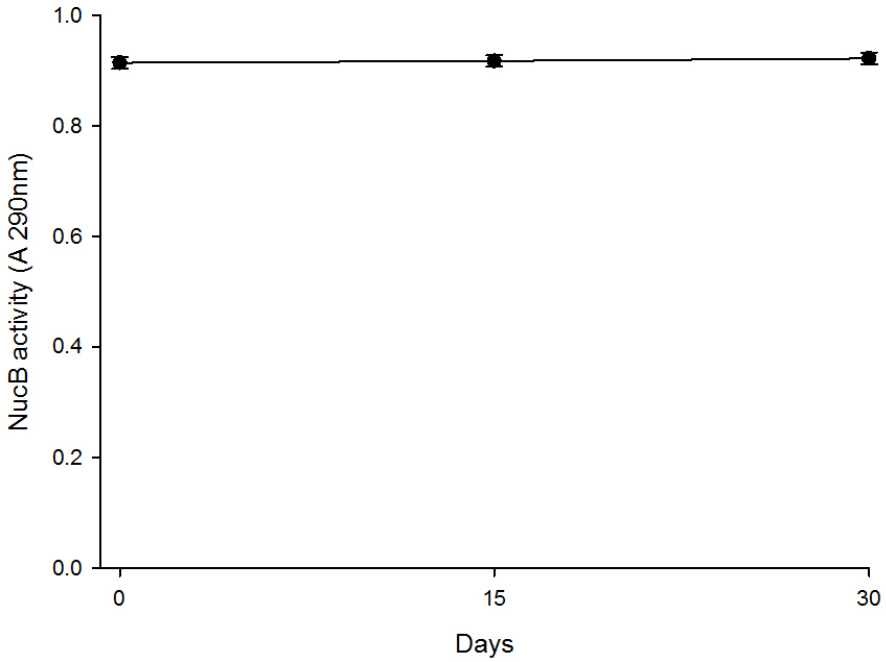


Figure 2-5 **NucB activity assayed after storage for up to 30 days.** In each experiment, 10ng of NucB stored over time was used to digest 125µg of CT DNA for 60 minutes. Experiments were performed in triplicates. Mean and standard deviations are represented in the graph

2.3.5 NucB enzymatic activity in the presence of bacterial cells by agarose gel electrophoresis

The ability of NucB to digest DNA in the presence of bacterial cells was assessed by gel electrophoresis. Figure 2-6 demonstrates that NucB is capable of digesting DNA in the presence of bacterial cells and bacterial supernatant. Different concentrations of bacterial cells were tested (10µl, 50µl and 100µl) in different preparations (washed once, washed twice and unwashed) as well as two different concentrations of bacterial supernatant (10µl and 100µl). The DNA was digested in all 13 tested preparations demonstrating that NucB remains active in the presence of bacterial cells.

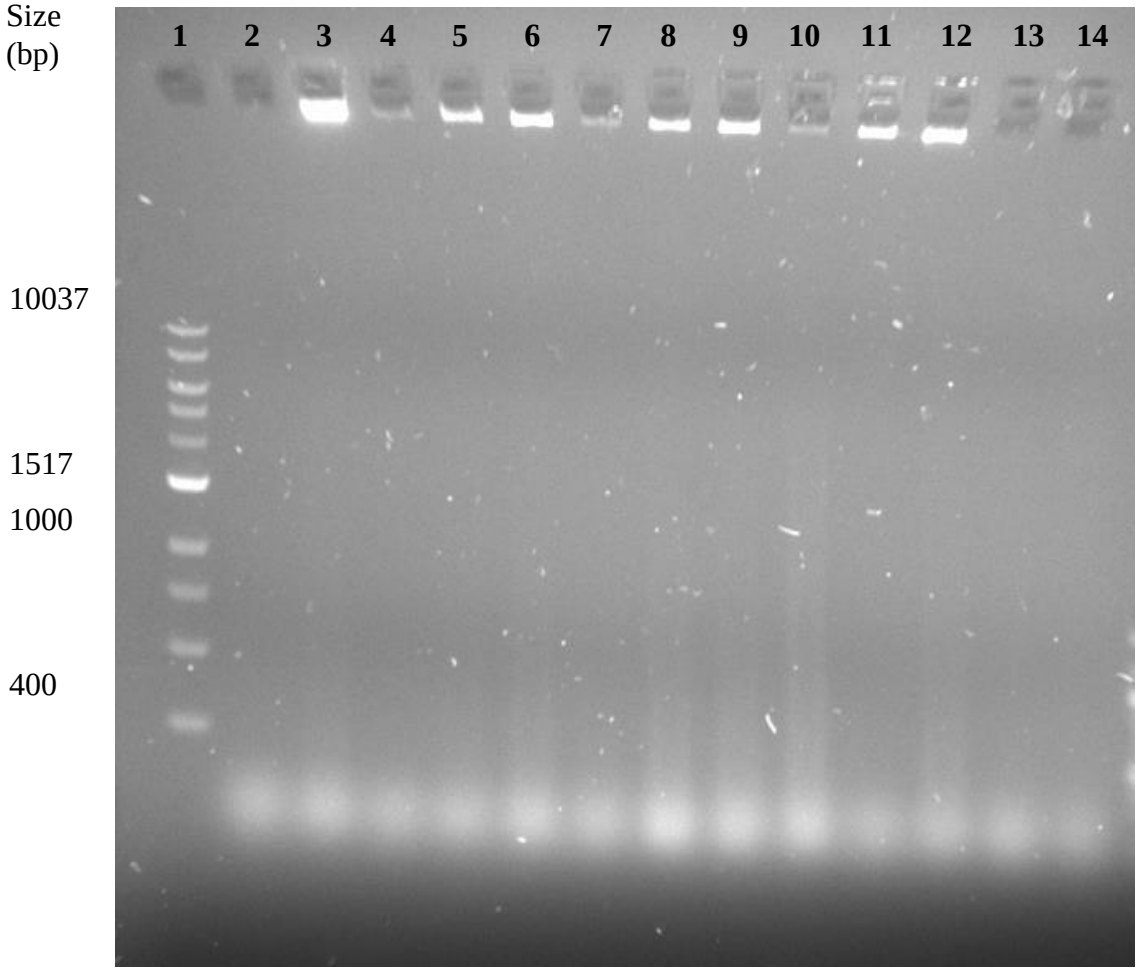


Figure 2-6 Enzymatic activity of NucB in the presence of bacterial cells. Row 1 represents the Hyperladder 1 kb Plus. Row 2 is the control well with DNA and NucB but no cells. Rows 3 to 12 represent NucB digestion of DNA in the presence of different bacterial cells concentration and preparation. Rows 13 and 14 represent NucB digestion of DNA in the presence of 10µl and 100µl of bacterial supernatant respectively. The different bacterial concentrations and preparations in each row is specified in the table below.

Rows	Content
1	*Hyperladder 1kb Plus*
2	*TSB + DNA + NucB + 5mM MnSO4 - No cells*
3	*TSB + DNA + NucB + 5mM $MnSO_4$ + pelleted cells*
4	*TSB + DNA + NucB + 5mM $MnSO_4$ + 10µl pelleted cells washed x1*
5	*TSB + DNA + NucB + 5mM $MnSO_4$ + 50µl pelleted cells washed x1*
6	*TSB + DNA + NucB + 5mM $MnSO_4$ + 100µl pelleted cells washed x1*
7	*TSB + DNA + NucB + 5mM $MnSO_4$ + 10µl pelleted cells washed x2*
8	*TSB + DNA + NucB + 5mM $MnSO_4$ + 50µl pelleted cells washed x2*
9	*TSB + DNA + NucB + 5mM $MnSO_4$ + 100µl pelleted cells washed x2*
10	*TSB + DNA + NucB + 5mM $MnSO_4$ + 10µl unwashed cells*
11	*TSB + DNA + NucB + 5mM $MnSO_4$ + 50µl unwashed cells*
12	*TSB + DNA + NucB + 5mM $MnSO_4$ + 100µl unwashed cells*
13	*TSB + DNA + NucB + 5mM $MnSO_4$ + 10µl bacterial supernatant*
14	*TSB + DNA + NucB + 5mM $MnSO_4$ + 100µl bacterial supernatant*

2.5 Discussion

The ability of these clinical strains to form biofilm in-vitro had not been previously assessed. Single colonies were used. The principle of isolating and using single colonies, which contain millions of individual cells, is well established in Microbiology (Sanders, 2012). Biofilms were grown using a modified technique based on the methodology described by Christensen et al. in 1985. The methodology of Christensen et al. has been widely used over the past three decades and provides a quick and reproducible way of growing biofilms consistently in an in-vitro setting (Cusumano *et al.*, 2019; Silha *et al.*, 2019). The use of a 96-well plates allows a large number of strains to be tested at once under the same growth conditions.

Figure 2-1 demonstrates the ability of all 19 clinical strains to grow biofilm in a 96 well polystyrene plate confirming previous studies (Arciola *et al.*, 2006; Manandhar *et al.*, 2018; Sugimoto *et al.*, 2018b). *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms have been successfully grown in different media (Seidl *et al.*, 2008; Cihalova *et al.*, 2015; Uribe-Alvarez *et al.*2015; Wijesinghe *et al.*, 2019). It was deemed necessary to identify the most appropriate growth media as well as the incubation time for the clinical strains tested. Staphylococci will often produce less biofilm than when grown on complex media, for this reason complex media were chosen (Wijesinghe *et al.*, 2019). The key aim was to carry out a straightforward comparison of three complex media to find the best biofilm growth in terms of quantities of biofilm. A detailed investigation of sensitivity and specificity at this stage was not needed. Two *Staphylococcus aureus* strains and two *Staphylococcus epidermidis* strains were selected for the next experimental phase. These strains were *Staphylococcus aureus* 76933, *Staphylococcus aureus* 518F, *Staphylococcus epidermidis* 76933 and *Staphylococcus epidermidis* 096R. No reference strains were used since clinical strains behave very differently to reference strains. Biofilms of all four strains grew strongly in TSB media compared to LB or NB (Figure 2-2). A possible explanation for this is the presence of glucose in the growth media. Glucose is part of the composition of TSB media and has been shown to enhance biofilm growth of *Staphylococcus aureus* and *Staphylococcus epidermidis* in vitro (Agarwal and Jain, 2013; Waldrop *et al.*, 2014). Although our early in-vitro experiments do not mimic in vivo growth conditions, they are an important step to understanding the process of biofilm formation by these clinical strains and, more importantly, the impact of NucB. Future experiments developing a model that also simulates synovial fluid as growth media will be needed. Equine and porcine synovial fluid has been used successfully in in-vitro models of *Staphylococcus aureus* biofilm (Gilbertie *et al.*, 2019).

Although possible, this methodology will require ethical approval and likely will incur a high cost. Synovial fluid can be manufactured synthetically to simulate in-vivo conditions so perhaps is a more preferable alternative (Bortel *et al.*, 2015).

More than 50% of the pathogens involved in PJI are Gram positive *Staphylococcus*, particularly *Staphylococcus aureus* and *Staphylococcus epidermidis* (Parikh and Anthony, 2016). But a multitude of other pathogens can be the causative organism of PJI. This work concentrates on the two most common strains of Gram positive bacteria. NucB has been previously tested against Gram negative bacteria such as *E.coli* and demonstrated to be capable of removing such biofilms (Nijland *et al.*, 2010). Therefore it is likely that NucB will also be capable of disrupting clinical strains of Gram negative pathogens. To fully assess the efficacy of NucB in PJI further experiments involving non Gram positive strains will be necessary.

The study of biofilms in-vitro has a number of advantages, they are low cost, easy to set up and easily reproducible which facilitates their use for research purposes. They are able to mimic certain environmental properties of in-vivo biofilms but not all, and often are unable to replicate important parameters including the host factors such as the innate immune response and certain environmental factors that will invariably affect the biofilm (Lebeaux *et al*., 2013). It has been demonstrated that the 3D structure of the biofilm differs in in-vitro conditions, for example the characteristic mushroom structures of *P. aeruginosa* biofilm formation, has yet to be observed in-vivo (Roberts et al., 2015). We have to bear this in mind and be aware that results observed in in-vitro experiments may not be replicated with the same results in vivo, but nevertheless in-vitro biofilm studies are necessary as a first step towards understanding the behaviour of biofilms under certain conditions and to establish a foundation of knowledge upon which to build.

The time in which a pathogen forms biofilm varies depending on the species. This was well demonstrated by Oliveira et al. who grew over 55 clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis,* and showed that biofilm growth varied significantly with some stains taking up to 72 hours to form established biofilms (Oliveira *et al.*, 2007). Our four clinical strains were capable of producing biofilm as early as 6 hours. The strongest biofilm was achieved at 24hours in all strains apart from *Staphylococcus aureus* 518F which showed similar levels of biofilm formation at all four different times points (Figure 2-3). We did not observe more rapid growth with *Staphylococcus aureus* compared to *Staphylococcus epidermidis* which is at variance with a previous publication (Stewart *et al.*, 2017). Given that all four strains grew strong biofilm at 24hours this was the incubation time chosen for

subsequent experiments. A possible limitation of the study is that 24h biofilm may not represent clinical conditions where more mature biofilms (days, weeks and even months) are established. Further experiments assessing biofilms of different ages will be necessary to draw stronger conclusions.

Before proceeding with assessing the efficacy of NucB against clinical strains it was necessary to ensure the stability of NucB activity as well as establish its efficacy in a bacterial environment. Figure 2-4 and Figure 2-5 demonstrates NucB remains active with very little variation over time. The toxicity of NucB against bacterial cells had been previously assessed (Shakir *et al.*, 2012; Shields *et al.*, 2013). Shakir et al. demonstrated NucB did not affect the bacterial growth of clinical strains from chronic rhinosinusitis and this was later on supported by Shields et al. who assessed the toxicity of NucB against bacterial cells isolated from tracheoesophageal speech valves and found no bacterial growth inhibition when bacterial cultures when incubated in the presence of NucB. NucB is a stable enzyme capable of re-folding its structure and regaining activity after exposure to high temperatures (Basle *et al.*, 2018). We demonstrated that the enzyme remains active in the presence of staphylococcal cells and supernatant. It is known that *Staphylococcus* are capable of secreting a number of proteases including including two cysteine proteases (staphopain A, ScpA, and staphopain B, SspB), a serine protease (V8 or SspA), serine protease–like proteins (Spls) and a metalloproteinase (aureolysin, Aur) (Pietrocola *et al.*, 2017). These proteases play a role in the evasion of the host immune response (Prokesová *et al.*, 1992; Smagur *et al.*, 2009). As proteases, they have the potential to degrade enzymes such a NucB and this could therefore affect its efficacy against biofilms of this particular bacterial family. The confirmation of enzymatic activity despite bacterial presence is a fundamental requirement for this enzyme to be potentially developed as a treatment modality for prosthetic joint infections. Synthesis of extracellular proteases takes place during the exponential growth phase (Karlsson and Arvidson, 2002) and therefore these enzymes should also be present during the process of biofilm formation.

The marine nuclease NucB has been shown to maintain its enzymatic activity over time with virtually no degradation in its activity. The activity is not influenced by the presence of live bacterial cells. Clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* from patients undergoing treatment for prosthetic joint infections have been shown to be capable of producing biofilms in-vitro. The growth media and incubation time have been optimised. These steps were essential to be able to ensure reproducibility and

move forward to assess the efficacy of NucB in prevention and dispersal of biofilms of prosthetic joint infections.

Chapter 3. The effect of NucB on biofilm formation by clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* grown on prosthetic join materials.

3.1 Introduction

Currently the materials used in arthroplasty are divided in three categories: metals, ceramics and polymers. Titanium alloys, stainless steel, cobalt chrome, alumina, zirconia and ultra-high molecular weight polyethylene (UHMWPE) are most commonly used materials, the latter in the acetabular component of the total hip replacement (Merola and Affatato, 2019). Stainless steel used to be a common material used for hip prosthesis due to its resistance to oxidation and ease of manufacture but has now lost popularity due to poor biocompatibility (Hu and Yoon, 2018). Despite this, the Exeter stem, remains one of the most implanted and successful hip prosthesis in United Kingdom and is made of stainless steel (Kazi *et al.*, 2019). Cobalt Chrome has favourable strength, corrosion and wear properties which make it the implant material of choice, particularly for cemented hip stems (Hu and Yoon, 2018). Titanium alloys have also been used for stem implants as well as cementless acetabular components due to low density but high mechanical strength, corrosion, resistance properties and osteointegration (Head *et al.*, 1995).

Bacterial adhesion is complex and multifactorial. Artificial surface properties play a role in the initial attachment and biofilm formation (Song *et al.*, 2015). Metal surfaces have surface oxides that bind to glycoproteins and possibly to bacteria. Surgical alloys have high energy surface that promotes the interaction with colonising cells (Gristina, 1987). Surface roughness and topography of the substrate also plays a role in bacterial adhesion and biofilm formation, but when it comes to polymers, surface energy appears to have a more determinant role for bacterial attachment than surface roughness (Hallab *et al.*, 2001).

In 1987 Anthony Gristina described “a race for the surface” (Gristina, 1987). When a biomaterial is implanted in the human body, the host cells and bacteria compete against each other to colonise the new implanted surface. The hope is that the tissue cells wins the race and colonises the surface of the material defending it from bacterial attachment.

Over the years, research has focused on developing new strategies that will facilitate the host cell to win the race against bacteria. However, despite these efforts, pathogens such as *Staphylococcus aureus* are well-known to form biofilms on materials such as stainless steel, titanium and polyethylene (Smith and Hunter, 2008; Merghni *et al.*, 2017; Moley *et al.*,

2018). Various approaches have been assessed to inhibit biofilms including antibacterial coating of surfaces (Nielsen *et al.*, 2018), excretions of maggots (Cazander *et al.*, 2010) and modification of surface properties (Kruszewski *et al.*, 2013). However, the potential of enzymes for treating biofilms has not been investigated in detail. Therefore, this Chapter set out to develop a model for investigating biofilm formation on the surfaces of relevant materials and for assessing the ability of NucB to control those attached and clinically relevant biofilms.

The objectives of this chapter were:

1. To assess whether NucB can prevent or inhibit biofilm formation by *Staphylococcus aureus and Staphylococcus epidermidis.*
2. To assess whether NucB can prevent or inhibit biofilms grown on cobalt chrome, titanium, stainless steel and UHMWPE.

3.2 Materials and methods

3.2.1 Effect of NucB on biofilm formation. An in-vitro microtiter plate assay

The protocol for assessment of anti-biofilm activity of NucB was based on the in vitro model of Shakeri et al (Shakeri *et al.*, 2007) and was as described in Chapter 2, with the following changes: A 100µl of NucB (1µg/ml) were added to the sample wells, whereas 100µl of TSB was added to control wells of a 96 well plate. Overnight bacterial broth was added to each well to achieve OD_{600} = 0.1. 5mM $MnSO_4 \bullet H_2O$ was added to all wells. The plates were covered and incubated statically at 37°C for 24 hours. The biofilms were stained and measured as previously described in section 2.2.3. Each assay was performed in triplicate, three independent times.

3.2.2 Sourcing of simulated joint materials

Titanium, cobalt chrome, stainless steel and high molecular weight polyethylene were used as surfaces to simulate the materials of a joint replacement in vitro. These are materials commonly used in total hip and total knee replacements. Cobalt-Chrome-Mo Alloy ASTM 75 and titanium 6Al4V ELI were purchased from Acnis International, Lyon (France) and high molecular weight polyethylene discs were obtained from Biosurface Technologies Corporation, Montana (USA). These material are the same used in approved and marketed arthroplasty implants (Bezuidenhout *et al.*, 2015; Ahearne and Baron, 2017).

3.2.3 Effect of NucB on biofilm formation on metal and polyethylene surfaces

The same four clinical strains selected for previous experiments (section 2.3.3) were selected for this experiment: *Staphylococcus aureus* 76901, *Staphylococcus aureus* 518F, *Staphylococcus epidermidis* 76933 and *Staphylococcus epidermidis* 096R. To assess the effect of NucB on biofilm formation on titanium, cobalt chrome, stainless stell and HMWPE discs as a substrate, discs were washed in 1% virkon and 70% ethanol and rinsed with sterile distilled water to remove any residues, then autoclaved. Individual discs were placed into separate Falcon tubes; each well contains 5mL total volume. Control tubes contained bacterial cultures of overnight growth at OD_{600} of 0.1, 50mM Tris-HCl, 5mM $MnSO_4.H_2O$ (pH 8.0). Treatment tubes contained bacterial culture of overnight growth at OD_{600} of 0.1, NucB at 1µg/ml in 50mM Tris-HCl, 5mM $MnSO_4 \bullet H_2O$ (pH 8.0). The tubes were incubated at 37°C statically for 24hours. Discs were removed and excess biofilms was washed with sterile PBS. Once dry, samples were transferred to individual Falcon tubes with 5ml of 0.1% crystal violet (CV) for 20 minutes. After washing, the discs were transferred to a sterile

polystyrene 6-multiwell plate and the crystal violet dye was solubilized in 5ml of 33% acetic acid for 30 minutes. 200µl of the solution of each well were transferred to a new 96 well polystyrene plate and the CV absorbance at 595nm was measured using a Fluostar Optima plate reader (BMG Labtech, Bucks, UK), using the MARS software package (BMG Labtech). Each assay was done in triplicate and repeated three times.

3.2.4 Biofilm imaging

To assess the effect of NucB on biofilm formation on titanium surfaces, discs were prepared and sterilized as previously described. Individual discs were placed into sterile polystyrene 6-multiwell plate. *Staphylococcus aureus* 76901 culture was grown overnight as previously described. Control wells had bacterial culture of overnight growth at OD_{600} of 0.1, 50mM Tris-HCl, 5mM $MnSO_4 \bullet H_2O$ (pH 8.0). Treatment wells contained bacterial culture of overnight growth at OD600 of 0.1, NucB at 1µg/ml in 50mM Tris-HCl, 5mM $MnSO_4 \bullet H_2O$ (pH 8.0). The tubes were incubated at 37°C statically for 24 hours. The supernatant was then aspirated and discarded, and discs were washed by pipetting sterile PBS solution and air dried. Live/Dead® BacLight™ (Thermo Fisher Scientific) bacterial viability kit was used to stain the biofilm(Shen *et al.*, 2010). This kit uses SYTO® 9 stain and the propidium iodide nucleic acid stain. Live bacteria with intact cell membranes are stained with fluoresce green, dead bacteria with compromised membranes with fluoresce red. Bacteria were stained using the manufacturer's protocol. 1.5µl of SYTO® 9 stain and propidium iodide stain were added into a microfuge tube and thoroughly mixed. SYTO® 9 stain and propidium iodide stain were stored at -20°C protected from light. 200µl of the dye mixture was dropped on each of the discs and at room temperature in the dark for 15 minutes. The excess dye was rinsed off by pipetting and discarding sterile water 3 times on the discs then allowed to air dry. Discs were fully immersed in sterile water and the plate was covered with foil to maintain darkness during the transfer to the imaging room. Images were obtained using a Leica TCS SP2 UV AOBS MP point scanning confocal microscope with a 40x water dipping objective. SYTO® 9 excitation/emission was 480/500nm and propidium iodide was 490/635nm. Biomass and biofilm thickness were measured using Comstat2 (Technical University of Denmark). Quantification of live (green colour) and dead (red colour) bacterial cells was done using MATLAB R2017a.

3.2.5 Statistical analysis

Statistical analysis was performed with SPSS (IMB SPSS Statistics-version 22) by using a non-parametric Mann-Whitney U test. A non-parametric test was used as the sample number is small and normal distribution cannot be assumed. To calculate significant differences between control and experimental samples, p values below 0.05 were considered statistically significant.

3.3 Results

3.3.1 The effect of NucB on biofilm formation of clinical isolates.

Eleven clinical strains of *Staphylococcus aureus* and eight clinical strains of *Staphylococcus epidermidis* and were tested. Following 24 hours of static incubation, a varying effect of NucB was observed (Table 3-1). The difference in biofilm growth was in some cases visible to the naked eye (Figure 3-1).

Figure 3-2 demonstrates the effect of NucB on biofilm formation of eleven clinical strains of *Staphylococcus aureus.* NucB had a variable effect and was capable of reducing biofilm formation in six of the eleven strains. The maximum reduction in biofilm formation was seen in SA 378S (56% less biofilm formed in the presence of NucB, $p=0.009$), followed by SA 649D (54%, $p<0.001$).

Figure 3-3 shows that NucB had a variable effect and was capable of reducing biofilm formation in five of the eight clinical strains of *Staphylococcus epidermidis*. The inhibition effect observed varied from a 28 to 55% reduction of biofilm formation in the presence of NucB compared to control samples where no NucB was present. The maximum reduction of biofilm formation was 55% observed with strain SE 414W ($p=0.006$), followed by 52% with strain SE 096R ($p<0.001$).

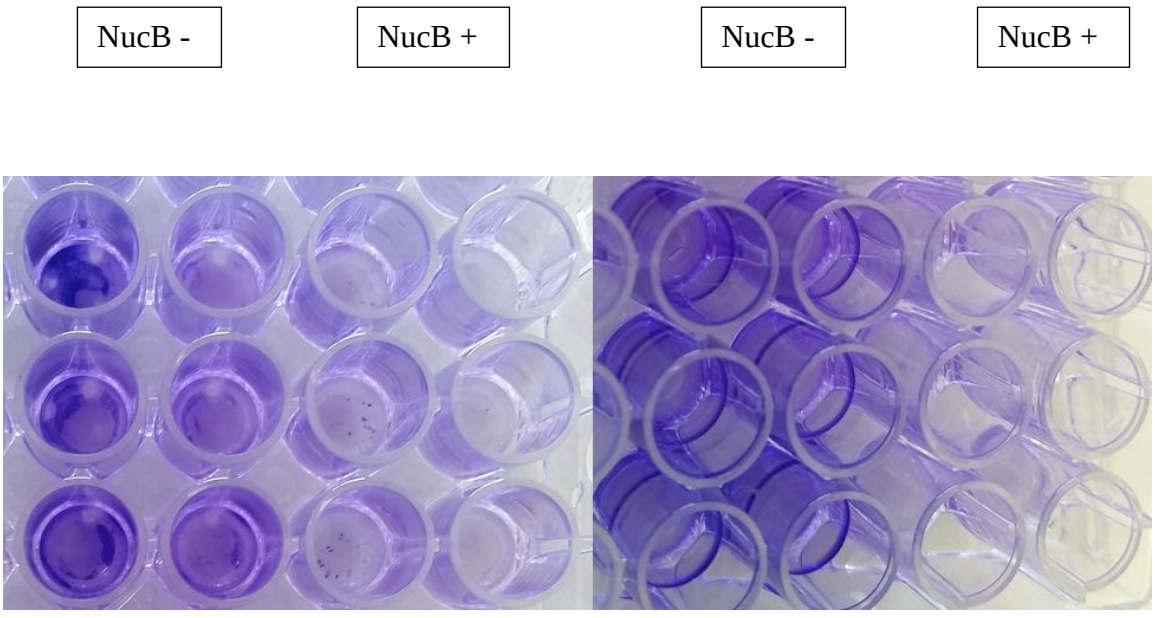


Figure 3-1 Biofilms grown on a 96 well plate. Biofilms grown had been grown in the presence (NucB+) and absence (NucB-) of NucB and stained with 0.1% crystal violet. Biofilms grown in the absence of NucB could be seen with the naked eye in the side and bottom of the 96 well plate prior solubilisation with acetic acid.

Table 3-1 Effect of NucB on biofilm formation of clinical strains. Absorbance measured at 595nm. * Statistical significance was determined using a Mann-Whitney U test $p<0.05$. Experiments were performed in triplicates.

Strain	Biomass NucB-	Biomass NucB+	Reduction in formation	p value
SA559C	1.73	1.56	10%	0.589
SA722P	1.38	1.08	22%	0.589
SA76901	1.90	1.44	25%	0.240
SA717T	2.69	1.89	30%	0.008*
SA089G	3.11	2.39	23%	0.040 *
SA476A	3.16	2.90	8%	0.394
SA518F	3.00	1.72	43%	0.009 *
SA171F	2.85	2.06	28%	0.240
SA649D	2.52	1.20	52%	0.024 *
SA378F	1.69	0.74	56%	0.001 *
SA107H	2.19	1.37	37%	0.008 *
SE286G	1.66	1.19	28%	<0.001*
SE76933	2.24	1.99	10%	0.190
SE033G	0.63	0.38	40%	0.011*
SE684X	0.71	0.61	14%	0.222
SE150T	1.92	1.46	23%	0.063
SE096R	0.58	0.27	52%	<0.001*
SE248X	0.61	0.45	26%	0.024*
SE414W	1.28	0.57	55%	<0.001*

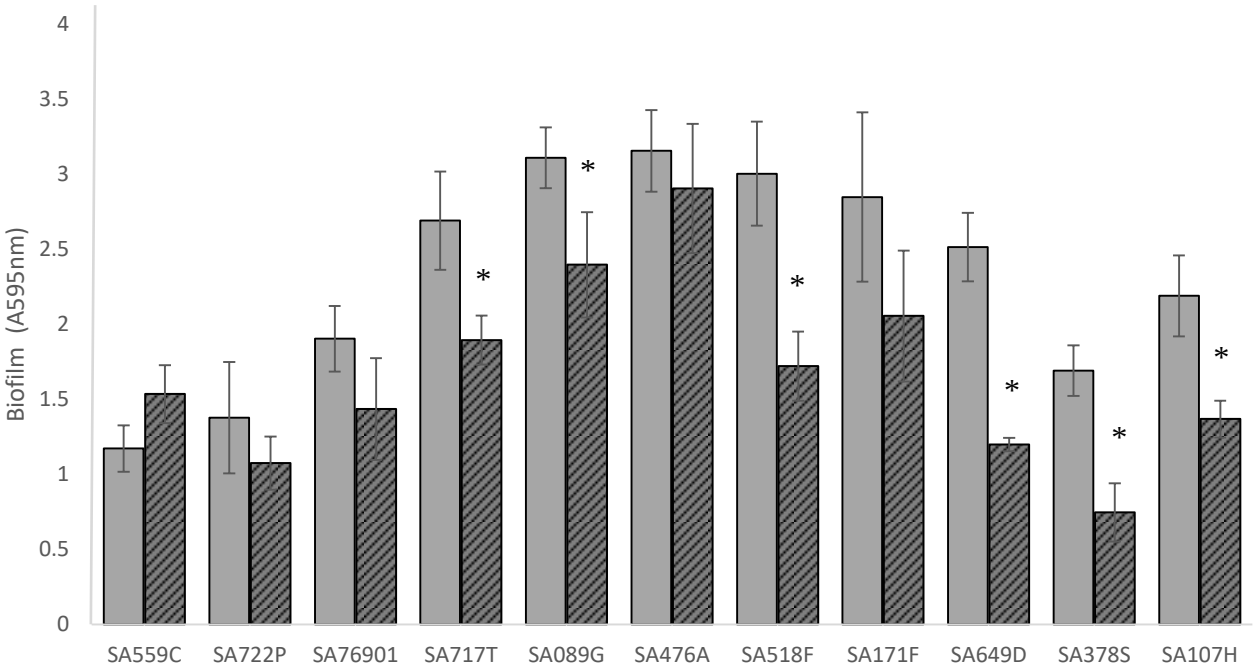


Figure 3-2 NucB effect on prevention of biofilm formation of clinical strains of *Staphylococcus aureus* grown in a 96 well plate. Control biofilms, are represented in solid grey bars. Biofilms grown in the presence of NucB are represented in stripped dark grey bards. Biofilms of strains SA717T, SA089G, SA518F, SA649D, SA378S and SA107H grew significantly less when NucB was present at the time of incubation. NucB had no effect on biofilm growth of SA559C, SA722P, SA76901, SA476A and SA171F. Mean and standard deviation are represented in the graphs. *= Statistical significance Mann-Whitney U test $p<0.05$. Experiments were performed in triplicates, three independent times.

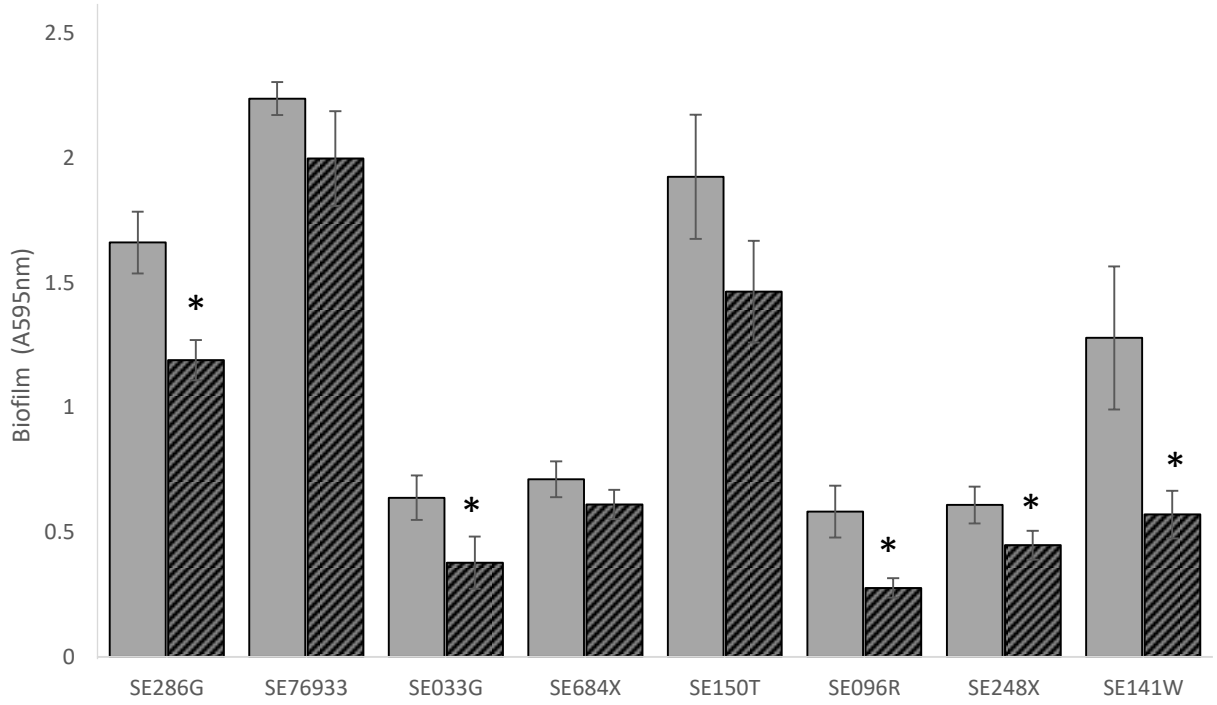


Figure 3-3 NucB effect on prevention of biofilm formation of clinical strains of *Staphylococcus epidermidis* grown in a 96 well plate. In the presence of NucB (dark columns) significantly less biofilm of SE286G, SE033G, SE096R, SE248X and SE414W was formed compared to control biofilms grown without NucB. NucB had no effect on biofilm growth of SE76933, SE684X and SE150T. Mean and standard deviation are represented in the graphs. *= Statistical significance Mann-Whitney U test $p<0.05$. Experiments were performed in triplicates, Three independent times.

3.3.2 Preparation of Simulated artificial joint surfaces

Titanium 6Al4V ELI bar was cut into discs in the School of Mechanical Engineering at Newcastle University (UK). Due to the hardness of cobalt-Chrome-Mo Alloy ASTM 75 it was not possible to cut the bar into discs in-house, and thus cutting was outsourced to a specialist company. The cobalt chrome bar was sent to Tecomet, Inc. (Sheffield, UK) for processing and cutting. HMWPE discs were polished using silicon carbide fine grit discs on a Metaserv rotary pregrinder (Buehler UK, Coventry, UK) with water cooling. Discs were pressed using circular movements against the silicon carbide fine grit discs that had been previously cut to the appropriate measurement to fit the pregrinder (Figure 3-4). Water was used continuously during the polishing process to cool the disc surfaces. Following polishing, disc height and circumference measurements were obtained using a micrometer (Table 3-2). The surface roughness was evaluated using a calibrated stylus contact optical surface profiler at room temperature and mean roughness (Ra) recorded (Table 3-3).

Figure 3-4 Rotary pregrinder (Buehler UK, Coventry, UK) used for manually polishing the HMWPE discs.

Table 3-2 Size and surface area of simulated artificial joint surfaces.

	Average radius (mm)	Average height (mm)	Surface area (mm^2)
Titanium	8.97	3.95	728.17 +/- 1.88
Cobalt-Chrome	8.99	5.08	794.76 +/- 3.18
Stainless Steel	1.13	2.52	57.79 +/- 2.17
HMWPE	10.6	5.81	1003.12 +/- 1.38

Table 3-3 Surface roughness of simulated joint discs.

	Surface Roughness	
	RA (µm)	RQ
Titanium	0.551 +/- 0.172	0.698 +/- 0.059
Cobalt-Chrome	0.196 +/- 0.017	0.255 +/- 0.028
Stainless Steel	0.613 +/- 0.075	0.754 +/- 0.057
HMWPE	0.669 +/- 0.100	0.789 +/- 0.059

The stainless discs were significantly smaller than the other materials. The stainless steel discs were already available in our department and although we attempted to obtain similar size discs of the other materials, this was not possible due to manufacturing limitations. The smoothest surface was cobalt chrome with an RA of 0.196, followed by titanium with an RA of 0.551, stainless steel with an RA of 0.613, and the roughest material was HMWPE with an RA of 0.699 (Table 3-3). Once the materials were characterised, biofilm formation on these materials was tested.

3.3.3 Effect of NucB on biofilm formation on metal and polyethylene surfaces

To test the effect of NucB on biofilm grown on different surfaces commonly used in arthroplasty, the same four strains previously selected (section 2.3.3) were used in this experiment: strains SA 518F, SA76901, SE096R and SE76933. Due to time constraints and availability of materials (NucB being extremely expensive to produce), it was not possible to test all four strains on all four surfaces, so two strains capable of average biofilm formation were selected. SA518F and SE096R were tested on HMWPE, titanium, cobalt chrome and

stainless steel while SA76901 and SE76933 were only tested on HMWPE, titanium and cobalt chrome. Biofilms were grown in the presence and absence of NucB. Table 4.3 summarises the biomass formation on artificial surfaces of all four clinical strains.

Table 3-4 Effect of NucB on biofilm formation of clinical strains on artificial surfaces. Absorbance measured at 595nm. *= Statistical significance Mann-Whitney U test $p<0.05$. Experiments were performed in triplicate, three independent times.

Strain	Surface	Biomass NucB-	Biomass NucB+	P value
SA518F	HMWPE	0.523	0.548	0.666
	Titanium	0.126	0.072	0.161
	Cobalt Chrome	0.178	0.033	<0.001*
	Stainless Steel	0.232	0.054	0.002*
SE096R	HMWPE	0.754	0.403	<0.001*
	Titanium	0.110	0.026	<0.001*
	Cobalt Chrome	0.111	0.038	0.001*
	Stainless Steel	0.154	0.028	<0.001*
SA76901	HMWPE	0.545	0.239	0.014*
	Titanium	0.609	0.428	0.190
	Cobalt Chrome	0.347	0.126	0.019*
SE76933	HMWPE	1.234	0.338	<0.001*
	Titanium	0.866	0.174	0.003*
	Cobalt Chrome	0.588	0.219	0.077

Staphylococcus aureus 518F showed significantly less biofilm formation on metal surfaces when NucB was present at the time of inoculation (Figure 3-5). We observed 81% less biofilm growth on cobalt chrome surfaces ($p<0.001$) and 77% less biofilm formation on stainless steel surfaces ($p=0.002$) when biofilms were grown in the presence of NucB compared to control samples incubated without NucB. NucB had no effect on *Staphylococcus aureus* 518F grown on HMWPE and titanium discs (Figure 3-5).

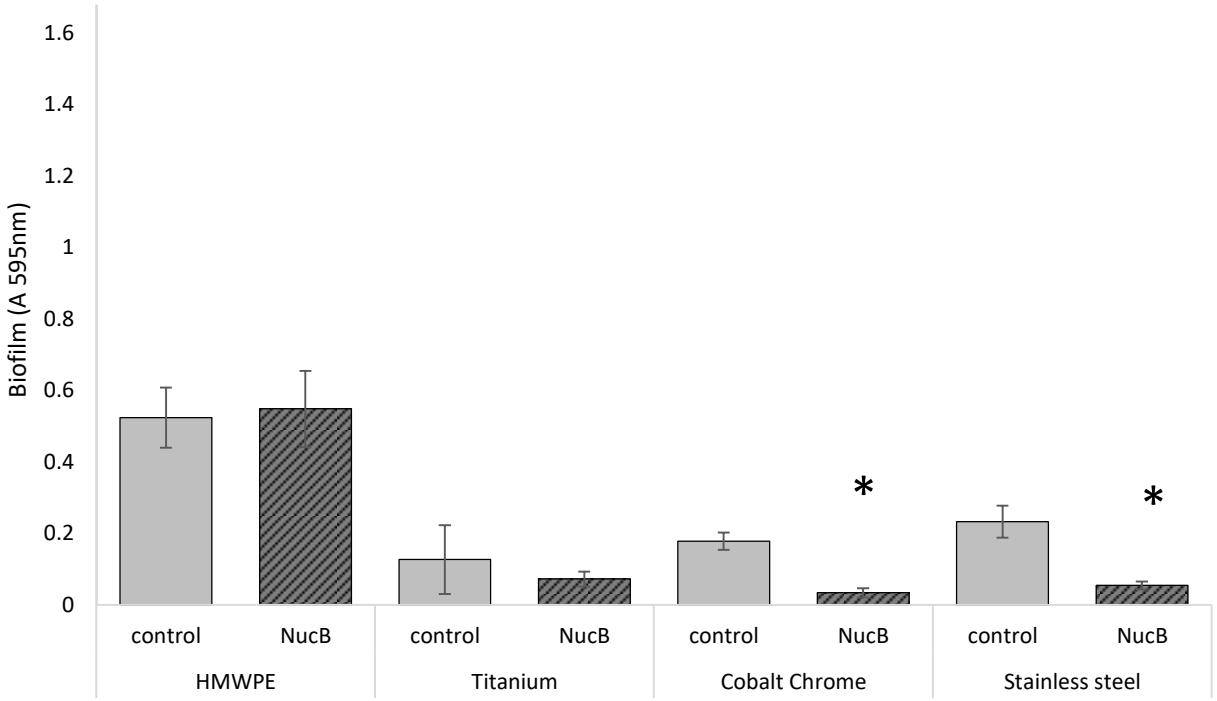


Figure 3-5 Effect of NucB on *Staphylococcus aureus* 518F biofilm formation. NucB (1µg/mL) was added at the time of inoculation. NucB significantly prevented biofilm formation on cobalt chrome by 81% ($p<0.001$) and stainless steel surfaces by 77% ($p=0.002$). No effect was demonstrated on HMWPE ($p=0.666$) and titanium surfaces ($p=0.161$). Mean values and standard error bars are represented in the graph. * = Statistical significance Mann-Whitney U test $p<0.05$. Experiments were performed in triplicate, three independent times.

NucB was also tested against *Staphylococcus epidermidis* SE096R and shown to be effective preventing biofilm formation on all four surfaces. After 24 hours of static incubation we observed 47% less biofilm formation on HMWPE discs ($p<0.001$), 76% less biofilm formed on titanium discs ($p<0.001$) and a 65% and 81% less biofilm formed on cobalt chrome ($p=0.001$) and stainless steel surfaces ($p<0.001$) when NucB was present at the time of incubation compare to control samples without NucB (Figure 3-6).

The effect of NucB on biofilm formation of *Staphylococcus aureus* 76901 on artificial surfaces was also tested. Biofilms grown on HMWPE and cobalt chrome discs had 56% and 67% less biofilm formed respectively when NucB was present (p=0.014, p=0.019). In this strain NucB had no effect on biofilm grown on titanium surfaces (Figure 3-7).

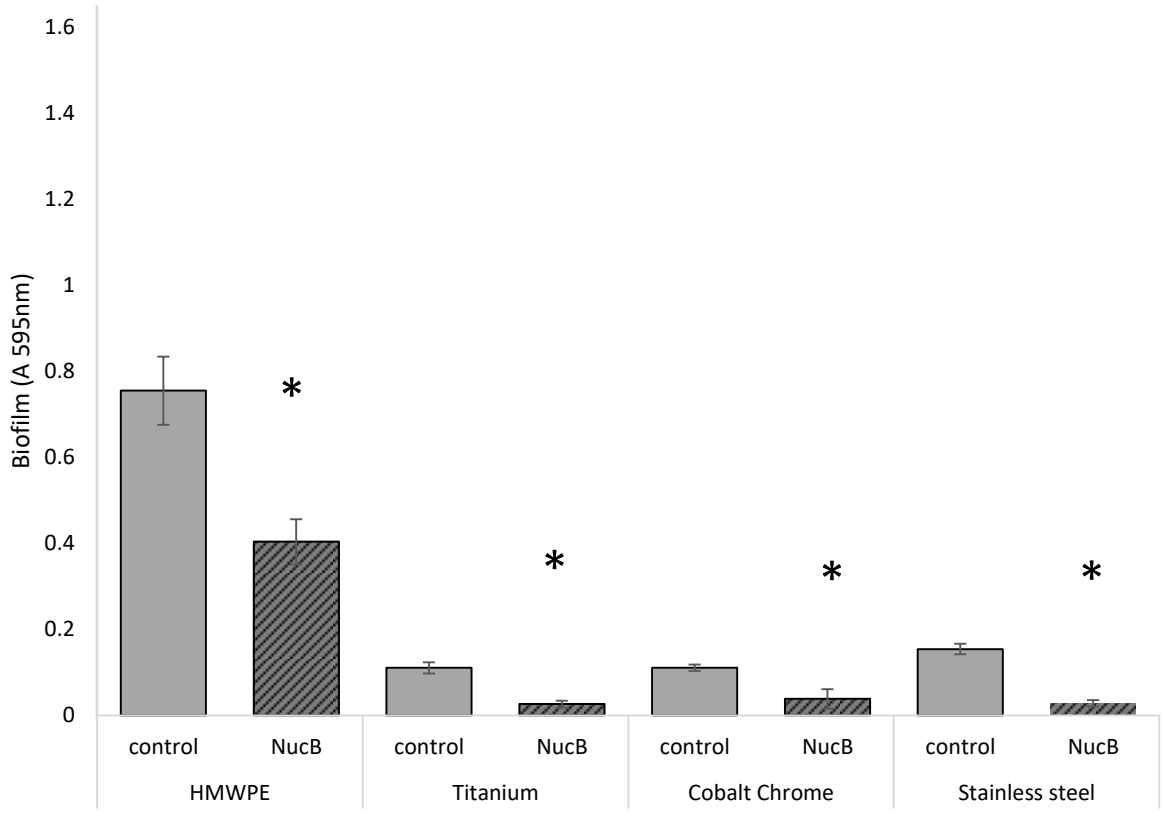


Figure 3-6 Effect of NucB on *Staphylococcus epidermidis 096R* biofilm formation. NucB (1μg/mL) was added at the time of inoculation. NucB significantly prevented biofilm formation on all four artificial surfaces: HMWPE (P<0.001), titanium (p<0.001), cobalt chrome (p=0.001) and stainless steel (p<0.001). Mean values and standard error bars are represented in the graph. *= Statistical significance Mann-Whitney U test p<0.05. Experiments were performed in triplicate, three independent times.

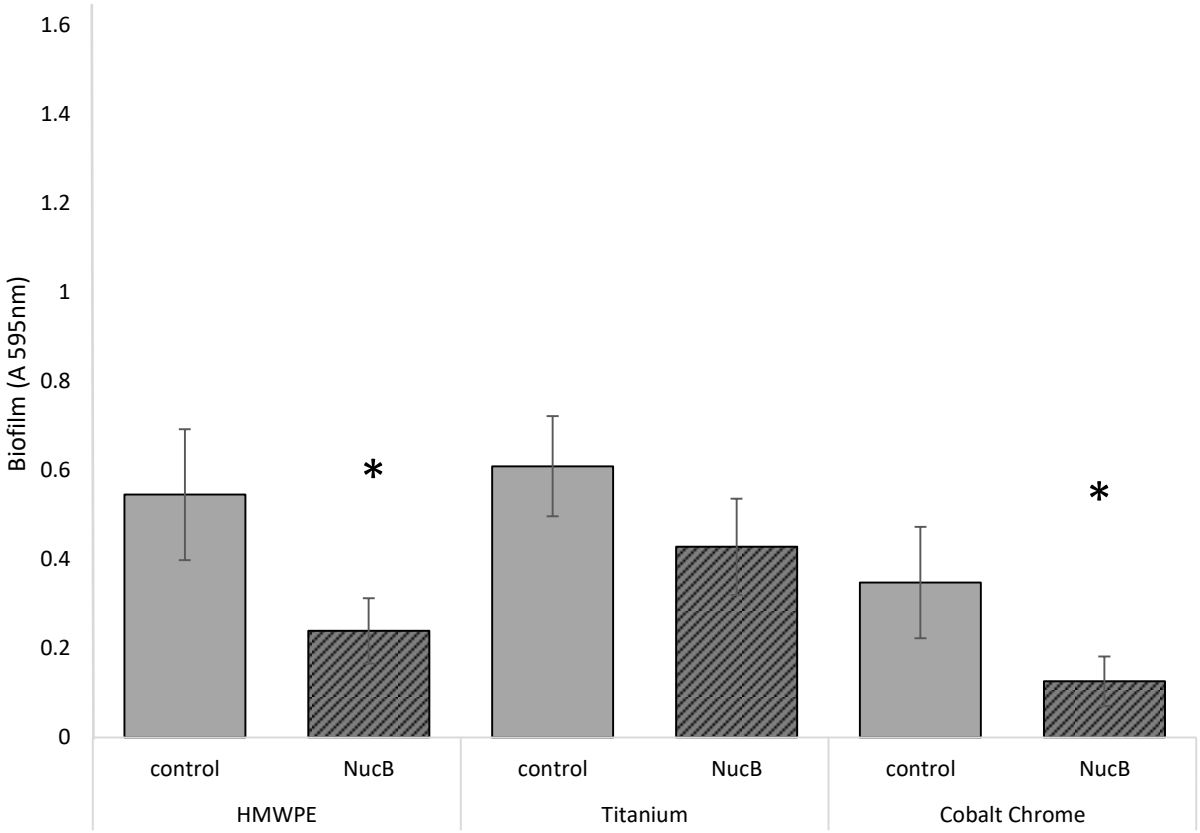


Figure 3-7 Effect of NucB on *Staphylococcus aureus* 76901 biofilm formation. NucB (1µg/mL) was added at the time of inoculation. NucB significantly prevented biofilm formation on HMWPE (p=0.014) and cobalt chrome (p=0.019) surfaces. NucB had no effect on biofilm formation on titanium surfaces (p=0.190) Mean values and standard error bars are represented in the graph. *= Statistical significance Mann-Whitney U test $p<0.05$. Experiments were performed in triplicate, three independent times.

When NucB was present at the time of inoculation, *Staphylococcus epidermidis* 76933 biofilms grown on HMWPE had 73% less biofilm formed than control samples ($p<0.001$). Similarly, biofilm grown on titanium had significantly less biofilm (80%) than those grown without NucB present (p=0.003). No significant effect was seen on biofilm formation of this strain on cobalt chrome surfaces (p=0.077).

While in the initial experiments NucB had no effect on biofilm formation of clinical strains SA76901 and SE76933 grown on polystyrene plates (Figure 3-2 and Figure 3-3), NucB significantly reduced the formation of biofilm of SA76901 on HMWPE and cobalt chrome surfaces and on HMWPE and titanium in strain SE76933 (Figure 3-7 and Figure

3-8). NucB did not inhibit formation of SA76901 biofilms grown on titanium surfaces or biofilms of SE76933 grown on cobalt chrome surfaces.

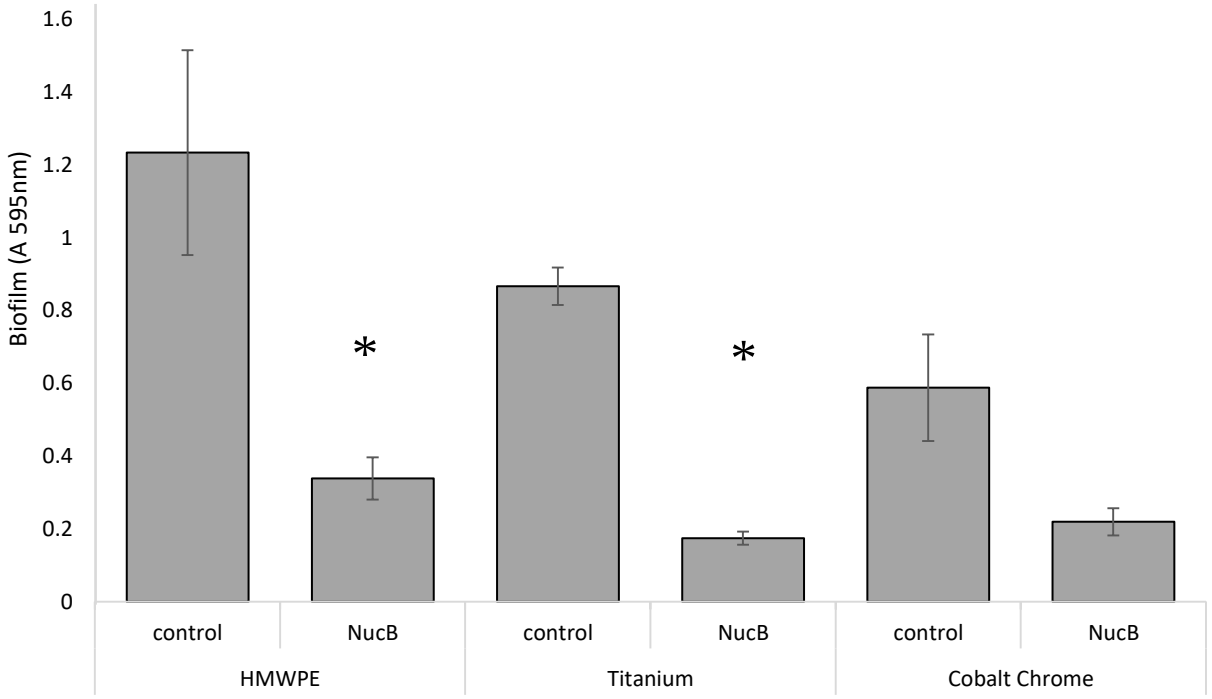


Figure 3-8 Effect of NucB on *Staphylococcus epidermidis* 76933 biofilm formation. Biofilms grown significantly less in HMWPE (p=<0.001) and titanium (p=0.003) surfaces when NucB was present at the time of inoculation compared to control samples where NucB was not present. No effect was seen on biofilms grown on cobalt chrome surfaces (p=0.077). Mean values and standard error bars are represented in the graph. *= Statistical significance Mann-Whitney U test p<0.05. Experiments were performed in triplicate, three independent times.

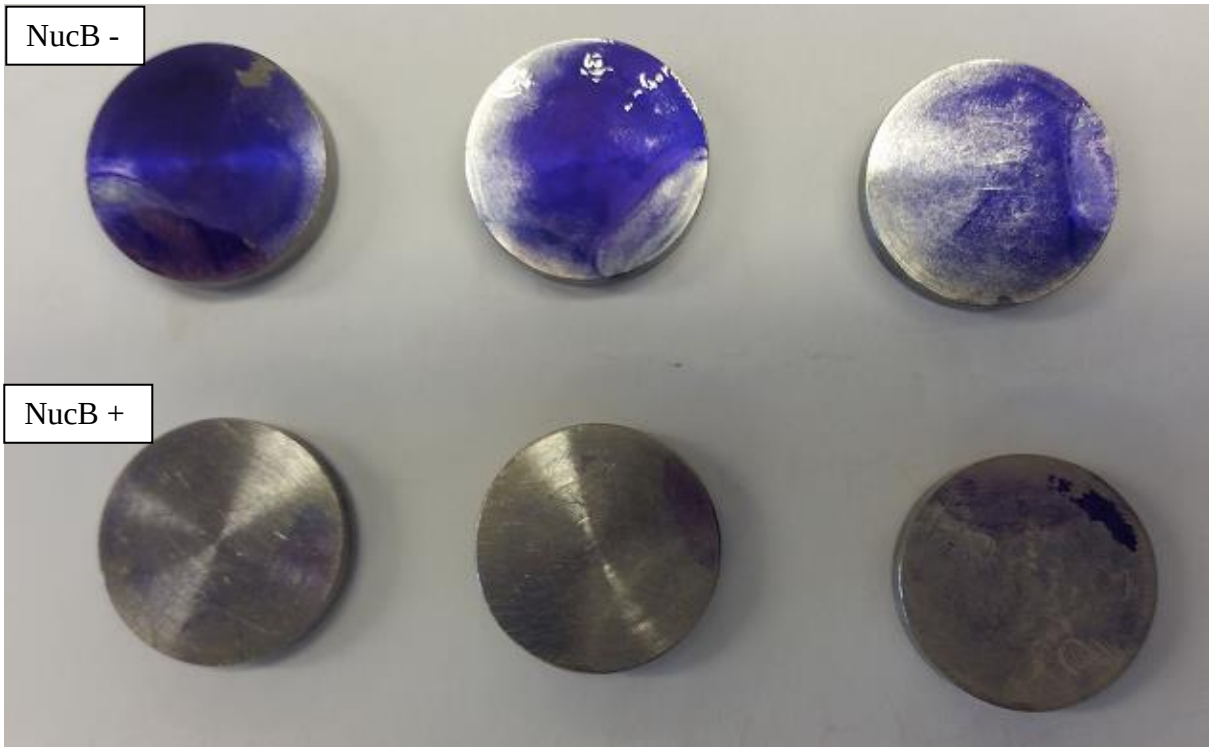


Figure 3-9 Effect of NucB on the prevention of formation of biofilms grown on titanium surfaces. Biofilms of SE 76933 were grown without NucB (NucB -) and with NucB (NucB +) added at inoculation time. Biofilms were grown for 24 hours and stained with 0.1% crystal violet.

3.3.4 Biomass visualization using CLSM

Experiments were performed solely by the author of this thesis. The author underwent training on the use of CLSM prior to performing experiments at the bio imaging unit at Newcastle University. The strain of choice for this experiment was *Staphylococcus aureus* 76901 as it formed typical levels of biofilm. CLSM was used to obtain images of biofilms of SA76901 grown in the presence and absence of NucB on titanium discs and visualized using CLSM. Biofilms grown with NucB appeared to show an overall reduced number of cells compared to the biofilm grown in the absence of NucB (Figure 3-10).

Biomass and biofilm thickness was measured using Comstat2 (Heydorn *et al.*, 2000; Vorregaard, 2008) base on three images. The average biomass of biofilm grown in the presence of NucB was 1.4$\mu m^3/\mu m^2$ which is considerably less than biofilm grown without

NucB present (average biomass=5.8µm^3/µm$^{2)}$. Biofilm thickness was also considerably reduced in the presence of NucB (21.4 µm vs 5.8µm).

Measurement of live/dead cells was performed using MATLAB R2017a.

Table 3-5 shows the amount of live and dead cells in each biofilm. There was a tree

	Control	NucB
Live (green) % cover	18.3	5.2
Dead (red) % cover	4.2	0.6

fold reduction in the number of live cells on the titanium discs when biofilms were grown in the presence of NucB compared to control but the number of dead cells remains similar. We did not expect the number of dead cells to vary between both samples as NucB has no bactericidal effect.

The experiment was performed with the intent of visualizing the efficacy of NucB on biofilm formation previously indicated by CV staining results. We are aware that more consistent experiments in which all strains and all surfaces are tested and image and measurements are obtained with CLSM are necessary to be able to draw stronger conclusions.

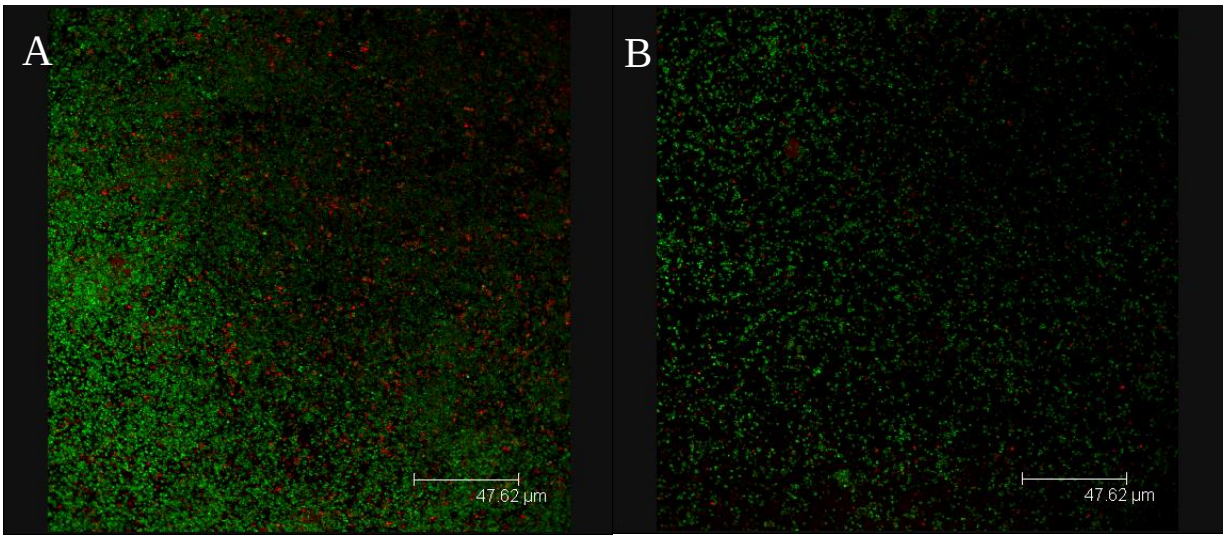


Figure 3-10 *Staphylococcus aureus* 76901 biofilms grow on titanium discs without NucB (A) and with addition of NucB at the time of inoculation (B). There is a higher number of bacterial cells present in biofilms grown in the absence of NucB (A) compare to those grown with NucB present (B)

Table 3-5 Percentage of live/dead cells of *Staphylococcus aureus* 76901 biofilm grown on titanium discs in the presence and absence of NucB.

	Control	NucB
Live (green) % cover	18.3	5.2
Dead (red) % cover	4.2	0.6

3.4 Discussion

DNAse I and recombinant human DNAse can reduce biofilm formation of several bacterial strains including clinical strains of *Staphylococcus aureus* from skin and *Staphylococcus epidermidis* from patients with implanted catheter infections (Eckhart *et al.*, 2007; Qin *et al.*, 2007; Kaplan *et al.*, 2012). In this work, eleven clinical strains of *Staphylococcus aureus* and eight clinical strains of *Staphylococcus epidermidis* were incubated in the presence and absence of 1μg/ml NucB for 24 hours. The results showed that NucB was capable of inhibiting biofilm formation on a 96 well plate with a maximum inhibition effect of 56% in *Staphylococcus aureus* and 55% in *Staphylococcus epidermidis.* Eckhart et al. demonstrated that DNaseI and recombinant human DNase1L2 could inhibit biofilm growth of a non-clinical strain of *Pseudomonas aeruginosa* and skin isolates of *Staphylococcus aureus* by 90% (Eckhart *et al.*, 2007). Although the enzymatic activity of DNAse1L2 appears to be more successful that NucB in reducing biofilm formation, Eckhart's experiments were carried using reference laboratory strains which are non-pathogenic and may have lost important pathophysiological characteristics, or be producing weaker biofilms (Fux *et al.*, 2005). The quantity of enzyme added at the time of inoculation was twenty times higher than NucB (20μg/ml vs 1μg/ml) and this may have an effect on the enzymatic efficacy observed. Reference strains and clinical strains behave differently and it has been suggested that genes could be lost after numerous sequential passages of planktonic sub-culturing and therefore their behaviour in-vitro differs from clinical strains (Fux *et al.*, 2005). The rate at which biofilm grows has been shown to differ between them. The difference in genetic material and behaviour in-vitro may explain the higher reduction in biofilm formation of DNAse1L-2 compared to the results obtained in this work by NucB (Ali *et al*., 2013).

DNase I was also effective in preventing *Staphylococcus epidermidis* biofilm formation from reference strains as well as strains isolated from patients with implanted catheter infections (Qin *et al.*, 2007). Qin's results also showed a varying effect between 75% and 95% in preventing biofilm formation, the amount of DNase I inoculated at the time of

incubation was significantly higher than the quantities of NucB used here, (2mg/mL vs 1µg/mL) (Qin *et al.*, 2007). The hypothesis that eDNA is essential for the formation of biofilms by a variety of staphylococcal strains is supported by a number of studies in the literature. Kaplan et al. showed that 4ng/ml of rhDNase inhibited biofilm formation of *Staphylococcus aureus* by 90% and 1µg/ml of rhDNase detached *Staphylococcus aureus* biofilms in 4 minutes (Kaplan *et al.*, 2012).

Similarly, efficacy of NUC1, an endonuclease produced by *Staphylococcus aureus* was assessed by Tang et al. and demonstrated to reduce biofilm formation of several non-clinical strains including a reference strain of *Pseudomonas aeruginosa* by 2.5 fold (Tang *et al.*, 2011). Previously, NucB was shown to have an effect on biofilm formation of organisms isolated from patients suffering chronic rhinosinusitis (Shields *et al.*, 2013), until now its effect was unknown for PJI associated strains. Shields et al. demonstrated the effectiveness of NucB at a concentration of 3µg/ml, while the enzymatic concentration used in this work was lower. This lower concentration of 1µg/ml was effective in reducing biofilm formation from clinical isolates of prosthetic joint infections. With NucB having the potential to be developed into a therapy for preventing PJI, identifying a lower enzymatic concentration that successfully reduces biofilm formation will reduce cost. One of the limitations of this study is that direct comparison between other nucleases and NucB was not carried out due to cost implications. Although direct comparison will always be difficult as enzyme purity will never be 100% (in the case of NucB the enzyme was present at >95% purity), further experiments directly comparing the effect of NucB and other DNases will be useful in future, to ascertain the comparative activities of other available nucleases.

Shields demonstrated that NucB was more effective against nuclease-producing strains (Shields *et al.*, 2013); the production of nucleases has been shown to have an effect on biofilm formation (Beenken *et al.*, 2012). Although the presence of NucB reduced biofilm formation in most of the strains, this effect was not seen in all 19 strains tested (Table 3-1). We did not assess the ability of these strains to produce nucleases and perhaps this may be a possible explanation for the lack of effect seen in certain strains. The nuclease production activity of bacterial strains has been assessed by Berends et al. using a nuclease assay (Berends *et al.*, 2010). Developing similar experiments, identifying if the tested strains produce nucleases and correlating the outcome of those experiments with the ability of NucB to reduce biofilm formation may provide answers as to why not all staphylococcal strains were susceptible to NucB. The composition of the extracellular matrix of biofilms also varies significantly between species and this includes the quantity of extracellular DNA present on

each biofilm (Qin *et al.*, 2007; Izano *et al.*, 2008; Tang *et al.*, 2013). The inconsistent effect of NucB on biofilm formation observed in this study was therefore expected, and could also be explained by the variable amount of extracellular DNA present in staphylococcal biofilms. This has been shown to have an effect on the ability of nucleases to prevent biofilm formation by such species (Sugimoto *et al.*, 2018a).

The RA of manufactured arthroplasty implants varies significantly depending on implant design. The Exeter Trauma Stem (ETS), one of the most common hip implants in the UK, is made of stainless steel has an average RA of 0.235µm (Petheram *et al.*, 2013). Cobalt Chrome femoral heads have been shown to have an RA of 0.012 µm and RQ of 0.016µm for non-implanted prosthesis but the RA significantly increases to an average of 0.380µm and RQ of 0.540µm in an implanted prosthesis due to wear (Eberhardt *et al.*, 2009). Cobalt Chrome stems vary significantly from <1µm to as high as 5 µm depending on the implant design and manufacturer (Verdonschot, 2005). The morphology of the implant also varies depending of the size of the implant as well as the weight of the individual (Battaglia *et al.*, 2014). Battaglia et al. demonstrated that larger UHMWPE tibia inserts have two-fold increased volumetric wear with respect to the smaller ones but undergo less morphological changes on a molecular scale (Battaglia *et al.*, 2014). The surface roughness and morphology of the implant is in constant change due friction, wear and load. Therefore, we did not deem it necessary to adjust the discs used in this work to a particular RA, although it was important to know the RA used.

Although some previous studies have been published which investigate biofilm formation on metal and polyethylene surfaces (Coraca-Huber *et al.*, 2012), this study is the first to investigate the use of a nuclease to prevent biofilm formation on clinically relevant surfaces. NucB was effective at reducing biofilm formation by *Staphylococcus epidermidis* 96R on all four surfaces tested (Figure 3-6). The significant effect in reduction on biofilm formation was only seen on two surfaces on the second *Staphylococcus epidermidis* strain SE76933: HMWPE and titanium (Figure 3-7). While NucB had an effect of the formation of *Staphylococcus aureus* 518F on cobalt chrome and stainless steel surfaces, no effect was seen on HMWPE and titanium discs (Figure 3-5). Interestingly NucB did have an effect on biofilms grown HMWPE on the other *Staphylococcus aureus* strain tested SA76901 (Figure 3-7) but no effect was seen in either of the staphylococcal strain biofilms grown on titanium surfaces. Despite NucB not having an effect on biofilm formation of SE76933 and SA76901 grown on polystyrene 96 well plate (Table 3-1); it was capable of reducing biofilm formation of those strains grown on different materials. Surfaces such as Titanium, cobalt chrome,

stainless steel and HMWPE have different properties (Koseki *et al.*, 2014) therefore the interactions between the bacteria, the enzyme and the surface will be different. Bacterial attachment to a surface is multifactorial. The material properties including surface roughness and surface charge as well as the bacterial cell properties have an essential role in the initial bacterial attachment and subsequent biofilm development (F.M. AlAbbas, 2012; Lorenzetti *et al.*, 2015). We observed that *Staphylococcus epidermidis* has higher adhesion to polymers than S*taphylococcus aureus* ($p=0.01$) which reinforces previous findings in the literature (Gristina, 1987; Barth *et al.*, 1989). Although it has been demonstrated that *Staphylococcus aureus* has a preference for metal surfaces we did not observe any difference in adherence to metal surfaces between species. Further experiments to study the particular relationship and interaction between biofilm, NucB and different materials will be necessary to clarify reasons for this variability. Nevertheless the observation of significant reductions in biofilm formation in the presence of NucB reiterates the important role of extracellular DNA in the initial phases of biofilm formation.

Titanium, cobalt chrome, HMWPE and stainless steel were the materials chosen for these experiments as they are common materials used in joint replacements, but they are not the only ones. Ceramic replacements, particularly ceramic on polyethylene, have been gaining popularity over the past few years (National Joint Registry, 2019). Also, a large proportion of joint replacement are implanted with PMMA cement. To be able to obtain a better understanding of the effect of NucB on PJI biofilms such surfaces should also be assessed in future.

We were able to visualise the effect of NucB on biofilm formation using CLSM. The results support the reduction observed with the crystal violet experiments. We observed a 3 fold reduction of biofilm formation of *Staphylococcus aureus* 76901 biofilms grown on titanium discs. Although CLSM microscopy with additional strains and materials would be useful, this result is promising.

It is likely that the reduction of biofilm formation is due to breaking down extracellular DNA that has been shown to play a role in the initial attachment phase of bacteria to surfaces (Das *et al.*, 2010; Harmsen *et al.*, 2010). It is unclear why NucB has had a variable effect in reducing biofilm depending on the strain and/or surface. Due to the complexity and the multifactorial nature of biofilm formation, further experiments will be required.

This chapter has demonstrated that NucB can be effective in reducing biofilm formation from some PJI associated strains of *Staphylococcus aureus* and *Staphylococcus*

epidermidis on four different clinically relevant surfaces. An enzyme that can reduce biofilm formation by ~50% may be clinically useful. In addition, it is possible that there are more subtle effects on other strains, for example degrading some biofilm matrix material might sensitise strains to other antibacterial approaches.

NucB appears to have the potential to be developed into a therapeutic agent for reducing biofilms associated with PJI. A recent study targeting different extracellular matrix components to prevent biofilm formation has concluded that treatment with multiple extracellular matrix-degrading enzymes, would be a promising approach to inhibiting biofilms (Sugimoto *et al.*, 2018a). The addition of NucB during the initial implantation either as a solution prior surgical closure or as a coating of the implant alone or in combination with other matrix degrading enzymes are two possible methods of delivery. When looking at a coating as a method of delivery for NucB, this could be applied to the fixation or articulating surfaces of the implant. Although applying NucB to the whole implant would be ideal, applying it to the articulating surfaces may alter the properties of such surfaces and affect the longevity of the implant. The articulating surfaces are also modular and therefore can often be exchanged during the DAIR procedure, so focusing on a coating of NucB to improve prevention of biofilm formation on fixed surfaces may the best way to start.

In-vitro experiments do not fully mimic the in-vivo environment, and therefore the results obtained may not be replicated in-vivo. Without in-vivo experiments it is difficult to understand the significance that the prevention of formation of biofilm by NucB observed in this work will have in the clinical setting. Results presented in this chapter lay the foundations for further development of this enzyme with in-vivo models of PJI.

Chapter 4. The effect of NucB on the dispersal of established biofilms

4.1 Introduction

The development of biofilms on the surface of artificial joints remains a challenge to the treatment of PJI (Antony and Farran, 2016). The antibiotic therapy used in the treatment of prosthetic joint infection is often guided by the causative organism and their antimicrobial sensitivities, tested against planktonic bacteria in a standardised model. In those cases where the causative organism is not identified, broad-spectrum antibiotics are used. Common antibiotics used in the treatment of prosthetic joint infection are gentamicin, vancomycin and teicoplanin amongst others. Gentamicin is an aminoglycoside antibiotic commonly used as a prophylactic antibiotic in joint replacement surgery (Popat *et al.*, 2007) and antibiotic loaded cement spacers used in the gold standard 2 stage revision surgery for the treatment of prosthetic joint infections (Chen and Parvizi, 2014). Especially active against Gram-negative bacteria, gentamicin is given intravenously or intramuscularly due to poor absorbance through the gastrointestinal tract (Germovsek *et al.*, 2016). It is only effective against aerobic bacteria. Its bactericidal effect is due to the interruption of protein synthesis by binding to the 30S subunit of the bacterial ribosome (Popat *et al.*, 2007; Germovsek *et al.*, 2016). Another common antibiotic used in the treatment of prosthetic joint infections, especially in patients with true penicillin allergy, is vancomycin (Tan *et al.*, 2016b). This antibiotic belongs to the glycopeptide family. It inhibits the synthesis of the bacterial wall by binding to disaccharide pentapeptides and preventing the synthesis of peptidoglycan in the bacterial cell wall (Bourguignon *et al.*, 2016). Teicoplanin is also a glycopeptide antibiotic used to prevent and treat serious Gram positive infections including MRSA (Somma *et al.*, 1984; Soriano *et al.*, 2006; Tornero *et al.*, 2015). It is obtained by fermentation of certain strains of *Actinoplanes teichomyceticus* (Boix-Montanes and Garcia-Arieta, 2015) and consists of a combination of five structurally related components with similar polarity (Somma *et al.*, 1984; Boix-Montanes and Garcia-Arieta, 2015). The main characteristic that distinguishes teicoplanin from other glycopeptide antibiotics is the occurrence of glucosamine as the basic sugar and the presence of aliphatic acid residues (Somma *et al.*, 1984). Teicoplanin works by inhibiting bacterial cell wall synthesis (Somma *et al.*, 1984). It has been shown to have successful outcomes in the treatment of prosthetic joint infections either alone or in combination therapy (Peeters *et al.*, 2016).

Standard antibiotic therapy is often not sufficient as the presence of biofilm increases resistance to most antibiotic treatments (Hoiby *et al.*, 2010; Molina-Manso *et al.*, 2013). The interaction of the antibiotics with certain components of the extracellular matrix such as eDNA may inactivate the antibiotic inside the biofilm matrix (Mulcahy *et al.*, 2008; Chiang *et al.*, 2013). Bacteria within a biofilm typically experience nutrient limitation and physiological changes. These factors have been suggested to also play a role in antibiotic resistance (Mah and O'Toole, 2001). As well as the lack of oxygen in the deeper layers of the biofilm that may affect antibiotic activity, particularly of aminoglycosides (Stewart and Costerton, 2001), it is likely that those cells embedded in a biofilm can reversibly enter a slow-growing phase (Jolivet-Gougeon and Bonnaure-Mallet, 2014). Some antibiotics act in actively growing bacteria like those that target cell wall synthesis or replication. Those cells with reduced metabolic activity therefore will be more resistant to antibiotic therapy (Lewis, 2012; Ciofu *et al.*, 2017). It has also been demonstrated that bacteria in biofilms can also activate stress response genes that promote antibiotic resistance (Jolivet-Gougeon and Bonnaure-Mallet, 2014) as well as uptake of resistance genes by horizontal gene transfer (Mah, 2012). It is apparent that the mechanism of antibiotic resistance in biofilms is multifactorial and complex and those mechanisms vary depending not only on the antimicrobial agent but also the particular biofilm and its growth conditions (Hall and Mah, 2017). Research is necessary to try to overcome the mechanism of antibiotic resistance in biofilms to be able to improve the treatment of biofilm related infections.

In an attempt to tackle the problem, research has been focused on developing new ways to disrupt pre-formed biofilms. The extracellular matrix of the biofilm promotes bacterial adherence to surfaces as well as cell-cell bond and aggregation (Flemming and Wingender, 2010). Targeting the extracellular matrix could be therefore an effective therapeutic approach against bacterial biofilms. The difficulty targeting the extracellular matrix lies in its variability and interaction against its components that add new challenges and complexity to the already intricate subject of biofilm dispersal (Hobley *et al.*, 2015; Peterson *et al.*, 2015). Since the discovery of eDNA, multiple enzymatic approaches to disperse pre-formed biofilms have been investigated. Some nucleases such as rhDNase, are already used to treat specific medical conditions. This enzyme is being used in combination with antibiotics to treat cystic fibrosis (Manzenreiter *et al.*, 2012). *Neisseria gonorrhoeae*, the pathogen causative of gonorrhea, produces a thermonuclease called Nuc that is capable to disperse their own biofilms by degrading DNA of the biofilm matrix (Steichen *et al.*, 2011). No successful treatment for infected arthroplasty has yet been identified, and these examples

of effective use of nucleases against biofilm related infection encourages research to continue exploring the possibility of developing an enzymatic approach to orthopaedic prosthetic joint infections.

Several nucleases have been tested and proven to be effective in disrupting pre-formed biofilms by releasing the bacteria back into their planktonic form (Kaplan *et al.*, 2004; Tang *et al.*, 2011). This change of the state of the bacteria could enhance antimicrobial susceptibility as enzymes such as recombinant human DNAse (Kaplan *et al.*, 2012). Donelly et al. demonstrated that Dispersin B, an enzyme that targets poly-*N*-acetyl-1,6-beta-glucosamine (PNAG) which mediates bacterial intercellular adhesion and is part of the extracellular matrix of the biofilm was capable of dispersing biofilms and enhancing antibiotic activity against staphylococcal biofilms (Donelli *et al.*, 2007). As well as releasing the bacteria into a planktonic form, the effect of nucleases in degrading the matrix potentially may allow the antibiotics to improve their penetration into deeper and difficult to reach layers of the biofilm. It has been demonstrated that NucB is capable or disperse biofilms from tracheoesophageal speech valves (Shakir *et al.*, 2012) and is capable of disrupting established biofilms with higher efficacy than bovine pancreatic DNase I (Nijland *et al.*, 2010). Here, the ability of NucB to disperse pre-formed biofilms from orthopaedically relevant surfaces was explored.

We hypothesize that the exposure of pre-established biofilms grown on prosthetic joint replacement materials to NucB will disrupt biofilms from prosthetic joint materials by breaking down extracellular DNA of the extracellular matrix releasing the bacteria into a planktonic form and therefore potentially improving access to antibiotics such as gentamicin, vancomycin and teicoplanin for the improved eradication of these infections.

The objectives of this chapter were therefore:

1. To assess the effect of NucB on the dispersal of pre-established biofilms of clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* grown on microtitre plates.
2. To assess the effect of NucB on the dispersal of pre-established biofilms of clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* grown on cobalt chrome, titanium, stainless steel and UHMWPE.
3. To assess the effect of NucB on the efficacy of the antibiotics gentamicin, vancomycin and teicoplanin

4.2 Materials and methods

4.2.1 Effect of NucB on biofilm dispersal. An in-vitro microtiter plate assay

Biofilms of all 19 clinical strains were grown in 96-well plates as previously described in section 2.3.2. To evaluate the efficacy of NucB, biofilms were washed with 200µl sterile isotonic phosphate buffered saline (10mM PBS, pH 7.4), 200µl NucB (1µg/ml in TSB, 5mM $MnSO_4 \cdot H_2O$) was added to each well, and incubated at 37°C for 1 hour. 200µl TSB with 5mM $MnSO_4 \cdot H_2O$ was added as control. Liquid medium with planktonic cells was removed from the wells. Plates were gently washed with sterile PBS. The remaining biofilms were stained with 200µl of 0.1% crystal violet (CV) as previously described in section 2.2.3.

4.2.2 Effect of NucB on dispersal of biofilms grown on metal and HMWPE surfaces

The same four clinical strains selected for previous experiments (section 2.3.3) were selected for this experiment: *Staphylococcus aureus* 76901, *Staphylococcus aureus* 518F, *Staphylococcus epidermidis* 76933 and *Staphylococcus epidermidis* 096R. Biofilms were grown as previously described 3.2.3. Discs were removed with sterile forceps with special care to not disrupt the biofilm and washed by immersion in sterile PBS solution. Discs were air dried on a rack to allow drying and fixation of the biofilm on both surfaces. Once dried, discs were transferred to a new sterile 50ml falcon tube. Five ml of NucB solution at 1µg/ml in 50mM Tris-HCl, 5mM $MnSO_4 \cdot H_2O$ (pH 8.0) was added as treatment. A solution without NucB was used as control (1ml of 50mM Tris-HCl, 5mM $MnSO_4 \cdot H_2O$ (pH 8.0). Falcon tubes were then incubated at 37°C statically for 1hour. Metal discs were removed with sterile forceps and washed thoroughly with sterile PBS and air dried. Dry discs were transferred to a sterile polystyrene 6-multiwell plate and stained with 5ml 0.1% crystal violet (CV) for 20 minutes. The excess dye was rinsed off by immersing the discs in PBS solution and then allowed to air-dry. Discs were transferred to a new sterile polystyrene 6-multiwell plate and the crystal violet dye was solubilised in 5ml of 33% acetic acid for 30 minutes. 200µl of the solution of each well was transferred to a 96 well polystyrene plate and absorbance at 595nm was read. Three independent experiments were done with each assay done in triplicate.

4.2.3 Biofilm imaging

To assess the effect of NucB on pre-established biofilms, biofilms were grown on HMWPE surfaces as previously described in section 3.2.4. Five ml of a solution made of

NucB at 1μg/ml in 50mM Tris-HCl, 5mM $MnSO_4 \cdot H_2O$ (pH 8.0) was added as treatment. A solution without NucB was used as the control (1ml of 50mM Tris-HCl, 5mM $MnSO_4 \cdot H_2O$ (pH 8.0). The plates were incubated at 37°C statically for 1 hour. The supernatant was aspirated, discarded and discs were washed by pipetting sterile PBS solution and air-dried. Once dry they were stained with Live/Dead® BacLight™ bacterial viability kit (Thermo Fisher Scientific)(Shen *et al.*, 2010). The excess dye was rinsed off by pipetting sterile water 3 times then allowed to air-dry. Discs were fully immersed in sterile water and the plate was covered with foil to prevent light penetration. Images were obtained using a Leica TCS SP2 UV AOBS MP point scanning confocal microscope with a 40x water dipping objective. SYTO® 9 excitation/emission was 480/500nm and propidium iodide was 490/635nm.

4.2.4 Effect of NucB on the minimal inhibitory concentration (MIC) of commonly used antibiotics

Gentamicin sulphate salt (Sigma-Aldrich, Dorset, UK), teicoplanin (Sigma-Aldrich, Dorset, UK) and vancomycin hydrochloride (Sigma-Aldrich, Dorset, UK) were used. MICs against *Staphylococcus aureus* 518F and *Staphylococcus epidermidis* 096R were determined by broth dilution as previously described (Wiegand *et al.*, 2008). Serial dilutions of antibiotics from 256 μg/ml to 0.5 μg/ml were prepared in TSB medium and 100μl of each dilution was inoculated sequentially into a 96-well polystyrene microtiter plate. Overnight cultures of each strain were diluted and 100μl was added using an Eppendorf BioPhotometer to each of the 96 well plate which were inoculated with antibiotic dilutions to obtain an optical density at 600nm (OD_{600}) of 0.1. Control samples had 50mM of Tris-HCL while intervention samples contained NucB (1μg/ml). Wells with TSB only, bacterial broth only and bacterial broth and NucB only with no antibiotics were added as controls. Wells with bacterial broth and NucB (1μg/ml) were also included in the experiment to confirm NucB alone had no bactericidal effect. The plates were then incubated statically for 24 hours at 37°C. The MICs were defined as per Wiegand et al. (Wiegand *et al.*, 2008).

4.2.5 Effect of NucB in combination with antibiotics against biofilms. An in-vitro microtiter plate assay.

Biofilms of each strain were grown in 96-well plates as previously described. To evaluate the combined effects of NucB and teicoplanin biofilms were measured using a metabolic dye (2, 3-Bis-(2-Methoxy-4-Nitro-5-Sulfophenyl)-2H-Tetrazolium-5-Carboxanilide, also known as XTT) (Pierce *et al.*, 2008). It was decided to use a metabolic

dye to quantify live cells within the biofilm and therefore assess the efficacy of antibiotic treatment. Previous experiments were performed using crystal violet dye that does not discriminate between live/dead bacteria and therefore this technique was deemed not appropriate for this experimental design.

These experiments were carried out on microtiter plate grown biofilms to reduce variability as biofilms could be grown and tested against different antibiotic doses simultaneously in the same microtiter plate. An assay was developed based on the methodology of Cerca et al. (Cerca *et al.*, 2005) and Claessens et al. (Claessens *et al.*, 2015). *Staphylococcus epidermidis* 096R and *Staphylococcus aureus* 518F were selected for these experiments. Of the four main strains that had been tested in this work, two strains that were more susceptible to NucB treatment were selected. Biofilms were grown in a 96-well plate as previously described. After 24 hours, biofilms were washed and different antibiotic concentrations were added (from 1024µg/ml to 2µg/ml) with or without NucB (1µg/ml) and incubated for a further 24 hours at 37°C. The incubation time in this particular experiment is longer than the 1hour in previous experiments in order to allow sufficient time for the antibiotic to penetrate and act on the living cells (Claessens *et al.*, 2015). The supernatant was discarded and cells were washed with PBS. An XTT solution (0.2mg/ml XTT, 0.02 mg/ml phenazine methosulphate (PMS); Sigma-Aldrich) was prepared in 1% TSB. Biofilms were incubated with the staining solution (75µl of solution in 150µL of 1% TSB for each well) for 3 hours at 37°C in the dark with a gentle oscillation. 100µl of all solutions were transferred into a new sterile microtiter plate and the absorbance of the supernatant was measured at 450nm using a plate reader. Each assay was performed twice with triplicate samples.

4.2.6 Statistical analysis

Statistical analysis was performed with SPSS (IMB SPSS Statistics-version 22) by using a non-parametric Mann-Whitney U test. A non-parametric test was used as the sample number is small and normal distribution cannot be assumed. To calculate significant differences between control and experimental samples, p values below 0.05 were considered statistically significant.

4.3 Results

4.3.1 Dispersal of pre-established biofilms by NucB

NucB was able to statistically disperse pre-formed biofilms in 64% (7/11) of the tested strains after one hour of treatment. The extent of the biofilm dispersed was between 21 and 61% of pre-formed biofilms from clinical strains of *Staphylococcus aureus* (Table 4-1). NucB dispersed 24 hour biofilms of strains SA559C by 47% ($p<0.001$) as well as SA722P, SA76901 and SA717T by 40%, 21% and 30% respectively ($p=0.011$, $p=0.031$, $p=0.011$) (Figure 4-1). The maximum dispersal effect was observed on strain SA518F where NucB was able to disperse 61% of the pre-formed biofilm ($p=0.004$), followed by 58 % dispersal of pre-formed biofilms of SA476A ($p=0.014$) (Figure 4-1). Finally, NucB had also a significant effect in dispersing 29% of 24 hour biofilms of SA466A after one hour of treatment ($p=0.014$) (Figure 4-1). NucB had no statistically significant ability to disperse biofilms from strains SA171H, SA649D, SA378S and SA171H (Figure 4-1).

Similar experiments were performed using eight clinical strains of *Staphylococcus epidermidis.* NucB significantly dispersed biofilms formed by four of the eight clinical strains. NucB was able to disperse between 21 and 50% of pre formed biofilms after 1 hour of treatment (Table 4-1). The maximum dispersal effect was 50% and it was observed on strain SE248X ($p<0.001$), followed by 31% of SE76933. NucB was also capable of significantly dispersing SE286G biofilms by 24% ($p=0.011$) and SE 033G biofilm by 21% ($p=0.006$) (Figure 4-2). NucB had no significant effect on dispersing 24 hour biofilms of strains SE684X, SE150T, SE096R and SE 414W (Figure 4-2).

Table 4-1 Effect of NucB on biofilm dispersal of clinical strains. Absorbance at 595nm.

Strain	Biomass NucB-	Biomass NucB+	Biofilm dispersal	p value
SA559C	1.113	0.595	47%	<0.001 *
SA722P	0.752	0.445	40%	0.011*
SA76901	1.077	0.847	21%	0.031*
SA717T	0.577	0.399	30%	0.011*
SA089G	0.843	0.601	29%	0.024*
SA476A	1.134	0.473	58%	0.014*
SA171F	0.968	0.800	17%	0.190
SA518F	1.371	0.533	61%	0.004*
SA649D	1.828	1.960	+2.2%	1.00
SA378S	1.133	0.722	36%	0.161
SA107H	0.702	0.481	31%	0.063
SE286G	1.390	1.061	24%	0.011*
SE76933	0.433	0.299	31%	0.031*
SE033G	2.893	2.273	21%	0.006*
SE684X	2.776	2.761	0.5%	0.605
SE150T	2.429	2.221	8.6%	0.190
SE096R	0.717	0.464	35%	0.077
SE248X	0.613	0.307	50%	<0.001*
SE414W	0.492	0.477	3.1%	0.931

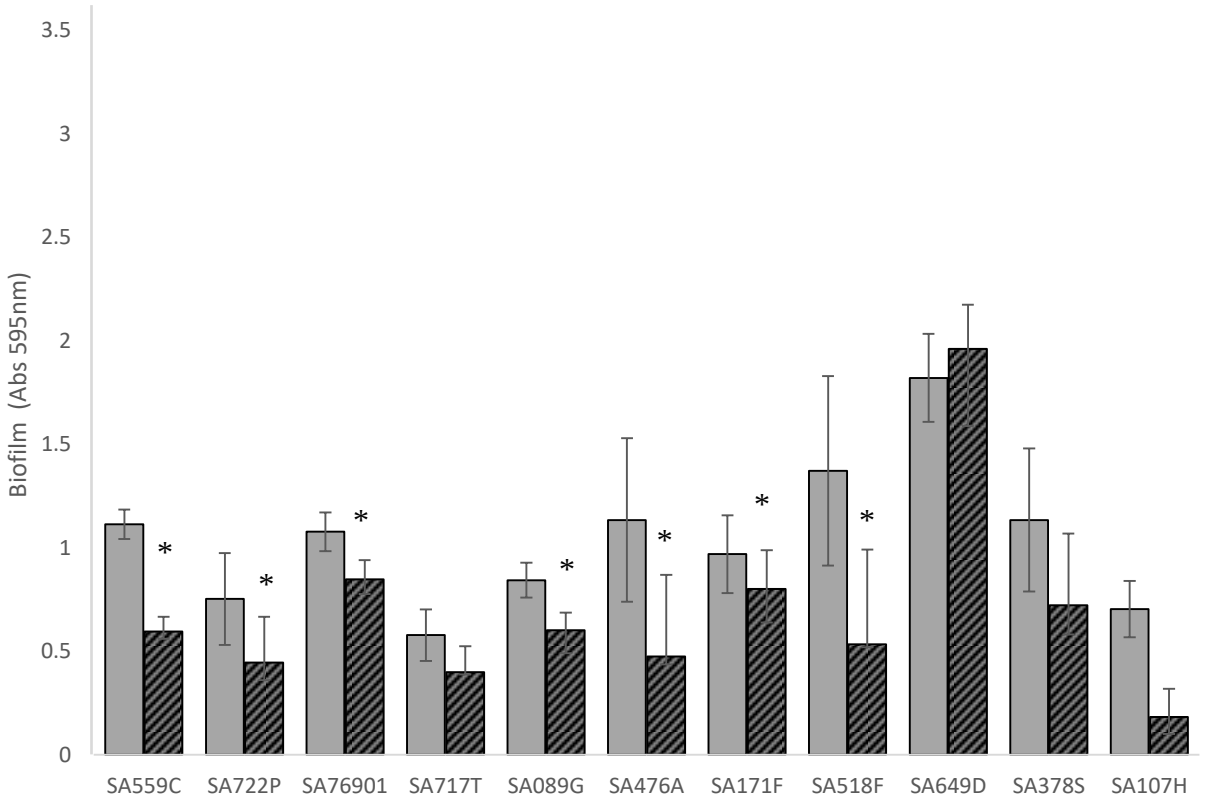


Figure 4-1 The effect of NucB on dispersal of pre-formed biofilms of clinical strains of *Staphylococcus aureus* grown in a 96 well plate. NucB was capable of significantly dispersing the 24 hour biofilm of SA559C by 47%, SA722P by 40%, SA76901 by 21% and SA717T by 30%. SA089G by 29%, SA476A by 58% and SA518F by 61% after one hour of treatment. NucB had no significant effect dispersing biofilms of strains SA 171F (17%, p=0.190), SA649D (+2.2%, p=1), SA376S (36%, p=0.161) and SA107H (31%, p=0.063) after one hour of treatment. Mean and standard deviation are represented in the graphs. *= Statistical significance Mann-Whitney U test $p<0.05$. Experiments performed in triplicates, three independent times.

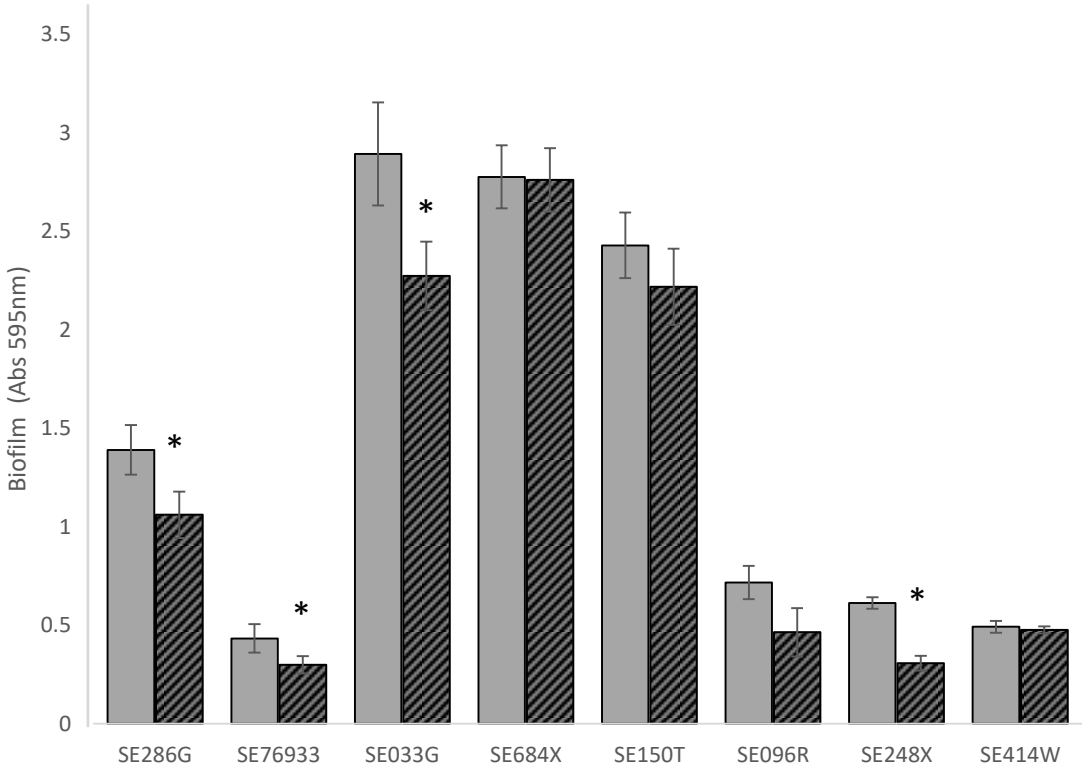


Figure 4-2 NucB effect on dispersal of pre-formed biofilms of clinical strains of *Staphylococcus epidermidis* grown in a 96 well plate. Nuc B was capable of significantly disperse 24 hour biofilms of strains SE286G by 24% (p=0.011), SE76933 by 31% (p=0.031), SE033G by 21% (p=0.006) and SE248X by 50% (p<0.001). NucB had no significant effect dispersing biofilms of strains S684XF (0.5%, p=0.605), SE150T (8.6%, p=0.190), SE096R (35%, p=0.077) and SE414W (3.1%, p=0.931) after one hour of treatment Mean and standard deviation are represented in the graphs. *= Statistical significance Mann-Whitney U test p<0.05. Experiments performed in triplicates, three independent times.

4.3.2 Quantification of the effect of NucB on biofilm dispersal of clinical isolates grown on clinically relevant surfaces

For consistency, the same four clinical strains used in previous experiments (section 2.2.4 and 3.2.3) were used to assess the effect of NucB on biofilm dispersal on metal and HMWPE surfaces. NucB had a statistically significant dispersal effect on all four strains in all surfaces tested. NucB was capable of dispersing a variable amount of biofilms grown on metal and polyethylene surfaces, between 39 and 92% of these four clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* (Table 4-2).

NucB dispersed 76% of 24 hour biofilm on SA518F grown on HMWPE ($p<0.001$), similar dispersal effect was seen on biofilms grown on stainless steel (76%, $p=0.002$) while the dispersal effect observed on titanium and cobalt chrome was 63 and 66% respectively ($p=0.002$, $p<0.001$) (Figure 4-3).

Biofilms of *Staphylococcus epidermidis* 096R were also effectively dispersed by NucB in all four surfaces tested. Biofilms grown on HMWP show a reduction of biomass of 74% ($p<0.001$). A more moderate reduction but still significant was observed on titanium surfaces with a 39% dispersal effect ($p=0.002$). The highest dispersal effect was observed on chrome surfaces were biofilms were dispersed by 92% ($p<0.001$) while those grown on stainless steel surfaces had a biofilm dispersal of 79% ($p<0.001$) (Figure 4-4).

Staphylococcus aureus SA76901 was also susceptible to NucB treatment. This effect could be observed by the naked eye (Figure 4-5) Biomass of pre-formed biofilms of SA76901 were significantly reduced on all surfaces following NucB treatment compared with controls by 76% on HMWPE discs ($p<0.001$)**,** 75% on titanium surfaces ($p<0.001$) and 79% on cobalt chrome surfaces ($p<0.001$) (Figure 4-6).

NucB was able to disperse 24 hour biofilms *Staphylococcus epidermidis* strain SE76933 biofilms grown on all three surfaces. The percentage of biomass dispersed after 1 hour treatment varied between depending on the surface: 54% of the biofilms was dispersed from HMWPE surfaces ($p=0.001$), 53% from titanium surfaces ($p=0.031$) and 71% of the biofilm was dispersed from cobalt chrome surfaces ($p<0.001$) (Figure 4-7).

Interestingly, biofilms grown on a 96-well polystyrene plates as well as those grown on clinically relevant orthopaedic surfaces from strains SA 518F, SA76901 and SE76933 were significantly dispersed by NucB after one hour treatment (Table 4-1, Table 4-2) but biofilms from SE096R did not behave in the same way. NucB significantly reduced the biomass of biofilms from SE096R grown on artificial surfaces from prosthetic joints (Table 4-2) but no statistically significant dispersal effect was seen in the polystyrene surfaces of the 96-well plate (Table 4-1). It is unclear why NucB appears to have a variable effect on biofilm dispersal on biofilms grown on different surfaces. Based on our limited observation of four strains, NucB appears to be more effective at dispersing biofilms grown on metal and polyethylene than on 96-well plate polystyrene surfaces. Further experiments on the effect of NucB on pre-formed biofilms on artificial joint replacement surfaces from the remaining clinical strains tested on a 96-well plate are being planned for future studies, to further assess this observation.

Table 4-2 Effect of NucB on pre-formed biofilms of *Staphylococcus aureus* and *Staphylococcus epidermidis* grown on metal and HMWPE surfaces. Biomass quantified by absorbance measurement at 595nm. *=statistical difference. Experiments performed in triplicates, three independent times.

Strain	Surface	Biomass NucB-	Biomass NucB+	P value
SA518F	HMWPE	1.311	0.313	<0.001*
	Titanium	0.114	0.042	0.002*
	Cobalt Chrome	0.265	0.089	<0.001*
	Stainless Steel	0.229	0.054	0.002*
SE096R	HMWPE	0.441	0.118	<0.001*
	Titanium	0.212	0.130	0.002*
	Cobalt Chrome	0.261	0.020	<0.001*
	Stainless Steel	0.138	0.029	0.001*
SA76901	HMWPE	0.984	0.240	<0.001*
	Titanium	0.197	0.533	<0.001*
	Cobalt Chrome	1.892	0.399	<0.001*
SE76933	HMWPE	0.990	0.456	0.001*
	Titanium	0.597	0.279	0.031*
	Cobalt Chrome	0.465	0.135	<0.001*

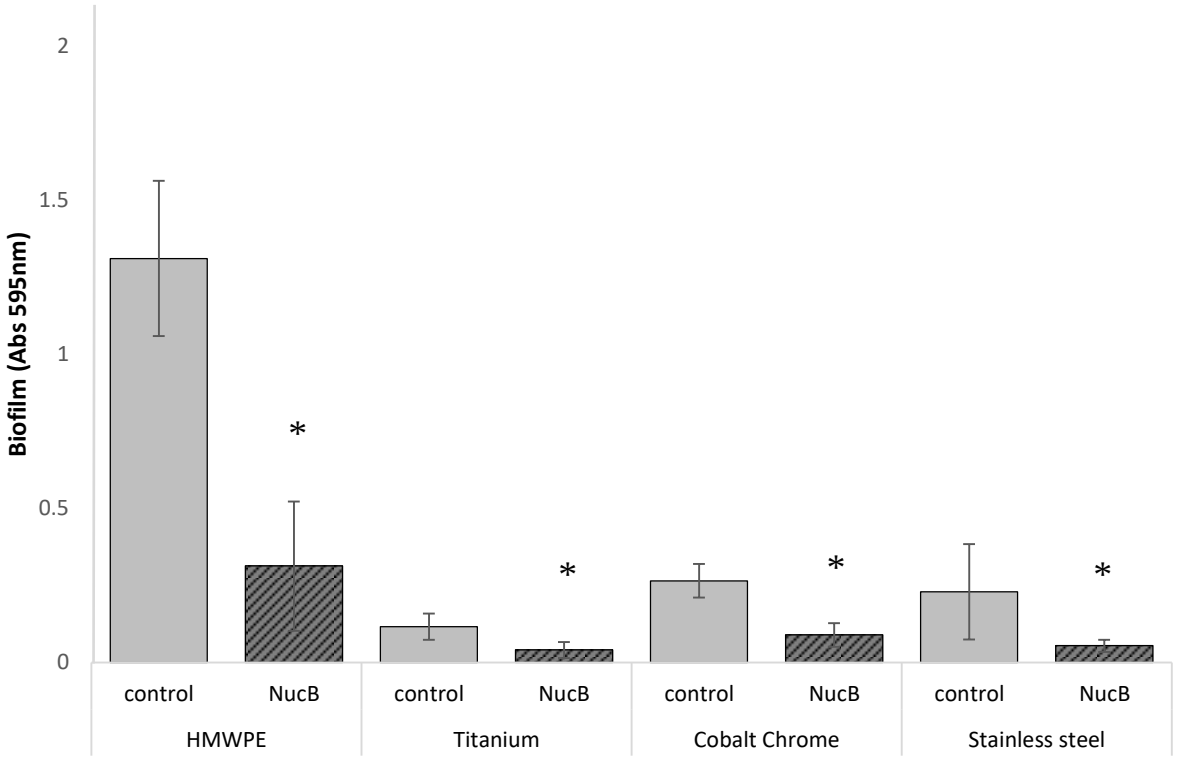


Figure 4-3 Effect of NucB on *Staphylococcus aureus* 518F biofilm dispersal. NucB significantly dispersed biofilms on all four surfaces tested. We observed 76% biofilm dispersal on HMWPE surfaces ($p<0.001$), 63% biofilm dispersal on titanium discs ($p=0.002$), 66% on cobalt chrome surfaces ($p<0.001$) and 76% on stainless steel surfaces ($p=0.002$). Mean values and standard error bars are represented in the graph. *= Statistical significance Mann-Whitney U test $p<0.05$. Experiments performed in triplicates, three independent times.

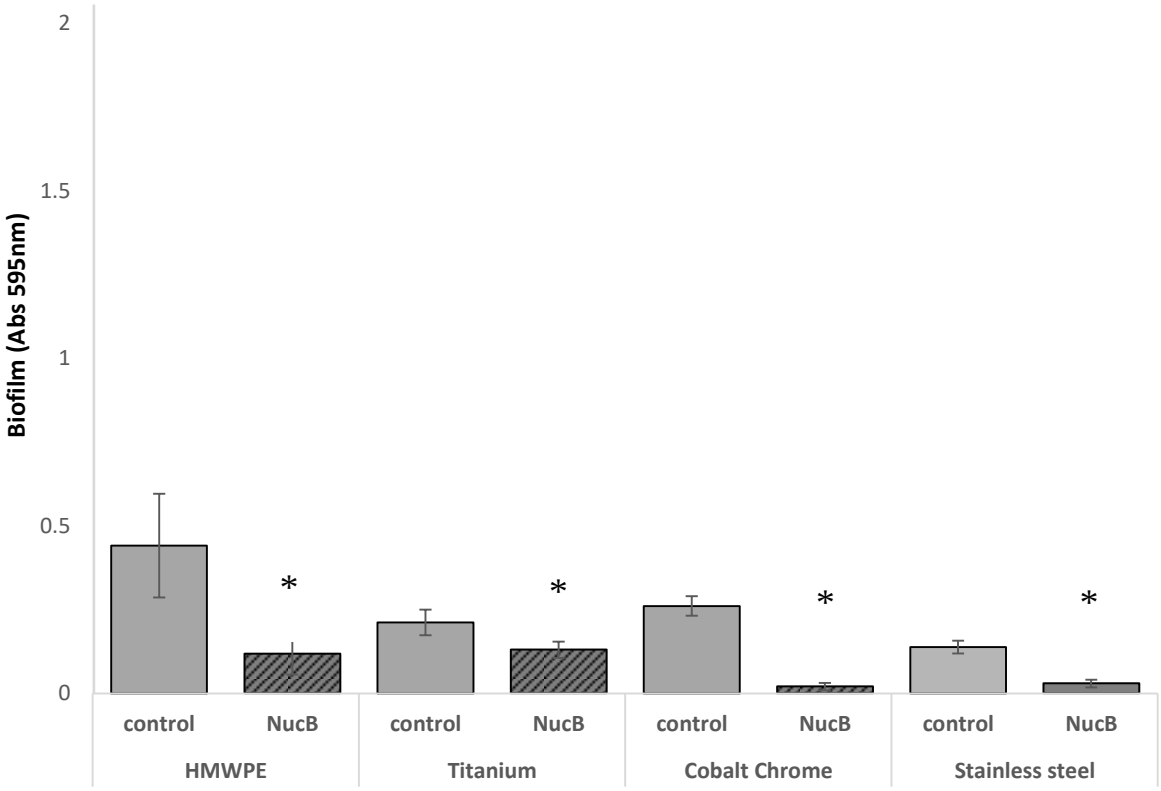


Figure 4-4 Effect of NucB on *Staphylococcus epidermidis* 096R biofilm dispersal. NucB significantly dispersed biofilms on all four surfaces tested. We observed 73% biofilm dispersal on HMWPE surfaces ($p<0.001$), 39% biofilm dispersal on titanium discs ($p=0.002$) and 92% on cobalt chrome surfaces ($p<0.001$) and 79% on stainless steel surfaces ($p=0.001$). Mean values and standard error bars are represented in the graph. * = Statistical significance Mann-Whitney U test $p<0.05$. Experiments performed in triplicates, three independent times.

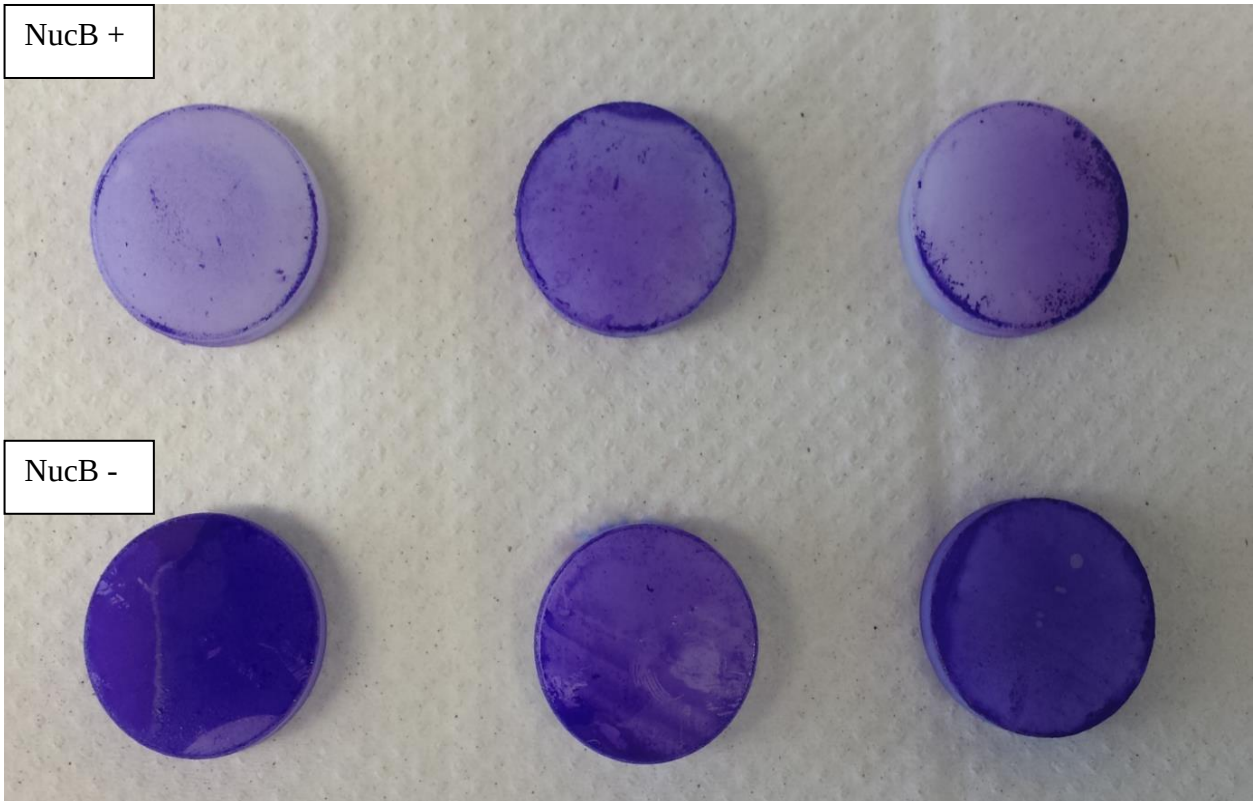


Figure 4-5 SA76901 biofilms grown on HMWPE discs of 20mm diameter. Biofilms have been grown on HMWPE discs for 24 hours and treated with and without NucB for 1hour. The remaining biofilm has been stained with 0.1% crystal violet and air dried. To the naked eye there is significantly less biofilm on discs treated with NucB (NucB+) compared to those treated with buffer Tris-HCL (NucB-).

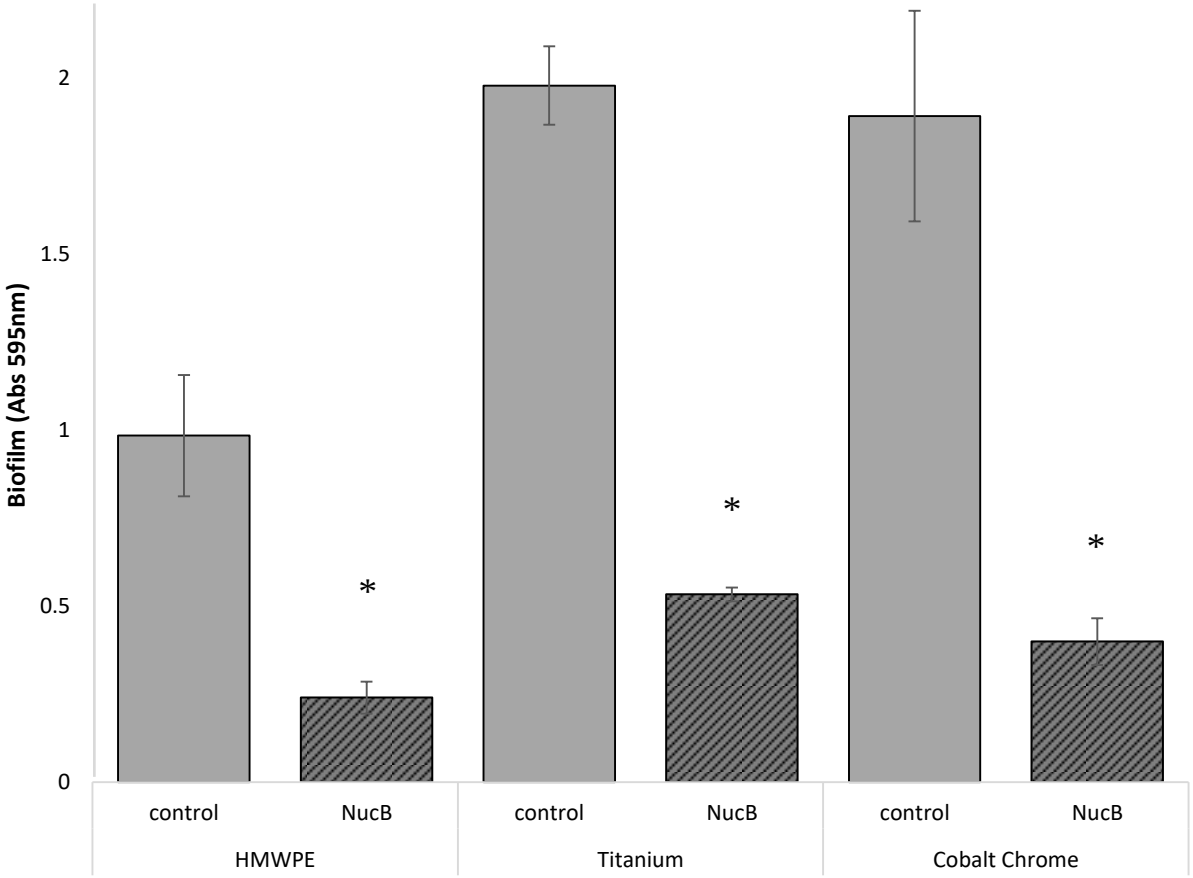


Figure 4-6 Effect of NucB on *Staphylococcus aureus 76901* biofilm dispersal. NucB significantly dispersed biofilms on all three surfaces tested. We observed 76% biofilm dispersal on HMWPE surfaces ($p<0.001$), 75% biofilm dispersal on titanium discs ($p<0.001$) and 79% on cobalt chrome surfaces ($p<0.001$). Mean values and standard error bars are represented in the graph.*****= Statistical significance Mann-Whitney U test $p<0.05$. Experiments performed in triplicates, three independent times.

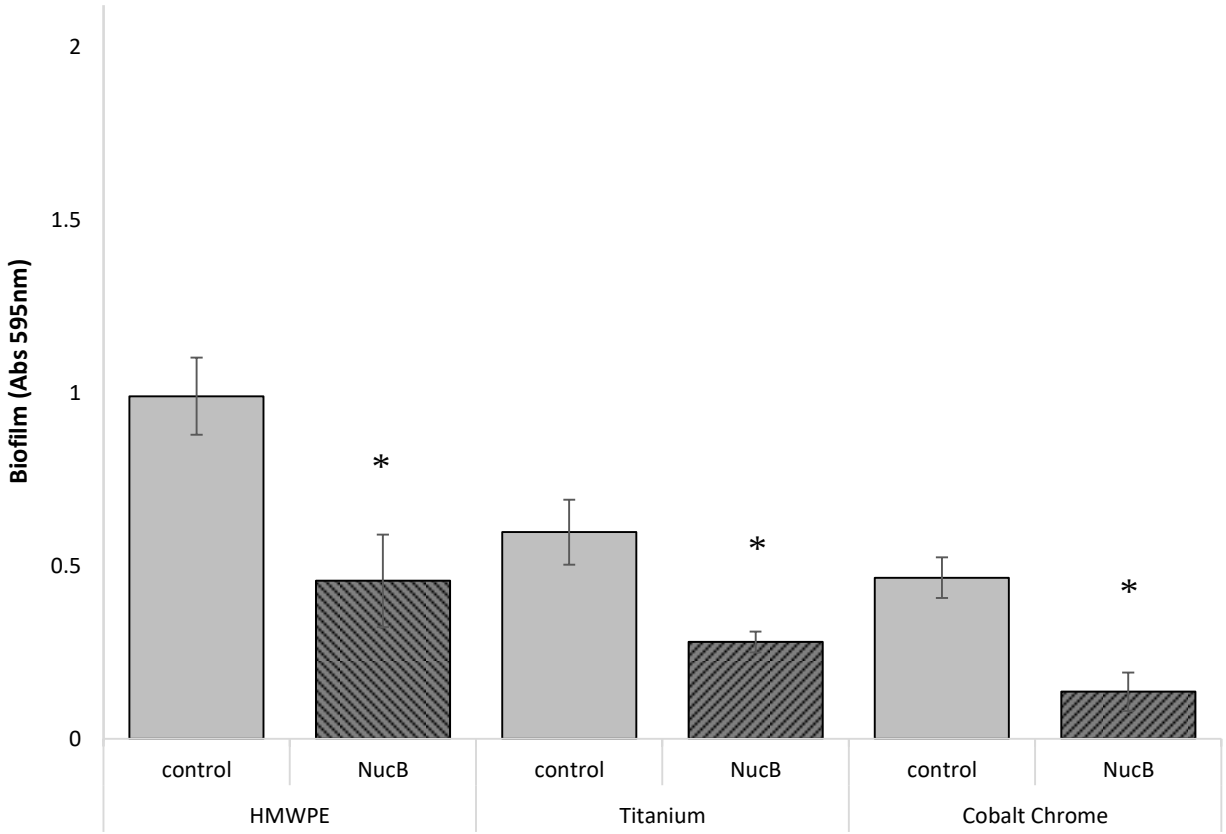


Figure 4-7 Effect of NucB on *Staphylococcus epidermidis* 76933 biofilm dispersal. NucB significantly dispersed biofilms on all three surfaces tested. We observed 54% biofilm dispersal on HMWPE surfaces ($p=0.001$), 53% biofilm dispersal on titanium discs ($p=0.031$) and 71% on cobalt chrome surfaces ($p<0.001$). Mean values and standard error bars are represented in the graph. * = Statistical significance Mann-Whitney U test $p<0.05$. Experiments performed in triplicates, three independent times.

4.3.3 Visualization of biofilm dispersal from surfaces using CLSM.

To visualise the effect of NucB on biofilm dispersal, *Staphylococcus aureus* 76901 was chosen as a typical strain. Biofilms of *Staphylococcus aureus* 76901 were grown on HMWPE and treated with or without NucB. To the naked eye there was a clear reduction in biomass of biofilms that had been treated with NucB compared to control. Biofilms were also stained with BacLight Live/Dead stain and visualised by confocal laser scanning microscopy (Figure 4-8). Biomass and biofilm thickness were measured using Comstat2 , measuring 3 fields (Heydorn *et al.*, 2000; Vorregaard, 2008) *Staphylococcus aureus* 76901 biofilm after 1 hour treatment with NucB had a remaining biomass of 0.16$\mu m^3/\mu m^2$ which was considerably smaller than the control (biomass=0.73$\mu m^3/\mu m^{2)}$. Biofilm thickness was also considerably reduced in the NucB treated biofilms compared to control (3.3 µm vs 0.08µm). Due to the limited data obtained due to time constraints, it was not possible to perform statistical analysis. However, the reduction in biomass and biofilm thickness observed with CLSM confirms the results seen with previous experiments using crystal violet staining where the reduction in biomass after 1 hour of NucB treatment was significant (Table 4-2).

Measurement of live/dead cells present in the biofilm after treatment was performed using MATLAB R2017a. The percentage of life cells present in the biofilm treated with NucB was 33 fold less than the control biofilm (16.81% vs 0.497 %) (Table 4-3). The number of dead cells was similar in both biofilms as expected since NucB has no killing effect (Table 4-3).

There was a 97% reduction in live cells when biofilms were treated with NucB which supports the results seen with the crystal violet experiments were a 75% reduction of biomass was observed in biofilms treated with NucB.

The aim of this experiment was to be able to visualise the effect of NucB that had been previously demonstrated in previous experiments. Further experiments including all surfaces tested in previous experiments with the remaining clinical strains are being undertaken. Due to constraints on both time and funding, such experiments and results could not be included in this work but are the subject of future studies.

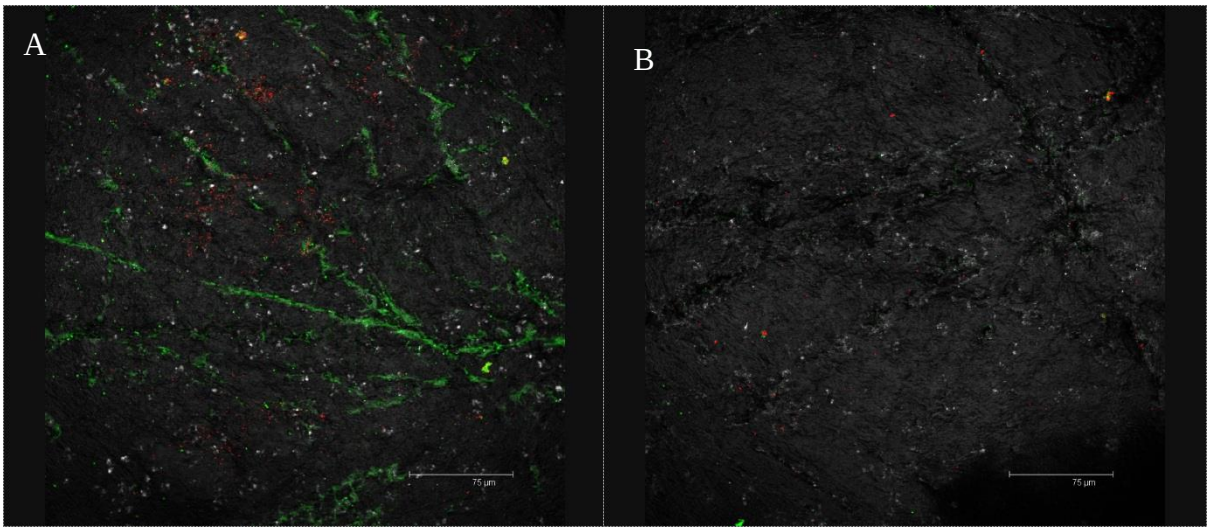


Figure 4-8 SA76901 biofilms grown on HMWPE surfaces. (A) Control biofilms treated with Tris-HCl buffer. Live cells are stained in green, dead cells are stained in red colour. (B) Remaining biofilms after one hour treatment with NucB. Scale bar is 75µm.

Table 4-3 Percentage of live/dead cells of a 24 hour *Staphylococcus aureus* 76901 biofilm grown on HMWPE treated with and without NucB for one hour.

	Control	NucB
Live (green) % cover	16.8	0.5
Dead (red) % cover	0.2	0.1

4.3.1 Effect of NucB on planktonic Staphylococcus aureus in the presence of antibiotics

Once it was established that NucB was effective in preventing and dispersing biofilms the combined effect of NucB with antibiotics was assessed. Due to time constrains and funding it was not possible to test all four strains previously used. All four strains had behaved similarly and therefore one clinical strain from each species was randomly selected. The strains of choice were *Staphylococcus aureus* 518F and *Staphylococcus epidermidis* 096R. It had previously been observed that the four tested strains behaved similarly in the presence of NucB with NucB having a significant ability to disperse biofilm of all four clinical strains tested. Therefore we randomly selected two of the four strains for further study here. It was hypothesised that NucB would be able to facilitate and enhance antibiotic activity against bacterial cells by two possible mechanism: breaking down the eDNA that held bacterial cells in clusters (Das *et al.*, 2013) and/or by breaking down the binding of eDNA to antibiotics which limits its activity (Doroshenko *et al.*, 2014) . The antibiotics of

choice for this particular experiment were teicoplanin, gentamicin and vancomycin as they are commonly used in the treatment of PJI. The MIC of these two clinical isolates of *Staphylococcus aureus* and *Staphylococcus epidermidis* were determined as previously described. *Staphylococcus aureus* 518F planktonic cells were tested against vancomycin, gentamicin and teicoplanin with and without the addition of NucB. When NucB was added in combination with teicoplanin or vancomycin a two-fold reduction of the MIC was observed (Table 4-4). NucB alone did not show any killing effect confirming that the reduction of cells was due to the antibiotic effect only as there was no difference in bacterial growth between the control wells when NucB was present or absent. *Staphylococcus epidermidis* 096R was also tested against antibiotics in the presence and absence of NucB. Similar results to those observed in *Staphylococcus aureus* 518F were observed. There was a two-fold reduction of the MIC of teicoplanin and vancomycin when NucB was added compared to those samples without NucB (Table 4-4). NucB had no effect in reducing the gentamicin MIC of both tested strains.

Table 4-4. Effect of NucB on MIC *of Staphylococcus aureus* 518F and *Staphylococcus epidermidis* 096R.

Strain	**NucB**	**Teicoplanin**	**Vancomycin**	**Gentamycin**
Staphylococcus aureus 518F	NucB -	4 µg/ml	8µg/ml	4µg/ml
	NucB+	2µg/ml	4µg/ml	4µg/ml
Staphylococcus epidermidis 096R	NucB -	128µg/ml	8µg/ml	32µg/ml
	NucB+	64µg/ml	4µg/ml	32µg/ml

4.3.2 In-vitro analysis of the combined effect of NucB and antibiotics for the treatment of biofilm related infection

Once it was investigated that NucB had the potential to increase antibiotic effect against planktonic cells, it was decided to assess the effect against bacterial cells within a biofilm. For consistency, the same two clinical strains were used in this experiment: *Staphylococcus epidermidis* 096R and *Staphylococcus aureus* 518F. Biofilms were treated with a variety of teicoplanin doses as previously described. Teicoplanin was chosen as the antibiotic of choice for this experiment as it is one of the treatment options of prosthetic join infection and it has been proven to be successful in monotherapy (Maiello *et al.*, 2005; Peeters *et al.*, 2016). The effect of antibiotic on the pre-existing biofilms was measured using XTT staining. Visual examination of biofilms stained with XTT showed a clear reduction in metabolic activity in combinations of NucB with teicoplanin compared with teicoplanin alone (Figure 4-9). These effects were quantified by spectrophotometry. In both strains, the addition of NucB increased the efficacy of action of teicoplanin against cells of biofilms of clinical isolates of both *Staphylococcus aureus* and *Staphylococcus epidermidis*. Biofilms of *Staphylococcus epidermidis* 096R treated with teicoplanin and the addition of NucB showed a reduction of viable cells when treated with doses of 8µg/ml and above compared to those only treated with the antibiotic only (Figure 4-10). Similar results were observed on biofilms of *Staphylococcus aureus* 518F treated with teicoplanin and with or without NucB. Biofilms treated with the addition of the enzyme showed a reduction of viable cells within the biofilm when treated with doses of 16µg/ml and above compared to those only treated with antibiotic only (Figure 4-11).

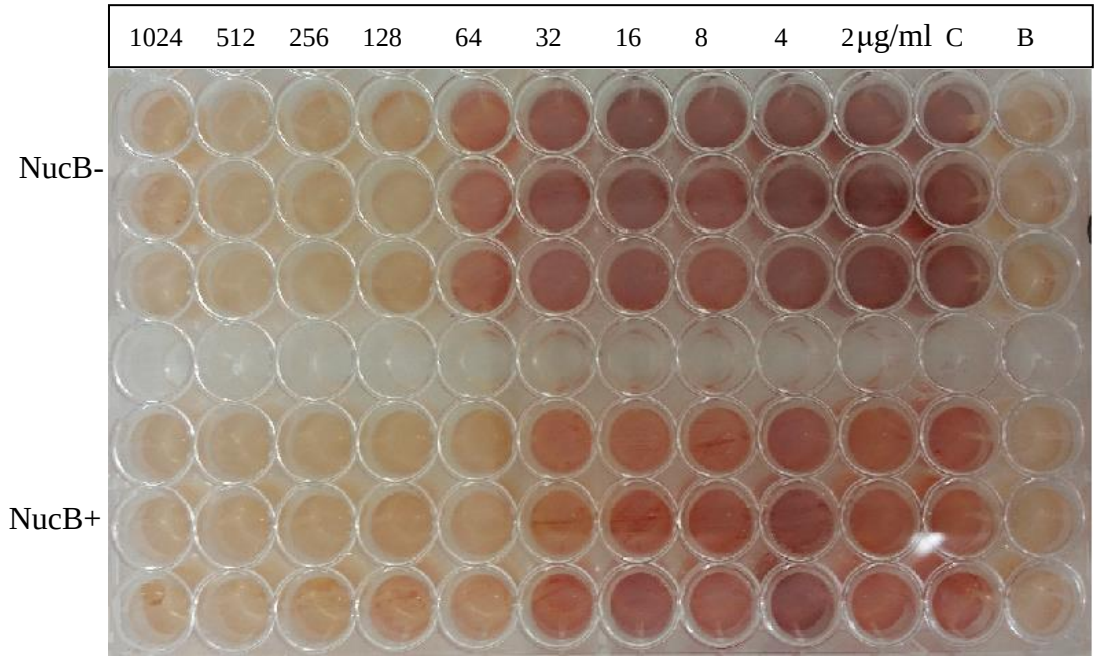


Figure 4-9. Synergistic effect of NucB and teicoplanin. *Staphylococcus epidermidis* 096R biofilms were treated with teicoplanin at different concentrations with and without the addition of NucB. Bacterial metabolism produces a Red colour which represents metabolic effect and therefore a higher number of active cells.

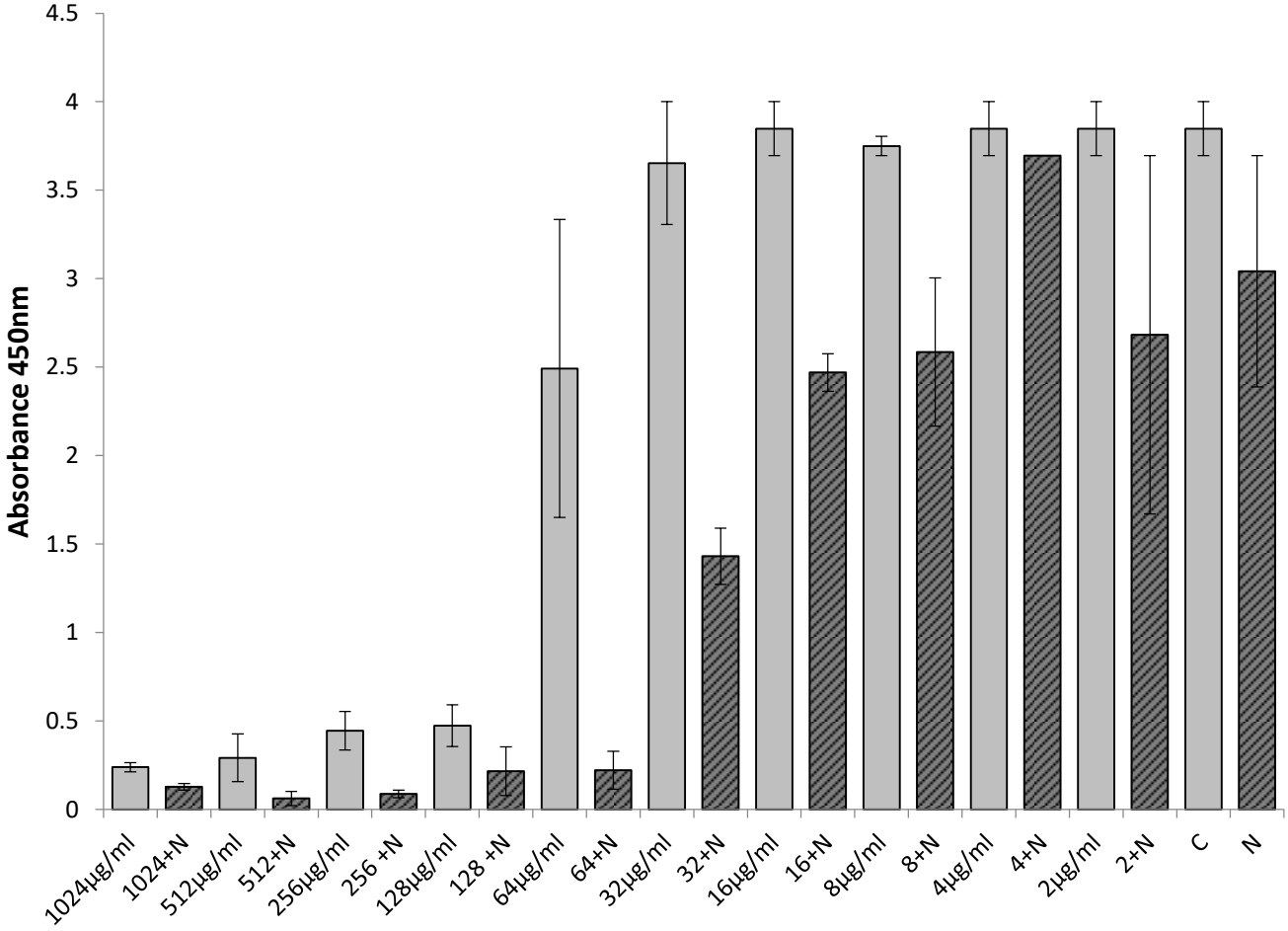


Figure 4-10. The effect of NucB on teicoplanin mediated cell killing of biofilms of clinical strain *Staphylococcus epidermidis* 096R. Biofilms treated with antibiotics alone are represented by solid grey bars while biofilms treated with a combination of antibiotics and NucB are represented by stripped dark grey bars. C = control cells, no treatment added. N = control NucB only added. Standard error bars are represented. C = control, no antibiotic added. N = control NucB only added. Biofilms treated with a combination of NucB and teicoplanin showed a reduction of viable cells in those biofilms treated with teicoplanin doses above 8µg/ml compared to those only treated with the same antibiotic dose without the addition of NucB.

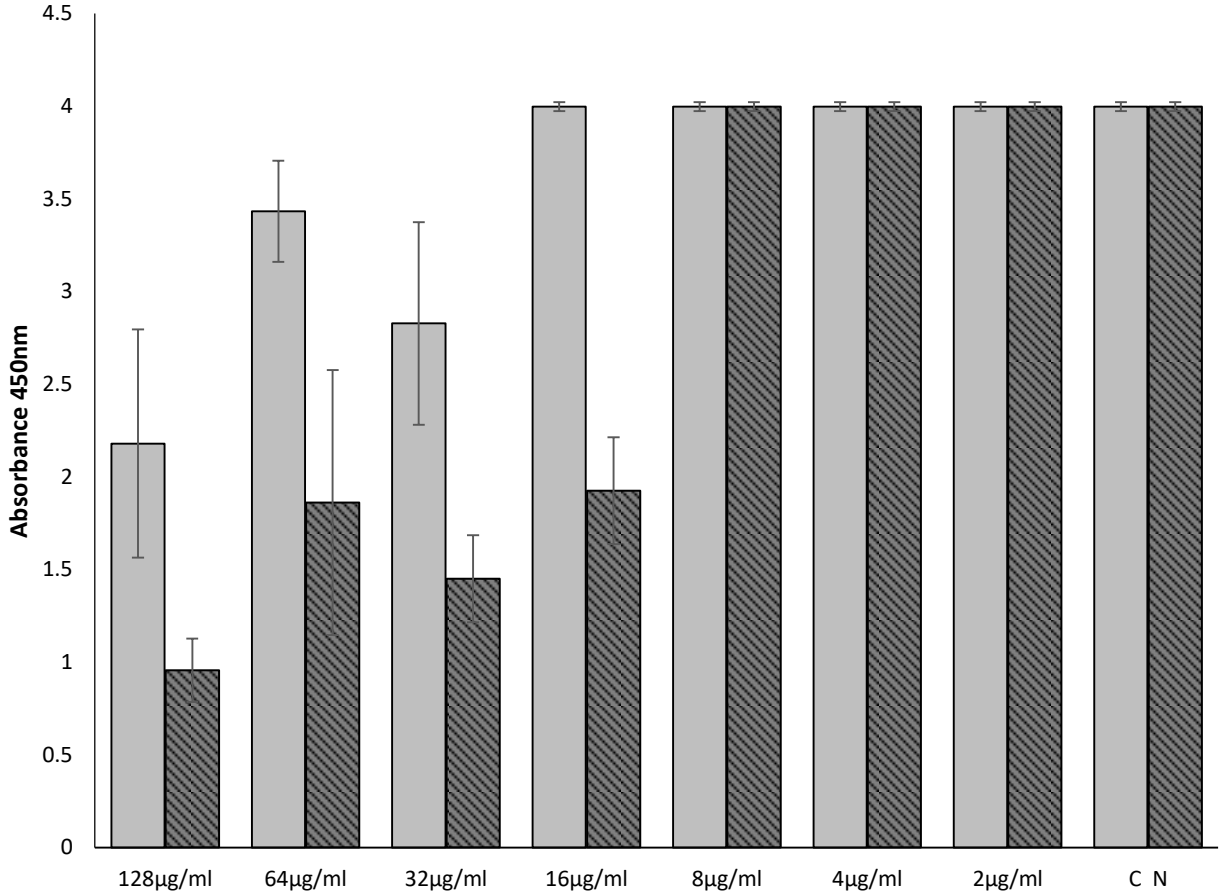


Figure 4-11. The effect of NucB on teicoplanin mediated cell killing of biofilms of clinical strain *Staphylococcus aureus* 518F. Biofilms treated with antibiotics alone are represented by solid grey bars while biofilms treated with a combination of antibiotics and NucB are represented by stripped dark grey bars. C = control cells, no treatment added. N = control NucB only added. Standard error bars are represented. C = control, no antibiotic added. N = control NucB only added. Biofilms treated with a combination of NucB and teicoplanin showed a reduction of viable cells in those biofilms treated with teicoplanin doses above 16µg/ml compared to those only treated with the same antibiotic dose without the addition of NucB.

4.4 Discussion

NucB has been previously tested on biofilms from explanted tracheoesophageal speech valves, where a 5.7-fold reduction in bacteria compared to control valves was observed (Shakir *et al.*, 2012). However, until now NucB has not been studied with respect to biofilms grown on joint replacement surfaces from clinical isolates. These results demonstrated that NucB was capable of dispersing between 39% and 92% of four clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms when they were grown on titanium, cobalt chrome, stainless steel and HMWPE surfaces. Lauderdale et al. successfully grew *Staphylococcus aureus* biofilms on titanium coupons inside a flow chamber and showed biofilm dispersal of between 35% and 85% using bovine DNase I (Lauderdale *et al.*, 2010). However, the results of both experiments cannot be directly compared due to significant differences in experimental design. Importantly, the bacterial strains used by Lauderlale et al. were not clinical strains and the dispersal effect was measured at 6 and 22 hours, which is significantly longer than studies presented here (NucB treatment was 1hour). In addition, differences in the definition of enzyme concentrations used make a direct comparison difficult.

When assessing the effect of NucB in dispersal of biofilms against 19 clinical strains, biofilms were exposed to NucB for 1h. The results demonstrated a variable dispersal effect (21 to 61%).To date there is no data on longer exposures of pre-formed biofilms to NucB to assess the dispersal effect - only 1h has been tested (Nijland *et al.*, 2010; Shields *et al.*, 2013). Lauderdale demonstrated higher dispersal effects of DNAseI with longer exposure to the nuclease (Lauderdale *et al.*, 2010) so there is a possibility that higher dispersal effect will be observed with longer exposures. Further experiments exposing pre-formed biofilms to NucB for longer times will be necessary to assess to be able to further understand the effect of NucB on biofilms dispersal.

Enzymatic approaches to the removal of biofilms of clinically relevant species have been studied in the literature with encouraging results. Mann et al. assessed the effect of DNase I against a clinical strain of *Staphylococcus aureus* that was the causative organism of osteomyelitis (Mann *et al.*, 2009). Biofilms were grown using a flow-cell biofilm model and exposed to 0.5units/ml of DNaseI. Here, the dispersal of biofilms were also assessed visually by CLSM, reinforcing the successful use of DNAses to disrupt pre-formed biofilms (Mann *et al.*, 2009). The dispersal effect of recombinant human DNase I (rhDNase) has also been tested against *Staphylococcus aureus* in vitro demonstrating an 80% dispersal effect (Kaplan *et al.*, 2012). The conditions differed significantly from the conditions described in this

chapter. Biofilms of non-clinical strains of *Staphylococcus aureus* were grown on a 96 well plate for 18h and treated with rhDNase at a concentration 10 times higher than NucB (10μg/ml vs 1μg/ml) and a short period of time (2-4 minutes) (Kaplan *et al.*, 2012).

One of the limitations of this study is that NucB was not directly compared with other available DNAses. Previous experiments performed by our group have demonstrated that NucB was effective dispersing biofilms at a fivefold lower concentration than DNAse I (Nijland *et al.*, 2010). While these experiments were not performed on clinical isolates or artificial joint surfaces, the results are encouraging, but direct comparison with clinical isolates will be necessary in future to draw concrete conclusions.

Other extracellular matrix components have been a research target to attempt to disperse biofilm formation. Lauderlale et al also successfully dispersed biofilms using Proteinase K. His results showed a dispersal effect between 93% and 100% of the pre-formed biofilms on a titanium surfaces using 2μg/ml (Lauderdale *et al.*, 2010). Boles et al. also showed biofilm dispersal of *Staphylococcus aureus* biofilms using Proteinase K (Boles and Horswill, 2008). In his experiments Boles et al. describes a rapid detachment of pre-established biofilms but quantification of the detached biomass was not given. Poly-*N*-acetylglucosamine surface polysaccharide (PNAG), a staphylococcal matrix biofilm polymer, has also been target of biofilm dispersal research. Dispersin B is the enzyme that targets such molecule. Izano et al. demonstrated Dispersin B was unable to disperse *Staphylococcus aureus* biofilms but capable to disperse more than 90% of a clinical strain of *Staphylococcus epidermidis* biofilm (isolated from an infected intravenous catheter) after 1 hour but the enzymatic concentration used was significantly higher concentration (20μg/mL Dispersin B vs 1μg/mL NucB) (Izano *et al.*, 2008). Mores successful results were demonstrated by Kaplan et al. who significantly dispersed clinical strains of *Staphylococcus epidermidis* grown on polystyrene rods and polyurethane and Teflon intravenous catheters. (Kaplan *et al.*, 2004).

These promising results confirm that targeting the extracellular matrix of the biofilm can help to remove pre-formed biofilms from clinical surfaces to a certain degree. Biofilm structure, including the composition of the extracellular matrix is highly variable depending on the species and the environment, it is likely that future treatment of biofilm related infection might include a combined enzymatic approach with different target enzymes working together to disrupt independent extracellular matrix components and increase the chances of success. It will be therefore necessary to assess the efficacy of NucB in combination with other enzymes to disrupt biofilms to test this hypothesis.

Although NucB was capable of dispersing a proportion of the biofilms grown on clinical surfaces from the four clinical strains tested, a more variable effect was seen on biofilms grown on 96 well polystyrene plates. Particularly, NucB had no effect on *Staphylococcus epidermidis* strain SE096R grown on a 96 well plate but had a significant dispersal effect when biofilms were grown on all four prosthetic joint replacement surfaces. This suggests that material-bacterial interactions may affect the structure and composition of the biofilm and perhaps the amount of extracellular DNA within the biofilm varies depending on the substrate. This material-bacterial interaction has been studied although not yet fully understood. Certain bacterial strains have more affinity to some biomaterials than others. *Staphylococcus epidermidis* has shown more affinity to titanium alloy and stainless steel than cobalt-chromium alloy (Koseki *et al.*, 2014; Malhotra *et al.*, 2019) although Patel el al. observed cobalt-chromium implants had a higher tendency for biofilm formation as compared to titanium alloy implants (Patel *et al.*, 2016). This variability in the literature is likely due to the other variables that influence biofilm adhesion and formation such as surface roughness, surface hydrophobicity and environment (Pavithra and Doble, 2008). Due to the multifactorial and complex nature of biofilm formation, it is difficult to identify the specific reason why biofilms from one species may be more susceptible to the effect of NucB than others. The variable results observed in our experiments reinforce current understanding that the composition of the extracellular matrix can vary significantly between closely related strains and species as well as with the environment and growth conditions (Tang *et al.,* 2013). (Nguyen and Burrows, 2014). NucB may only be effective on those biofilms with larger quantities of eDNA within the biofilm. Future experiments are being planned to quantify the eDNA present in the biofilms of the clinical strains tested in this work. The quantification of eDNA in the biofilm can be achieved by different methods including the use of fluorescent staining with 4’6-diamidino2-phenylindole (DAPI) as well as the eDNA extraction using a commercial kit such as the polymer mediated enrichment kit, and the use of confocal laser scanning microscopy to observe eDNA and its distribution in live biofilms (Zatorska *et al*., 2017) .

The substrate is also likely to play an important role and further experiments will be needed to clarify this bacterial-material-enzyme interaction. It will be interesting to include further experiments with other strains that were not susceptible to NucB in a 96 well plate to see if the efficacy of NucB changes when biofilms are grown on metal and polyethylene surfaces. This also raises the possibility that the very commonly used microtiter plate laboratory method may sometimes give misleading results.

NucB was capable of dispersing 24 hours biofilms on certain strains. Our results showed NucB was successful at reducing biofilm formation on 6/11 *Staphylococcus aureus* strains and 5/8 *Staphylococcus epidermidis* strains, and was effective at dispersing biofilms of 7/11 *Staphylococcus aureus* strains and 4/8 *Staphylococcus epidermidis* strains. Of all those, NucB was only effective at preventing biofilm formation and dispersing 24 hour biofilms in 2 *Staphylococcus aureus* strains (SA717T and SA089G) and 3 *Staphylococcus epidermidis* strains (SE286G, SE033G and SE248X). This variability is supported by other studies in the literature that suggest the age of the biofilm can have an effect on the efficacy of DNAses to disrupt biofilm (Okshevsky *et al.*, 2015). *Staphylococcus epidermidis* biofilms are more susceptible to DNAse treatment when less than 12 hours old (Qin *et al.*, 2007) and a similar effect has been noted on biofilms from *Pseudomonas aeruginosa* (Whitchurch *et al.*, 2002). Although results presented here, did not follow thi pattern, NucB did have a variable effect on different aged biofilms. While the reason why a biofilm may become resistant to DNAses has not yet been precisely identified, it has been hypothesised that perhaps other components of the extracellular matrix can structurally replace the existing eDNA or perhaps with time the eDNA binds to other components that prevent nucleases from successfully digesting it (Okshevsky *et al.*, 2015).

The dispersal effect was visualised using CLSM. HMWPE discs with 24 hour biofilms of Staphylococcus aureus 76901 biofilms were treated with and without NucB for 1 hour. The overall biomass and thickness was reduced in those biofilms treated with NucB as well as the number of live cells (Table 4-3). These results support those seen with the crystal violet experiments where the biofilm was significantly reduced compare to those treated with control (Table 3-4).

NucB has the potential to become a tool to improve treatment of biofilm related infections. A necessary step to move closer to the clinical setting is to ensure NucB can work in combination with antibiotics. Concerns have been raised regarding how the human body would respond to the increased influx of planktonic bacteria when pre-established biofilms are dispersed (Kaplan *et al.*, 2012). It is essential that antibiotics remain active despite the presence of nucleases and that they can cope with any increase in released cells. Since NucB has no bactericidal or bacteriostatic effect, experiments were needed to assess the efficacy of antibiotics in the presence of this novel nuclease.

We observed an enhanced activity of teicoplanin on *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms treated with NucB. The concentration of the antibiotic required to kill the bacteria within the biofilm reduced when NucB was present. The

combined effect of DNase and antibiotics has been previously described by Kaplan et al. who demonstrated that rhDNase enhanced tobramycin activity against *Staphylococcus aureus* planktonic cells (Kaplan *et al.*, 2012). DNase I was also observed to enhance susceptibility of *Hamemophilus influenzae* to ampicillin and ciprofloxacin showing that ampicillin in combination with DNase I resulted in a biofilm reduction of 54% and ciprofloxacin combined with DNase I decreased biofilm by 75% (Cavaliere *et al.*, 2014). Until now, no previous experiments had been performed assessing the effect of NucB in the presence of antibiotics. Our results support the evidence provided by Kaplan and Cavaliere that antibiotic activity can be enhanced by the addition of nucleases (Kaplan *et al.*, 2012; Cavaliere *et al.*, 2014). A possible explanation for this observation has been established by Chiang et al. who demonstrated that DNA binds aminoglycosides via electrostatic interactions and sequesters antibiotic molecules reducing their activity (Chiang and Chiu, 2012). It has also been shown that extracellular DNA reduces the effect of vancomycin against planktonic cells of *Staphylococcus epidermidis* (Doroshenko *et al.*, 2014). If DNA can bind to antibiotics reducing their activity, it is reasonable to speculate that the addition of DNases can break down these interactions increasing the efficiency of antibiotics on planktonic cells. Another possible explanation is that planktonic cells aggregate into small clusters by extracellular DNA (Das *et al.*, 2013) and the addition of a nuclease breaks down the aggregations allowing the antibiotics to attack the individual planktonic cells to be more efficiently.

In an era where antibiotic overuse and misuse has led to antibiotic resistance being a global concern and threat to public health (World Health Organization, 2014; Viganor *et al.*, 2016) it is important to carefully minimise the use of antibiotics to prevent further resistance. New therapies are being developed as an alternative to antibiotic treatment. P128, an anti-staphylococcal protein that has a cell wall-degrading enzymatic region and a *Staphylococcus*-specific binding region, has a strong bactericidal action against *Staphylococcus aureus* (Vipra *et al.*, 2012) has also been shown to have a synergistic effect with antibiotics including gentamicin and vancomycin (Nair *et al.*, 2016). Other novel strategies include bacteriophages, viruses capable of damaging the bacteria cell wall by replicating viral proteins and genomic material, resulting in death of the bacteria (Roach and Donovan, 2015). In place of intact bacteriophage, it is also possible to use individual components of bacteriophage particles such as lysins. These bacterial cell-wall-hydrolytic enzymes selectively kill specific Gram-positive bacteria (Fischetti, 2008) and can enhance antibiotic activity of gentamicin and penicillin against *Streptococcus pneumoniae* (Djurkovic *et al.*, 2005). In this research, it was observed that NucB has a supportive effect with teicoplanin

and vancomycin and is capable of reducing the MIC two-fold. The combined use of systemic and local antibiotic delivery has been shown to be more effective than systemic delivery alone (Rand et al., 2015). The synergistic effect of NucB and antibiotics will be advantageous as potentially smaller doses of antibiotics could be used and therefore will allow for two route combination therapy. Even if only used in a single route (either systemic or local delivery), the reduction in dose of antibiotics could potentially reduce side effects and antibiotic resistance. The presence of NucB did not modify the efficacy of gentamicin against planktonic cells. Previously it has been demonstrated that exogenous DNA increased minimal bactericidal concentration of gentamicin toward *Pseudomonas aeruginosa* biofilms suggesting that extracellular DNA reduces gentamicin activity (Chiang *et al.*, 2013). Therefore it is not irrational to think that the addition of nucleases could also enhance antibiotic activity in this case. Our experiments were performed with gentamicin and planktonic cells which are known to be phenotypically and behaviourally different to those involved in biofilm (Costerton *et al.*, 1995; Stoodley *et al.*, 2002). NucB was capable of reducing *Staphylococcus aureus* and *Staphylococcus epidermidis* MIC for vancomycin and teicoplanin. We are unsure of the reasons why NucB had no effect on gentamicin. A possible explanation is the physical interaction between eDNA and antibiotics. It has been shown that eDNA binds to vancomycin (Doroshenko *et al.*, 2014) and teicoplanin being also a glycopeptide may bind to eDNA in a similar manner. It will also be interesting to assess whether NucB can enhance gentamycin activity against staphylococcal biofilms as we observed with teicoplanin, therefore further experiments expanding on clinical isolates biofilms and antibiotics are currently being planned.

In this work antibiotics were used in monotherapy. This was the first attempt at assessing the effect of NucB in combination with antibiotics so it was important to start with single antibiotics to reduce the number of variables so that the results could be more carefully interpreted. It is well known that there is a synergistic effect between gentamicin and vancomycin by increasing intracellular penetration (Watanakunakorn and Bakie, 1973; Cottagnoud *et al*., 2003) and therefore it will be interesting to assess the efficacy of NucB in combination with gentamicin and vancomycin together to see if this synergistic effect is also manifested in the presence of NucB.

Experiments have shown an increased effect of antibiotic treatment against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms in the presence of NucB. Antibiotics were capable of killing bacteria more efficiently with the addition of NucB despite the increased influx of bacteria released from the biofilm into the supernatant.

Although NucB had no enhancing effect on gentamicin, it did not increase resistance of biofilm or planktonic cells during the experimental time. Our experiments assessed the efficacy of a single dose of antibiotic in a 24 hour period. Further experiments with extended antibiotic treatment may be necessary to ensure that resistance does not occur with longer exposures. An important limitation of this particular experiment is the limited number of strains and antibiotics tested. To be able to draw more meaningful conclusions it will be essential to repeat the experiments with a wider variety of clinical strains and antibiotics. However, these initial experiments are important as a proof of concept study, and have provided promising results. The overall aim of these experiments was to assess if NucB enhances or facilitates antibiotics activity against biofilms and therefore has the potential to improve current treatment of prosthetic joint infections.

The efficacy of NucB in combination with antibiotics has been assessed using a metabolic dye to quantify live cells. It would also be important to investigate the impact of antibiotics on total biomass, since it is possible that reductions in metabolic activity result in changes to biomass levels. This could be done, for example, using the crystal violet assay.

NucB can effectively disperse in-vitro biofilms formed by different clinical strains of *Staphylococcus aureus* and *Staphylococcus epidermidis.* Biofilms were more susceptible to NucB treatment when grown on clinically relevant surfaces such a titanium, cobalt chrome, stainless steel and HMWPE compared to those grown in a 96-well plate. NucB can enhance antibiotic activity of vancomycin and teicoplanin against *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms and planktonic cells and have demonstrated that antibiotics are able to neutralize the increased influx of bacterial release from the biofilm when NucB is added.

Chapter 5. Final perspectives

5.1 Thesis impact

In an era of increasing antibiotic resistance (World Health Organization, 2014), the current treatment for prosthetic joint infections is more challenging than ever. Biofilms are difficult to eradicate with current antibiotic therapy (Antony and Farran, 2016). Surgery causes high morbidity/mortality for patients and increasing costs for the health care system (Vanhegan *et al.*, 2012a). Evidence suggests that the seriousness of these problems and the number of surgical implants employed is only set to rise (Sedrakyan *et al.*, 2011).

This thesis began with the intention of developing new approaches to the treatment and prevention of biofilm related infections. The results provide a promising foundation for the further careful development of NucB as a possible tool to improve the management of prosthetic joint infections.

The discovery of extracellular DNA as a component of the extracellular matrix has suggested possibilities for the development of novel anti-biofilm agents. Several studies have tested nucleases as a method for prevention of biofilm formation as well as dispersal of pre-formed biofilms, and have confirmed that DNases are capable of weakening biofilms and increasing antibiotic penetrance (Tetz *et al.*, 2009; Kaplan *et al.*, 2012). Biofilms from *Staphylococcus aureus* had been previously grown and imaged on joint replacement surfaces (Coraca-Huber *et al.*, 2012) but no experiments had been done prior to this study using clinical strains from prosthetic joint infections. Our research group discovered a deoxyribonuclease isolated from a marine bacterium, *Bacillus licheniformis* (Nijland *et al.*, 2010). This nuclease is effective against single and multispecies biofilms in chronic rhinosinusitis and tracheoesophageal speech valves (Shakir *et al.*, 2012; Shields *et al.*, 2013) but has not yet been investigated in the orthopaedic field until now.

The variability observed in the effect of NucB adds to the existing knowledge of the inconsistency of eDNA production and DNase effectiveness between bacteria and also amongst different strains of the same bacterial species (Izano *et al.*, 2008). The results of chapter 3 and 4 provide an impetus to develop nucleases for treatment of prosthetic joint infections in addition to other implant related infections.

This thesis assessed the effect of NucB on biofilm formation as well as disruption of pre formed biofilms from the nineteen clinical strains as well as four of those strains grown on metal and polyethylene surfaces with promising results. Little research has been

performed on enzymatic treatment of biofilm in orthopaedic prosthetic infections (Donelli *et al.*, 2007); Kaplan, 2012 #79}. The results obtained in this thesis support the important role of the extracellular DNA as an essential component of the extracellular matrix and biofilm structure (Das *et al.*, 2013; Jakubovics *et al.*, 2013), and specifically improves our understanding of biofilm related orthopaedic infection. The results are encouraging, although the use of NucB alone will not be effective against all staphylococcal strains. Nevertheless NucB biofilm degradation may have important clinical benefits by improving the activity of conventional antimicrobials and by opening the door to further progress in a clinical setting. Methods for delivering the nuclease are now being developed and plans for in vivo animal studies will go ahead pending promising toxicological studies justify this.

This thesis also evaluates the in vitro effect of combining NucB and antibiotics. There is a potential concern regarding the use of biofilm dispersal agents alone as they might not be appropriate for clinical use due to the release of a large influx of planktonic cells into the bloodstream and therefore the possibility of increased risk of septicaemia. Enzymes have been proven to have a supportive effect with antibiotics against certain bacterial strains (Kaplan *et al.*, 2012; Cavaliere *et al.*, 2014). This is the first time that the combination of antibiotics and a nuclease has been tested against clinical strains from orthopaedic infections. NucB was shown to be effective releasing bacteria from the biofilm and subsequently to enhance the antibiotic activity, reducing the overall bacterial count and the concentration of antibiotics needed to treat planktonic cells. These novel results suggest that nucleases act to enhance antibiotic activity. This may improve antibiotic treatment of biofilm infections and also potentially non-biofilm associated infections.

There are limitations to the use of natural enzymes as a sole treatment for dispersing biofilms due to the limited activity time and recovery (Swartjes *et al.*, 2013). Therefore synthetic DNases such as DNase-mimetic artificial enzyme (DMAE) have been developed in an attempt to overcome those difficulties (Chen *et al.*, 2016). The future of successful treatment of biofilm related infections may be a combination of antibiotic therapy and several biofilm dispersal agents. This research has contributed to expanding on the possible use of nucleases as an effective anti-biofilm formation and biofilm-dispersal agent in the prevention and treatment of orthopaedic implant related infections.

Wider impact of this research:

This research has generated attention in the orthopaedic community, with the researcher being awarded Young Investigator 2015 by the British Orthopaedic Association. In addition, three invited lectures have been given nationally and internationally during the course of this research project.

The work presented in this thesis has also attracted attention from the Orthopaedic Industry. Results obtained in this thesis have provided industry with confidence to start a funded research project at Newcastle University aimed at further exploration of the utility of NucB in controlling infection in the clinic.

5.2 Limitations and future research

This thesis has explored the effect of NucB, a novel marine endonuclease, in the prevention and dispersal of strains of clinically relevant *Staphylococcus aureus* and *Staphylococcus epidermidis*. These experiments have been performed in vitro, growing biofilms in a 96-well microtiter plate and also on clinically relevant surfaces such as titanium, cobalt chrome and polyethylene discs. It has been difficult to compare the efficacy of NucB to other already available nucleases such as rhDNAse, or DNAse I or other biofilm dispersal agents such as Dispersin B due to the difference in reported concentrations and assay methodology (Donelli *et al.*, 2007; Kaplan *et al.*, 2012). Further experiments comparing the effect of these antibiofilm enzymes in the prevention and treatment of clinical biofilms grown on metal surfaces will be useful to fully assess the potential of NucB as a therapeutic agent. Further quantification and imaging using CLSM may be appropriate to determine the biofilm structure after each different enzymatic treatment.

We observed that NucB had a variable effect against the same biofilm depending on the surface it had been grown on, suggesting a possible interaction between the different materials, the enzyme and the bacteria. Due to time constraints it was not possible to explore these potential interactions. Further experiments will aim to further understand the mechanism of NucB action as well as biofilm and bacteria-surface interactions.

This thesis has shown that NucB can reduce the MIC of teicoplanin against certain bacterial strains. One possible explanation for why NucB enhances antibiotic activity against planktonic cells is due to the fact that extracellular DNA can bind to antibiotics reducing their activity (Chiang *et al.*, 2013). The addition of NucB breaks down those interactions improving the efficacy of the antibiotic. Another possible hypothesis is that planktonic cells are aggregated in clusters by extracellular DNA (Das *et al.*, 2013) and therefore the addition

of a DNase could break down those clusters making the individual planktonic cells more susceptible to antibiotic treatment. Further development of NucB as an adjunct to antimicrobial therapy may improve current antibiotic treatment for a number of infectious diseases, not limited to prostheses.

Another avenue for future research is studying possible methods of NucB delivery into the target area. The method of delivery will depend on the desired action of NucB. When considering using NucB as a prophylactic tool of PJI, the options of delivery include addition to cement (Hsu *et al.*, 2013; Matos *et al.*, 2014), or in a slow release hydrogel that can be inserted into the joint at the time of the primary surgery (Drago *et al.*, 2014), as well as part of the implant coating. When considering the use of NucB for the treatment of already established PJI, then them most appropriate use will be during the DAIR procedure delivering it directly into infected wounds supplementing surgical debridement, directly into the joint in combination with antibiotics (Whiteside *et al.*, 2011). Polymers such as polymethylmethacrylate (PMMA) also known as bone cement, have been used as a method for local delivery of antibiotics for over forty years (Marks *et al.*, 1976). One of the challenges encountered when attempting to deliver antibiotic via bone cement is that the polymerisation of bone cement is an exothermic reaction (Arora *et al.*, 2013) reaching temperatures of 80ºC. This makes the use of certain thermolabile antibiotics and other substances not suitable for this method of delivery (Perez-Jorge *et al.*, 2016). Preliminary experiments (data not shown) have confirmed that NucB is heat stable, able to re-fold and regain activity despite high temperatures of 80ºC and consequently cement remains a possible delivery method. Further research assessing the release mechanism of NucB within PMMA will be necessary.

So far NucB experiments have only been performed in vitro. Further in vitro studies are needed including the screening of more clinical strains and the synergistic effect of NucB and clinically relevant antibiotics. Further experiments on the mechanism of action against clinical strains and joint replacement materials are necessary before proceeding to animal studies to assess the effect and toxicity in an in-vivo environment prior to moving closer to the clinical setting.

5.3 Final conclusions

NucB has the potential to become a new tool in the prevention and treatment of PJI. This could be as part of an implant coating to prevent biofilm formation as well as a liquid form to be used during DAIR procedure where the surgical field could be soaked in the

solution to promote biofilm dispersal. The use of NucB as part of a treatment for PJI could translate into a reduction of the need for multiple surgical procedures, with potential enhancement of antibiotic treatment, which ultimately could help to improve patient care.

Annex

16S rRNA sequencing results

Strain	Source	Blast consensus	Identification
SA 559C	Freeman Hospital	*Staphylococcus aureus* strain ATCC 12600 16S ribosomal RNA, complete sequence	89%
SA 722P	Freeman Hospital	*Staphylococcus aureus* strain ATCC 12600 16S ribosomal RNA, complete sequence	94%
SA 76901	Northumbria Healthcare NHS Foundation Trust	*Staphylococcus aureus* strain ATCC 12600 16S ribosomal RNA gene, partial sequence	100%
SA 717T	Freeman Hospital	Staphylococcus petrasii strain CCM 8418 16S ribosomal RNA gene, partial sequence	96%
SA 089G	Freeman Hospital	*Staphylococcus aureus* strain ATCC 12600 16S ribosomal RNA gene, partial sequence	97%
SA 476A	Freeman Hospital	*Staphylococcus aureus* strain S33 R 16S ribosomal RNA, complete sequence	99%
SA 518F	Freeman Hospital	*Staphylococcus aureus* strain ATCC 12600 16S ribosomal RNA gene, partial sequence	98%
SA 171F	Freeman Hospital		
SA 649D	Freeman Hospital	*Staphylococcus aureus* strain S33 R 16S ribosomal RNA, complete sequence	99%
SA 378S	Freeman Hospital	*Staphylococcus aureus* strain ATCC 12600 16S ribosomal RNA gene, partial sequence	99%
SA 107H	Freeman Hospital	*Staphylococcus aureus* strain ATCC 12600 16S ribosomal RNA gene, partial sequence	99%

SE 286G	Freeman Hospital	*Staphylococcus epidermidis* strain Fussel 16S ribosomal RNA gene, partial sequence	97%
SE 76933	Northumbria Healthcare NHS Foundation Trust	*Staphylococcus epidermidis* strain Fussel 16S ribosomal RNA gene, partial sequence	98%
SE 033G	Freeman Hospital	*Staphylococcus epidermidis* strain Fussel 16S ribosomal RNA gene, partial sequence	99%
SE 684X	Freeman Hospital		
SE 150T	Freeman Hospital	*Staphylococcus epidermidis* strain Fussel 16S ribosomal RNA gene, partial sequence	98%
SE 096R	Freeman Hospital	*Staphylococcus epidermidis* strain Fussel 16S ribosomal RNA gene, partial sequence	99%
SE 248X	Freeman Hospital		
SE 414W	Freeman Hospital	*Staphylococcus epidermidis* strain SE95 chromosome	100%

References

Aboltins, C., Daffy, J., Choong, P. and Stanley, P. (2014) 'Current concepts in the management of prosthetic joint infection', *Intern Med J*, 44(9), pp. 834-40.

Aboltins, C.A., Page, M.A., Buising, K.L., Jenney, A.W., Daffy, J.R., Choong, P.F. and Stanley, P.A. (2007) 'Treatment of staphylococcal prosthetic joint infections with debridement, prosthesis retention and oral rifampicin and fusidic acid', *Clin Microbiol Infect*, 13(6), pp. 586-91.

Agarwal, A. and Jain, A. (2013) 'Glucose & sodium chloride induced biofilm production & ica operon in clinical isolates of staphylococci', *Indian J Med Res*, 138, pp. 262-6.

Aggarwal, V.K., Bakhshi, H., Ecker, N.U., Parvizi, J., Gehrke, T. and Kendoff, D. (2014) 'Organism profile in periprosthetic joint infection: pathogens differ at two arthroplasty infection referral centers in Europe and in the United States', *J Knee Surg*, 10, p. 10.

Ahearne, E. and Baron, S. (2017) 'Fundamental mechanisms in orthogonal cutting of medical grade cobalt chromium alloy (ASTM F75)', *CIRP J Manuf Sci Tech*, 19, pp. 1-6.

Ahl, T., Dalen, N., Jorbeck, H. and Hoborn, J. (1995) 'Air contamination during hip and knee arthroplasties. Horizontal laminar flow randomized vs. conventional ventilation', *Acta Orthop Scand*, 66(1), pp. 17-20.

Alam, F. and Balani, K. (2017) 'Adhesion force of *Staphylococcus aureus* on various biomaterial surfaces', *J Mech Behav Biomed Mater*, 65, pp. 872-880.

AlBuhairan, B., Hind, D. and Hutchinson, A. (2008) 'Antibiotic prophylaxis for wound infections in total joint arthroplasty: a systematic review', *J Bone Joint Surg Br*, 90(7), pp. 915-9.

Ali D. Alnuaimi, Neil M. O'Brien-Simpson, Eric C. Reynolds, Michael J. McCullough. (2013) 'Clinical isolates and laboratory reference *Candida* species and strains have varying abilities to form biofilms ', *FEMS Yeast Research*, 13, (7), pp 689–699.

Alijanipour, P., Bakhshi, H. and Parvizi, J. (2013) 'Diagnosis of periprosthetic joint infection: The threshold for serological markers', *Clinin Orthop and Relat Res*, 471(10), pp. 3186-3195.

Alijanipour, P. and Parvizi, J. (2014) 'Infection post-total knee replacement: current concepts', *Curr Rev Musculoskelet Med*, 7(2), pp. 96-102.

Allegrucci, M., Hu, F.Z., Shen, K., Hayes, J., Ehrlich, G.D., Post, J.C. and Sauer, K. (2006) 'Phenotypic characterization of *Streptococcus pneumoniae* biofilm development', *J Bacteriol*, 188(7), pp. 2325-2335.

Alvand, A., Rezapoor, M. and Parvizi, J. (2017) 'The role of biomarkers for the diagnosis of implant-related infections in orthopaedics and trauma', *Adv Exp Med Biol*, 971, pp. 69-79.

Alves, D.R., Gaudion, A., Bean, J.E., Perez Esteban, P., Arnot, T.C., Harper, D.R., Kot, W., Hansen, L.H., Enright, M.C. and Jenkins, A.T.A. (2014) 'Combined use of bacteriophage K and a novel bacteriophage to reduce biofilm formation', *Appl Enviro Microbiol*, 80(21), p. 6694.

Amanatullah, D.F., Landa, J., Strauss, E.J., Garino, J.P., Kim, S.H. and Di Cesare, P.E. (2011) 'Comparison of surgical outcomes and implant wear between ceramic-ceramic and ceramic-polyethylene articulations in total hip arthroplasty', *J Arthroplasty*, 26(6 Suppl), pp. 72-7.

Ammendolia, M.G., Di Rosa, R., Montanaro, L., Arciola, C.R. and Baldassarri, L. (1999) 'Slime production and expression of the slime-associated antigen by staphylococcal clinical isolates', *J Clin Microbiol*, 37(10), pp. 3235-8.

An, Y.H. and Friedman, R.J. (1998) 'Concise review of mechanisms of bacterial adhesion to biomaterial surfaces', *J Biomed Mater Res*, 43(3), pp. 338-48.

Anagnostakos, K. (2017) 'Therapeutic use of antibiotic-loaded bone cement in the treatment of hip and knee joint infections', *J Bone Jt Infect*, 2(1), pp. 29-37.

Anderl, J.N., Franklin, M.J. and Stewart, P.S. (2000) 'Role of antibiotic penetration limitation in *Klebsiella pneumoniae* biofilm resistance to ampicillin and ciprofloxacin', *Antimicro Agents Chemother*, 44(7), pp. 1818-1824.

Andersson, A.E., Bergh, I., Karlsson, J., Eriksson, B.I. and Nilsson, K. (2012) 'Traffic flow in the operating room: an explorative and descriptive study on air quality during orthopedic trauma implant surgery', *Am J Infect Control*, 40(8), pp. 750-5.

Anselme, K., Davidson, P., Popa, A.M., Giazzon, M., Liley, M. and Ploux, L. (2010) 'The interaction of cells and bacteria with surfaces structured at the nanometre scale', *Acta Biomater*, 6(10), pp. 3824-46.

Antoci, V., Jr., Adams, C.S., Parvizi, J., Davidson, H.M., Composto, R.J., Freeman, T.A., Wickstrom, E., Ducheyne, P., Jungkind, D., Shapiro, I.M. and Hickok, N.J. (2008) 'The inhibition of *Staphylococcus epidermidis* biofilm formation by vancomycin-modified titanium alloy and implications for the treatment of periprosthetic infection', *Biomaterials*, 29(35), pp. 4684-90.

Antony, S. and Farran, Y. (2016) 'Prosthetic joint and orthopedic devicer related infections. The role of biofilm in the pathogenesis and treatment', *Infect Disord Drug Targets*, 16(1), pp. 22-7.

Anwar, H. and Costerton, J.W. (1990) 'Enhanced activity of combination of tobramycin and piperacillin for eradication of sessile biofilm cells of *Pseudomonas aeruginosa'*, *Antimicrob Agents Chemother*, 34(9), pp. 1666-71.

Applerot, G., Lellouche, J., Perkas, N., Nitzan, Y., Gedanken, A. and Banin, E. (2012) 'ZnO nanoparticle-coated surfaces inhibit bacterial biofilm formation and increase antibiotic susceptibility', *RSC Advances*, 2(6), pp. 2314-2321.

Arciola, C.R., Campoccia, D., Baldassarri, L., Donati, M.E., Pirini, V., Gamberini, S. and Montanaro, L. (2006) 'Detection of biofilm formation in *Staphylococcus epidermidis* from implant infections. Comparison of a PCR-method that recognizes the presence of ica genes with two classic phenotypic methods', *J Biomed Mater Res A*, 76(2), pp. 425-30.

Argenson, J.N., Arndt, M., Babis, G., Battenberg, A., Budhiparama, N., Catani, F., Chen, F., de Beaubien, B., Ebied, A., Esposito, S., Ferry, C., Flores, H., Giorgini, A., Hansen, E., Hernugrahanto, K.D., Hyonmin, C., Kim, T.K., Koh, I.J., Komnos, G., Lausmann, C., Loloi, J., Lora-Tamayo, J., Lumban-Gaol, I., Mahyudin, F., Mancheno-Losa, M., Marculescu, C., Marei, S., Martin, K.E., Meshram, P., Paprosky, W.G., Poultsides, L., Saxena, A., Schwechter, E., Shah, J., Shohat, N., Sierra, R.J., Soriano, A., Stefansdottir, A., Suleiman, L.I., Taylor, A., Triantafyllopoulos, G.K., Utomo, D.N., Warren, D., Whiteside, L., Wouthuyzen-Bakker, M., Yombi, J. and Zmistowski, B. (2019) 'Hip and knee section, treatment, debridement and retention of implant: Proceedings of international consensus on orthopedic infections', *J Arthroplasty*, 34(2s), pp. S399-s419.

Arora, M., Chan, E.K., Gupta, S. and Diwan, A.D. (2013) 'Polymethylmethacrylate bone cements and additives: A review of the literature', *World J Orthop*, 4(2), pp. 67-74.

Artini, M., Cicatiello, P., Ricciardelli, A., Papa, R., Selan, L., Dardano, P., Tilotta, M., Vrenna, G., Tutino, M.L., Giardina, P. and Parrilli, E. (2017) 'Hydrophobin coating prevents *Staphylococcus epidermidis* biofilm formation on different surfaces', *Biofouling*, 33(7), pp. 601-611.

Babu, J.M., Kalagara, S., Durand, W., Antoci, V., Deren, M.E. and Cohen, E. (2019) 'Sarcopenia as a risk factor for prosthetic infection after total hip or knee arthroplasty', *J Arthroplasty*, 34(1), pp. 116-122.

Back, D.A., Scheuermann-Poley, C. and Willy, C. (2013) 'Recommendations on negative pressure wound therapy with instillation and antimicrobial solutions - when, where and how to use: what does the evidence show?', *Int Wound J*, 1, pp. 32-42.

Barth, E., Myrvik, Q.M., Wagner, W. and Gristina, A.G. (1989) 'In vitro and in vivo comparative colonization of *Staphylococcus aureus* and *Staphylococcus epidermidis* on orthopaedic implant materials', *Biomaterials*, 10(5), pp. 325-328.

Basle, A., Hewitt, L., Koh, A., Lamb, H.K., Thompson, P., Burgess, J.G., Hall, M.J., Hawkins, A.R., Murray, H. and Lewis, R.J. (2018) 'Crystal structure of NucB, a biofilm-degrading endonuclease', *Nucleic Acids Res*, 46(1), pp. 473-484.

Battaglia, S., Taddei, P., Tozzi, S., Sudanese, A. and Affatato, S. (2014) 'Toward the interpretation of the combined effect of size and body weight on the tribological performance of total knee prostheses', *Int Orthop*, 38(6), pp. 1183-1190.

Bayles, K.W. (2014) 'Bacterial programmed cell death: making sense of a paradox', *Nat Rev Microbiol*, 12(1), pp. 63-9.

Beenken, K.E., Spencer, H., Griffin, L.M. and Smeltzer, M.S. (2012) 'Impact of extracellular nuclease production on the biofilmpPhenotype of *Staphylococcus aureus* under in vitro and in vivo conditions', *Infect Immun*, 80(5), p. 1634.

Benito, N., Franco, M., Ribera, A., Soriano, A., Rodriguez-Pardo, D., Sorli, L., Fresco, G., Fernandez-Sampedro, M., Dolores Del Toro, M., Guio, L., Sanchez-Rivas, E., Bahamonde, A., Riera, M., Esteban, J., Baraia-Etxaburu, J.M., Martinez-Alvarez, J., Jover-Saenz, A., Duenas, C., Ramos, A., Sobrino, B., Euba, G., Morata, L., Pigrau, C., Coll, P., Mur, I. and Ariza, J. (2016) 'Time trends in the aetiology of prosthetic joint infections: a multicentre cohort study', *Clin Microbiol Infect*, 22(8), pp. 732.e1-8.

Berbari, E., Mabry, T., Tsaras, G., Spangehl, M., Erwin, P.J., Murad, M.H., Steckelberg, J. and Osmon, D. (2010) 'Inflammatory blood laboratory levels as markers of prosthetic joint infection: a systematic review and meta-analysis', *J Bone Joint Surg Am*, 92(11), pp. 2102-9.

Berends, E.T.M., Horswill, A.R., Haste, N.M., Monestier, M., Nizet, V. and von Köckritz-Blickwede, M. (2010) 'Nuclease expression by *Staphylococcus aureus* facilitates escape from neutrophil extracellular traps', *J Innate Immun*, 2(6), pp. 576-586.

Bezuidenhout, M.B., Dimitrov, D.M., van Staden, A.D., Oosthuizen, G.A. and Dicks, L.M.T. (2015) 'Titanium-based hip stems with drug delivery functionality through additive manufacturing', *BioMed Res Int*, 2015, pp. 134093-134093.

Bhattacharya, M., Wozniak, D.J., Stoodley, P. and Hall-Stoodley, L. (2015) 'Prevention and treatment of *Staphylococcus aureus* biofilms', *Expert Rev Anti Infect Ther*, 13(12), pp. 1499-516.

Bischoff, P., Kubilay, N.Z., Allegranzi, B., Egger, M. and Gastmeier, P. (2017) 'Effect of laminar airflow ventilation on surgical site infections: a systematic review and meta-analysis', *Lancet Infect Dis*, 17(5), pp. 553-561.

Bistolfi, A., Massazza, G., Verne, E., Masse, A., Deledda, D., Ferraris, S., Miola, M., Galetto, F. and Crova, M. (2011) 'Antibiotic-loaded cement in orthopedic surgery: a review', *ISRN Orthop*, 2011, p. 290851.

Bjarnsholt, T., Kirketerp-Moller, K., Kristiansen, S., Phipps, R., Nielsen, A.K., Jensen, P.O., Hoiby, N. and Givskov, M. (2007) 'Silver against *Pseudomonas aeruginosa* biofilms', *Apmis*, 115(8), pp. 921-8.

Blom, A.W., Brown, J., Taylor, A.H., Pattison, G., Whitehouse, S. and Bannister, G.C. (2004) 'Infection after total knee arthroplasty', *J Bone Joint Surg Br*, 86(5), pp. 688-91.

Bogut, A., Niedzwiadek, J., Strzelec-Nowak, D., Blacha, J., Mazurkiewicz, T., Marczynski, W. and Koziol-Montewka, M. (2014) 'Infectious prosthetic hip joint loosening: bacterial species involved in its aetiology and their antibiotic resistance profiles against

antibiotics recommended for the therapy of implant-associated infections', *New Microbiol*, 37(2), pp. 209-18.
Boix-Montanes, A. and Garcia-Arieta, A. (2015) 'Composition specification of teicoplanin based on its estimated relative bioavailability', *Drug Dev Ind Pharm*, 41(2), pp. 218-23.
Boles, B.R. and Horswill, A.R. (2008) 'Agr-mediated dispersal of *Staphylococcus aureus* biofilms', *PLoS Pathog*, 4(4), p. e1000052.
Boles, B.R. and Horswill, A.R. (2011) 'Staphylococcal biofilm disassembly', *Trends Microbiol*, 19(9), pp. 449-455.
Bori, G., Munoz-Mahamud, E., Cune, J., Gallart, X., Fuster, D. and Soriano, A. (2014) 'One-stage revision arthroplasty using cementless stem for infected hip arthroplasties', *J Arthroplasty*, 29(5), pp. 1076-81.
Bori, G., Navarro, G., Morata, L., Fernandez-Valencia, J.A., Soriano, A. and Gallart, X. (2018) 'Preliminary results after changing from two-stage to one-stage revision arthroplasty protocol using cementless arthroplasty for chronic infected hip replacements', *J Arthroplasty*, 33(2), pp. 527-532.
Borriello, G., Werner, E., Roe, F., Kim, A.M., Ehrlich, G.D. and Stewart, P.S. (2004) 'Oxygen limitation contributes to antibiotic tolerance of *Pseudomonas aeruginosa* in biofilms', *Antimicrob Agents Chemother*, 48(7), pp. 2659-2664.
Bortel, E., Charbonnier, B. and Heuberger, R. (2015) 'Development of a synthetic synovial fluid for tribological testing', *Lubricants*, 3, pp. 664-686.
Bourguignon, L., Cazaubon, Y., Debeurme, G., Loue, C., Ducher, M. and Goutelle, S. (2016) 'Pharmacokinetics of vancomycin in elderly patients aged over 80 years', *Antimicrob Agents Chemother*, 60(8), pp. 4563-7.
Boutin, P. (2014) 'Total arthroplasty of the hip by fritted alumina prosthesis. Experimental study and 1st clinical applications', *Orthop Traumatol Surg Res*, 100(1), pp. 15-21.
Bowden, M.G., Chen, W., Singvall, J., Xu, Y., Peacock, S.J., Valtulina, V., Speziale, P. and Hook, M. (2005) 'Identification and preliminary characterization of cell-wall-anchored proteins of *Staphylococcus epidermidis*', *Microbiology*, 151(Pt 5), pp. 1453-64.
Bowler, L.L., Zhanel, G.G., Ball, T.B. and Saward, L.L. (2012) 'Mature *Pseudomonas aeruginosa* biofilms prevail compared to young biofilms in the presence of ceftazidime', *Antimicrob Agents Chemother*, 56(9), pp. 4976-4979.
Bozhkova, S., Tikhilov, R., Labutin, D., Denisov, A., Shubnyakov, I., Razorenov, V., Artyukh, V. and Rukina, A. (2016) 'Failure of the first step of two-stage revision due to polymicrobial prosthetic joint infection of the hip', *J Orthop Traumatol*, 17(4), pp. 369-376.
Bozic, K.J., Lau, E., Kurtz, S., Ong, K. and Berry, D.J. (2012) 'Patient-related risk factors for postoperative mortality and periprosthetic joint infection in medicare patients undergoing TKA', *Clin Orthop Relat Res*, 470(1), pp. 130-7.
Branda, S.S., Vik, A., Friedman, L. and Kolter, R. (2005) 'Biofilms: the matrix revisited', *Trends Microbiol*, 13(1), pp. 20-26.
Brandt, C., Hott, U., Sohr, D., Daschner, F., Gastmeier, P. and Rüden, H. (2008) 'Operating room ventilation with laminar airflow shows no protective effect on the surgical site infection rate in orthopedic and abdominal surgery', *Ann Surg*, 248(5), pp. 695-700.
Brimmo, O., Ramanathan, D., Schiltz, N.K., Pillai, A.L., Klika, A.K. and Barsoum, W.K. (2016) 'Irrigation and debridement before a 2-stage revision total knee arthroplasty does not increase risk of failure', *J Arthroplasty*, 31(2), pp. 461-4.
Buchholz, H.W. and Engelbrecht, H. (1970) 'Depot effects of various antibiotics mixed with Palacos resins', *Chirurg*, 41(11), pp. 511-5.
Byren, I., Bejon, P., Atkins, B.L., Angus, B., Masters, S., McLardy-Smith, P., Gundle, R. and Berendt, A. (2009) 'One hundred and twelve infected arthroplasties treated with 'DAIR'

(debridement, antibiotics and implant retention): antibiotic duration and outcome', *J Antimicrob Chemother*, 63(6), pp. 1264-71.
Cafiso, V., Bertuccio, T., Santagati, M., Campanile, F., Amicosante, G., Perilli, M.G., Selan, L., Artini, M., Nicoletti, G. and Stefani, S. (2004) 'Presence of the ica operon in clinical isolates of *Staphylococcus epidermidis* and its role in biofilm production', *Clin Microbiol Infect*, 10(12), pp. 1081-8.
Campoccia, D., Montanaro, L. and Arciola, C.R. (2006) 'The significance of infection related to orthopedic devices and issues of antibiotic resistance', *Biomaterials*, 27(11), pp. 2331-9.
Cascioferro, S., Raffa, D., Maggio, B., Raimondi, M.V., Schillaci, D. and Daidone, G. (2015) 'Sortase A inhibitors: recent advances and future perspectives', *J Med Chem*, 58(23), pp. 9108-23.
Cash, D.J. and Khanduja, V. (2014) 'The case for ceramic-on-polyethylene as the preferred bearing for a young adult hip replacement', *Hip Int*, 24(5), pp. 421-7.
Catlin, B.W. (1956) 'Extracellular deoxyribonucleic acid of bacteria and a deoxyribonuclease inhibitor', *Sci (New York)*, 124(3219), pp. 441-2.
Cavaliere, R., Ball, J.L., Turnbull, L. and Whitchurch, C.B. (2014) 'The biofilm matrix destabilizers, EDTA and DNaseI, enhance the susceptibility of nontypeable Hemophilus influenzae biofilms to treatment with ampicillin and ciprofloxacin', *Microbiologyopen*, 3(4), pp. 557-67.
Cazander, G., van de Veerdonk, M.C., Vandenbroucke-Grauls, C.M., Schreurs, M.W. and Jukema, G.N. (2010) 'Maggot excretions inhibit biofilm formation on biomaterials', *Clin Orthop Relat Res*, 468(10), pp. 2789-96.
Cerca, N., Martins, S., Cerca, F., Jefferson, K.K., Pier, G.B., Oliveira, R. and Azeredo, J. (2005) 'Comparative assessment of antibiotic susceptibility of coagulase-negative staphylococci in biofilm versus planktonic culture as assessed by bacterial enumeration or rapid XTT colorimetry', *J Antimicrob Chemother*, 56(2), pp. 331-6.
Chang, C.Y., Chien, W.C., Chung, C.H., Tsao, C.H., Lin, F.H., Chang, F.Y., Shang, S.T. and Wang, Y.C. (2018) 'Protective effect of N-acetylcysteine in prosthetic joint infection: A nationwide population-based cohort study', *J Microbiol Immunol Infect*.
Charles, C., Gafni, A. and Freeman, E. (2011) 'The evidence-based medicine model of clinical practice: scientific teaching or belief-based preaching?', *J Eval Clin Pract*, 17(4), pp. 597-605.
Charnley, J. (1965) 'A biomechanical analysis of the use of cement to anchor the femoral head prosthesis ', *J Bone Joint Surg Br*, 47, pp. 354-63.
Charnley, J. (1968) 'Total prosthetic replacement of the hip', *Triangle*, 8(6), pp. 211-6.
Charnley, J. (1972) 'Postoperative infection after total hip replacement with special reference to air contamination in the operating room', *Clin Orthop Relat Res*, 87, pp. 167-87.
Charnley, J. (1974) 'Letter: Clean air operating room enclosures', *Br Med J*, 4(5938), pp. 224-5.
Charnley, J. (1976) 'A loophole in aseptic technique', *Br Med J*, 2(6051), pp. 1557-8.
Charnley, J. and Eftekhar, N. (1969) 'Postoperative infection in total prosthetic replacement arthroplasty of the hip-joint. With special reference to the bacterial content of the air of the operating room', *Br J Surg*, 56(9), pp. 641-9.
Chaudhari, P.R., Masurkar, S.A., Shidore, V.B. and Kamble, S.P. (2012) 'Effect of biosynthesized silver nanoparticles on *Staphylococcus aureus* biofilm quenching and prevention of biofilm Formation', *Nano-Micro Lett*, 4(1), pp. 34-39.
Chen, A.F. and Parvizi, J. (2014) 'Antibiotic-loaded bone cement and periprosthetic joint infection', *J Long Term Eff Med Implants*, 24(2-3), pp. 89-97.

Chen, Z., Ji, H., Liu, C., Bing, W., Wang, Z. and Qu, X. (2016) 'A multinuclear metal complex based DNase-mimetic artificial enzyme: matrix cleavage for combating bacterial biofilms', *Angew Chem Int Ed Engl*, 55(36), pp. 10732-6.

Chiang, C.C. and Chiu, F.Y. (2012) 'Cefuroxime-impregnated cement and systemic cefazolin for 1 week in primary total knee arthroplasty: an evaluation of 2700 knees', *J Chin Med Assoc*, 75(4), pp. 167-70.

Chiang, W.C., Nilsson, M., Jensen, P.O., Høiby, N., Nielsen, T.E., Givskov, M. and Tolker-Nielsen, T. (2013) 'Extracellular DNA shields against aminoglycosides in *Pseudomonas aeruginosa* biofilms', *Antimicrob Agents Chemother*, 57(5), pp. 2352-2361.

Chiew, A.L., Gluud, C., Brok, J. and Buckley, N.A. (2018) 'Interventions for paracetamol (acetaminophen) overdose', *Cochrane Database Syst Rev*, (2).

Christensen, G.D., Simpson, W.A., Younger, J.J., Baddour, L.M., Barrett, F.F., Melton, D.M. and Beachey, E.H. (1985) 'Adherence of coagulase-negative staphylococci to plastic tissue culture plates: a quantitative model for the adherence of staphylococci to medical devices', *J Clin Microbiol*, 22(6), pp. 996-1006.

Cihalova, K., Chudobova, D., Michalek, P., Moulick, A., Guran, R., Kopel, P., Adam, V. and Kizek, R. (2015) '*Staphylococcus aureus* and MRSA growth and biofilm formation after treatment with antibiotics and SeNPs', *Int J Mol Sci*, 16(10), pp. 24656-72.

Ciofu, O., Rojo-Molinero, E., Macià, M.D. and Oliver, A. (2017) 'Antibiotic treatment of biofilm infections', *APMIS*, 125(4), pp. 304-319.

Claessens, J., Roriz, M., Merckx, R., Baatsen, P., Van Mellaert, L. and Van Eldere, J. (2015) 'Inefficacy of vancomycin and teicoplanin in eradicating and killing *Staphylococcus epidermidis* biofilms in vitro', *Int J Antimicrob Agents*, 45(4), pp. 368-75.

Cobo, F., Rodriguez-Granger, J., Sampedro, A., Aliaga-Martinez, L. and Navarro-Mari, J.M. (2017) '*Candida* prosthetic joint infection. A review of treatment methods', *J Bone Jt Infect*, 2(2), pp. 114-121.

Collins English Dictionary (2009) HarperCollins Publishers.

Cook, L. and Dunny, G.M. (2013) 'Effects of biofilm growth on plasmid copy number and expression of antibiotic resistance genes in *Enterococcus faecalis*', *Antimicrob Agents Chemother*, 57(4), pp. 1850-1856.

Cooper, H.J. and Della Valle, C.J. (2013) 'The two-stage standard in revision total hip replacement', *Bone Joint J*, 95-B(11 Suppl A), pp. 84-7.

Coraca-Huber, D.C., Fille, M., Hausdorfer, J., Pfaller, K. and Nogler, M. (2012) '*Staphylococcus aureus* biofilm formation and antibiotic susceptibility tests on polystyrene and metal surfaces', *J Appl Microbiol*, 112(6), pp. 1235-43.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R. and Lappinscott, H.M. (1995) 'Microbial biofilms', *Ann Rev Microbiol*, 49, pp. 711-745.

Costerton, J.W., Lewandowski, Z., Debeer, D., Caldwell, D., Korber, D. and James, G. (1994) 'Biofilms, the customized microniche', *J Bacteriol*, 176(8), pp. 2137-2142.

Costerton, J.W., Stewart, P.S. and Greenberg, E.P. (1999) 'Bacterial biofilms: A common cause of persistent infections', *Science*, 284(5418), pp. 1318-1322.

Cottagnoud P, Cottagnoud M, Täuber MG. 'Vancomycin acts synergistically with gentamicin against penicillin-resistant pneumococci by increasing the intracellular penetration of gentamicin '. Antimicrob Agents Chemother. 2003 Jan;47(1), pp. 144-7.

Cramton, S.E., Gerke, C., Schnell, N.F., Nichols, W.W. and Gotz, F. (1999) 'The intercellular adhesion (ica) locus is present in *Staphylococcus aureus* and is required for biofilm formation', *Infect Immun*, 67(10), pp. 5427-33.

Cramton, S.E., Ulrich, M., Gotz, F. and Doring, G. (2001) 'Anaerobic conditions induce expression of polysaccharide intercellular adhesin in *Staphylococcus aureus* and *Staphylococcus epidermidis*', *Infect Immun*, 69(6), pp. 4079-85.

Crawford, R.J., Webb, H.K., Truong, V.K., Hasan, J. and Ivanova, E.P. (2012) 'Surface topographical factors influencing bacterial attachment', *Adv Colloid Interface Sci*, 179-182, pp. 142-9.
Crockarell, J.R., Hanssen, A.D., Osmon, D.R. and Morrey, B.F. (1998) 'Treatment of infection with debridement and retention of the components following hip arthroplasty', *J Bone Joint Surg Am*, 80(9), pp. 1306-13.
Cuckler, J.M. (2005) 'The rationale for metal-on-metal total hip arthroplasty', *Clin Orthop Relat Res*, 441, pp. 132-6.
Cunningham, D.J., Kavolus, J.J., 2nd, Bolognesi, M.P., Wellman, S.S. and Seyler, T.M. (2017) 'Specific infectious organisms associated with poor outcomes in treatment for hip periprosthetic infection', *J Arthroplasty*, 32(6), pp. 1984-1990.e5.
Cusumano, J.A., Caffrey, A.R., Daffinee, K.E., Luther, M.K., Lopes, V. and LaPlante, K.L. (2019) 'Weak biofilm formation among carbapenem-resistant *Klebsiella pneumoniae*', *Diagn Microbiol Infect Dis*, 95(4), p. 114877.
Darouiche, R.O., Green, G. and Mansouri, M.D. (1998) 'Antimicrobial activity of antiseptic-coated orthopaedic devices', *Int J Antimicrob Agents*, 10(1), pp. 83-6.
Das, T., Sehar, S., Koop, L., Wong, Y.K., Ahmed, S., Siddiqui, K.S. and Manefield, M. (2014) 'Influence of calcium in extracellular DNA mediated bacterial aggregation and biofilm formation', *PLoS One*, 9(3), p. e91935.
Das, T., Sehar, S. and Manefield, M. (2013) 'The roles of extracellular DNA in the structural integrity of extracellular polymeric substance and bacterial biofilm development', *Environ Microbiol Rep*, 5(6), pp. 778-786.
Das, T., Sharma, P.K., Busscher, H.J., van der Mei, H.C. and Krom, B.P. (2010) 'Role of extracellular DNA in initial bacterial adhesion and surface aggregation', *Appl Environ Microbiol*, 76(10), pp. 3405-3408.
de la Fuente-Nunez, C., Reffuveille, F., Haney, E.F., Straus, S.K. and Hancock, R.E. (2014) 'Broad-spectrum anti-biofilm peptide that targets a cellular stress response', *PLoS Pathog*, 10(5), p. e1004152.
Dean, S.N., Bishop, B.M. and van Hoek, M.L. (2011) 'Natural and synthetic cathelicidin peptides with anti-microbial and anti-biofilm activity against *Staphylococcus aureus*', *BMC Microbiol*, 11, p. 114.
Deirmengian, C., Hallab, N., Tarabishy, A., Della Valle, C., Jacobs, J.J., Lonner, J. and Booth, R.E., Jr. (2010) 'Synovial fluid biomarkers for periprosthetic infection', *Clin Orthop Relat Res*, 468(8), pp. 2017-23.
Deirmengian, C., Kardos, K., Kilmartin, P., Cameron, A., Schiller, K. and Parvizi, J. (2014) 'Diagnosing periprosthetic joint infection: has the era of the biomarker arrived?', *Clin Orthop Relat Res*, 472(11), pp. 3254-62.
Deirmengian, C., Kardos, K., Kilmartin, P., Gulati, S., Citrano, P. and Booth, R.E., Jr. (2015) 'The alpha-defensin test for periprosthetic joint infection responds to a wide spectrum of organisms', *Clin Orthop Relat Res*, 473(7), pp. 2229-35.
Deirmengian, C., Lonner, J.H. and Booth, R.E., Jr. (2005) 'The Mark Coventry Award: white blood cell gene expression: a new approach toward the study and diagnosis of infection', *Clin Orthop Relat Res*, 440, pp. 38-44.
Della Valle, C., Parvizi, J., Bauer, T.W., DiCesare, P.E., Evans, R.P., Segreti, J., Spangehl, M., Watters, W.C., 3rd, Keith, M., Turkelson, C.M., Wies, J.L., Sluka, P. and Hitchcock, K. (2011) 'American Academy of Orthopaedic Surgeons clinical practice guideline on: the diagnosis of periprosthetic joint infections of the hip and knee', *J Bone Joint Surg Am*, 93(14), pp. 1355-7.
Djurkovic, S., Loeffler, J.M. and Fischetti, V.A. (2005) 'Synergistic killing of *Streptococcus pneumoniae* with the bacteriophage lytic enzyme Cpl-1 and penicillin or gentamicin

depends on the level of penicillin resistance', *Antimicrob Agents Chemother*, 49(3), pp. 1225-8.
Donelli, G., Francolini, I., Romoli, D., Guaglianone, E., Piozzi, A., Ragunath, C. and Kaplan, J.B. (2007) 'Synergistic activity of dispersin B and cefamandole nafate in inhibition of staphylococcal biofilm growth on polyurethanes', *Antimicrob Agents Chemother*, 51(8), pp. 2733-40.
Dong, Y.L., Li, T., Xiao, K., Bian, Y.Y. and Weng, X.S. (2015) 'Ceramic on ceramic or ceramic-on-polyethylene for total hip arthroplasty: A systemic review and meta-analysis of prospective randomized studies', *Chin Med J (Engl)*, 128(9), pp. 1223-31.
Donlan, R.M. and Costerton, J.W. (2002) 'Biofilms: Survival mechanisms of clinically relevant microorganisms', *Clin Microbiol Rev*, 15(2), pp. 167-193.
Doroshenko, N., Tseng, B.S., Howlin, R.P., Deacon, J., Wharton, J.A., Thurner, P.J., Gilmore, B.F., Parsek, M.R. and Stoodley, P. (2014) 'Extracellular DNA impedes the transport of vancomycin in *Staphylococcus epidermidis* biofilms preexposed to subinhibitory concentrations of vancomycin', *Antimicrob Agents Chemother*, 58(12), pp. 7273-82.
Drago, L., Boot, W., Dimas, K., Malizos, K., Hansch, G.M., Stuyck, J., Gawlitta, D. and Romano, C.L. (2014) 'Does implant coating with antibacterial-loaded hydrogel reduce bacterial colonization and biofilm formation in vitro?', *Clin Orthop Relat Res*, 13, p. 13.
Drago, L., De Vecchi, E., Mattina, R. and Romano, C.L. (2013) 'Activity of N-acetyl-L-cysteine against biofilm of *Staphylococcus aureus* and *Pseudomonas aeruginosa* on orthopedic prosthetic materials', *Int J Artif Organs*, 36(1), pp. 39-46.
Drago, L., Vassena, C., Dozio, E., Corsi, M.M., De Vecchi, E., Mattina, R. and Romano, C. (2011) 'Procalcitonin, C-reactive protein, interleukin-6, and soluble intercellular adhesion molecule-1 as markers of postoperative orthopaedic joint prosthesis infections', *Int J Immunopathol Pharmacol*, 24(2), pp. 433-40.
Drevinek, M., Dresler, J., Klimentova, J., Pisa, L. and Hubalek, M. (2012) 'Evaluation of sample preparation methods for MALDI-TOF MS identification of highly dangerous bacteria', *Lett Appl Microbiol*, 55(1), pp. 40-6.
Dryden, M. (2014) 'Prosthetic joint infection: managing infection in a bionic era', *J Antimicrob Chemother*, 69 Suppl 1, pp. i3-4.
Duijf, S.V., Vos, F.J., Meis, J.F. and Goosen, J.H. (2015) 'Debridement, antibiotics and implant retention in early postoperative infection with *Enterococcus* sp', *Clin Microbiol Infect*, 21(5), pp. e41-2.
Dunne, W., Mason, E. and Kaplan, S.L. (1993) 'Diffusion of rifampin and vancomycin through a *Staphylococcus epidermidis* biofilm', *Antimicrob Agents Chemother*, 37(12), pp. 2522-2526.
Dürig, A., Kouskoumvekaki, I., Vejborg, R.M. and Klemm, P. (2010) 'Chemoinformatics-assisted development of new anti-biofilm compounds', *Appl Microbiol Biotechnol*, 87(1), pp. 309-317.
Eberhardt, A.W., McKee, R.T., Cuckler, J.M., Peterson, D.W., Beck, P.R. and Lemons, J.E. (2009) 'Surface Roughness of CoCr and ZrO(2) Femoral Heads with Metal Transfer: A Retrieval and Wear Simulator Study', *Int J Biomater*, 2009, pp. 185456-185456.
Eckhart, L., Fischer, H., Barken, K.B., Tolker-Nielsen, T. and Tschachler, E. (2007) 'DNase1L2 suppresses biofilm formation by *Pseudomonas aeruginosa* and *Staphylococcus aureus*', *Br J Dermatol*, 156(6), pp. 1342-5.
Edmiston, C.E., Jr., McBain, A.J., Roberts, C. and Leaper, D. (2015) 'Clinical and microbiological aspects of biofilm-associated surgical site infections', *Adv Exp Med Biol*, 830, pp. 47-67.
Eka, A. and Chen, A.F. (2015) 'Patient-related medical risk factors for periprosthetic joint infection of the hip and knee', *Ann Transl Med*, 3(16), p. 233.

El Helou, O.C., Berbari, E.F., Marculescu, C.E., El Atrouni, W.I., Razonable, R.R., Steckelberg, J.M., Hanssen, A.D. and Osmon, D.R. (2008) 'Outcome of enterococcal prosthetic joint infection: is combination systemic therapy superior to monotherapy?', *Clin Infect Dis*, 47(7), pp. 903-9.

Evans, J.T., Evans, J.P., Walker, R.W., Blom, A.W., Whitehouse, M.R. and Sayers, A. (2019) 'How long does a hip replacement last? A systematic review and meta-analysis of case series and national registry reports with more than 15 years of follow-up', *Lancet*, 393(10172), pp. 647-654.

Evans, R.P. (2011) 'Current concepts for clean air and total joint arthroplasty: laminar airflow and ultraviolet radiation: a systematic review', *Clin Orthop Relat Res*, 469(4), pp. 945-53.

Everts, R.J., Chambers, S.T., Murdoch, D.R., Rothwell, A.G. and McKie, J. (2004) 'Successful antimicrobial therapy and implant retention for streptococcal infection of prosthetic joints', *ANZ J Surg*, 74(4), pp. 210-4.

Eynon-Lewis, N.J., Ferry, D. and Pearse, M.F. (1992) 'Themistocles Gluck: An unrecognised genius', *Br Med J*, 305(6868), pp. 1534-1536.

F.M. AlAbbas, J.R.S., A. Kakpovbia, N.M. Balhareth, D.L. Olson, B. Mishra (2012) 'Bacterial attachment to metal substrate and its effects on microbiologically-influenced corrosion in transporting hydrocarbon pipelines', *J. Pipeline Eng,*, 11, pp. 63-72.

Fehring, T.K., Odum, S.M., Berend, K.R., Jiranek, W.A., Parvizi, J., Bozic, K.J., Della Valle, C.J. and Gioe, T.J. (2013) 'Failure of irrigation and debridement for early postoperative periprosthetic infection', *Clin Orthop Relat Res*, 471(1), pp. 250-7.

Ferry, T., Uckay, I., Vaudaux, P., Francois, P., Schrenzel, J., Harbarth, S., Laurent, F., Bernard, L., Vandenesch, F., Etienne, J., Hoffmeyer, P. and Lew, D. (2010) 'Risk factors for treatment failure in orthopedic device-related methicillin-resistant *Staphylococcus aureus* infection', *Eur J Clin Microbiol Infect Dis*, 29(2), pp. 171-80.

Fessler, M.B., Malcolm, K.C., Duncan, M.W. and Worthen, G.S. (2002) 'A genomic and proteomic analysis of activation of the human neutrophil by lipopolysaccharide and its mediation by p38 mitogen-activated protein kinase', *J Biol Chem*, 277(35), pp. 31291-302.

Figa, R., Muneton, D., Gomez, L., Matamala, A., Lung, M., Cuchi, E. and Corona, P.S. (2017) 'Periprosthetic joint infection by *Propionibacterium acnes*: Clinical differences between monomicrobial versus polymicrobial infection', *Anaerobe*, 44, pp. 143-149.

Fink, B., Schuster, P., Schwenninger, C., Frommelt, L. and Oremek, D. (2017) 'A standardized regimen for the treatment of acute postoperative infections and acute hematogenous infections associated with hip and knee arthroplasties', *J Arthroplasty*, 32(4), pp. 1255-1261.

Fischetti, V.A. (2008) 'Bacteriophage lysins as effective antibacterials', *Curr Opin Microbiol*, 11(5), pp. 393-400.

Flemming, H.C. and Wingender, J. (2010) 'The biofilm matrix', *Nat Rev Microbiol*, 8(9), pp. 623-33.

Fletcher, M. (1994) 'Bacterial biofilms and biofouling', *Curr Opin Biotechnol*, 5(3), pp. 302-6.

Fletcher, M. and Marshall, K.C. (1982) 'Bubble contact angle method for evaluating substratum interfacial characteristics and its relevance to bacterial attachment', *Appl Environ Microbiol*, 44(1), pp. 184-92.

Foster, T.J., Geoghegan, J.A., Ganesh, V.K. and Hook, M. (2014) 'Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*', *Nat Rev Microbiol*, 12(1), pp. 49-62.

Foulston, L., Elsholz, A.K., DeFrancesco, A.S. and Losick, R. (2014) 'The extracellular matrix of *Staphylococcus aureus* biofilms comprises cytoplasmic proteins that associate with the cell surface in response to decreasing pH', *MBio*, 5(5), pp. e01667-14.

French, M.L., Eitzen, H.E. and Ritter, M.A. (1976) 'The plastic surgical adhesive drape: an evaluation of its efficacy as a microbial barrier', *Ann Surg*, 184(1), pp. 46-50.

Fujii, T., Tsutsumi, S., Matsumoto, A., Fukasawa, T., Tabe, Y., Yajima, R., Asao, T. and Kuwano, H. (2010) 'Thickness of subcutaneous fat as a strong risk factor for wound infections in elective colorectal surgery: impact of prediction using preoperative CT', *Dig Surg*, 27(4), pp. 331-5.

Fux, C.A., Shirtliff, M., Stoodley, P. and Costerton, J.W. (2005) 'Can laboratory reference strains mirror "real-world" pathogenesis?', *Trends Microbiol*, 13(2), pp. 58-63.

Garrett, T.R., Bhakoo, M. and Zhang, Z. (2008) 'Bacterial adhesion and biofilms on surfaces', *Progr Nat Sci*, 18(9), pp. 1049-1056.

Gehrke, T., Alijanipour, P. and Parvizi, J. (2015) 'The management of an infected total knee arthroplasty', *Bone Joint J*, 97-b(10 Suppl A), pp. 20-9.

Gehrke, T. and Kendoff, D. (2012) 'Peri-prosthetic hip infections: in favour of one-stage', *Hip Int*, 22 Suppl 8, pp. S40-5.

Gehrke, T., Zahar, A. and Kendoff, D. (2013) 'One-stage exchange: it all began here', *Bone Joint J*, 95-B(11 Suppl A), pp. 77-83.

Geipel, U. (2009) 'Pathogenic organisms in hip joint infections', *Int J Med Sci*, 6(5), pp. 234-40.

Germovsek, E., Barker, C.I. and Sharland, M. (2016) 'What do I need to know about aminoglycoside antibiotics?', *Arch Dis Child Educ Pract Ed*.

Gilbertie, J.M., Schnabel, L.V., Hickok, N.J., Jacob, M.E., Conlon, B.P., Shapiro, I.M., Parvizi, J. and Schaer, T.P. (2019) 'Equine or porcine synovial fluid as a novel ex vivo model for the study of bacterial free-floating biofilms that form in human joint infections', *PloS one*, 14(8), pp. e0221012-e0221012.

Gillespie, J.A., Kennedy, J.W., Patil, S.R. and Meek, D.R. (2016) 'Noise production in ceramic-on-ceramic total hip arthroplasty is associated with lower patient satisfaction and hip scores', *J Orthop*, 13(4), pp. 282-4.

Gokcen, A., Vilcinskas, A. and Wiesner, J. (2013) 'Methods to identify enzymes that degrade the main extracellular polysaccharide component of *Staphylococcus epidermidis* biofilms', *Virulence*, 4(3), pp. 260-70.

Goksan, S.B. and Freeman, M.A. (1992) 'One-stage reimplantation for infected total knee arthroplasty', *J Bone Joint Surg Br*, 74(1), pp. 78-82.

Gomez, P.F. and Morcuende, J.A. (2005) 'Early attempts at hip arthroplasty--1700s to 1950s', *Iowa Orthop*, 25, pp. 25-29.

Goodman, S.D., Obergfell, K.P., Jurcisek, J.A., Novotny, L.A., Downey, J.S., Ayala, E.A., Tjokro, N., Li, B., Justice, S.S. and Bakaletz, L.O. (2011) 'Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins', *Mucosal Immunol*, 4(6), pp. 625-37.

Greene, C., McDevitt, D., Francois, P., Vaudaux, P.E., Lew, D.P. and Foster, T.J. (1995) 'Adhesion properties of mutants of *Staphylococcus aureus* defective in fibronectin-binding proteins and studies on the expression of fnb genes', *Mol Microbiol*, 17(6), pp. 1143-52.

Gristina, A.G. (1987) 'Biomaterial-centered infection: microbial adhesion versus tissue integration', *Science*, 237(4822), pp. 1588-95.

Gross, M., Cramton, S.E., Gotz, F. and Peschel, A. (2001) 'Key role of teichoic acid net charge in *Staphylococcus aureus* colonization of artificial surfaces', *Infect Immun*, 69(5), pp. 3423-6.

Gulhane, S., Vanhegan, I.S. and Haddad, F.S. (2012) 'Single stage revision: regaining momentum', *J Bone Joint Surg Br*, 94(11 Suppl A), pp. 120-2.
Gunn, J.S., Bakaletz, L.O. and Wozniak, D.J. (2016) 'What's on the outside matters: The role of the extracellular polymeric substance of Gram-negative biofilms in evading host immunity and as a target for therapeutic intervention', *J Biol Chem*, 291(24), pp. 12538-46.
Haasper, C., Buttaro, M., Hozack, W., Aboltins, C.A., Borens, O., Callaghan, J.J., de Carvalho, P.I., Chang, Y., Corona, P., Da Rin, F., Esposito, S., Fehring, T.K., Sanchez, X.F., Lee, G.C., Martinez-Pastor, J.C., Mortazavi, S.M., Noiseux, N.O., Peng, K.T., Schutte, H.D., Schweitzer, D., Trebse, R., Tsiridis, E. and Whiteside, L. (2014) 'Irrigation and debridement', *J Arthroplasty*, 29(2 Suppl), pp. 100-3.
Hall, C.W. and Mah, T.F. (2017) 'Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria', *FEMS Microbiol Rev*, 41(3), pp. 276-301.
Hallab, N.J., Bundy, K.J., O'Connor, K., Moses, R.L. and Jacobs, J.J. (2001) 'Evaluation of metallic and polymeric biomaterial surface energy and surface roughness characteristics for directed cell adhesion', *Tissue Eng*, 7(1), pp. 55-71.
Hammond, B.T. and Charnley, J. (1967) 'The sphericity of the femoral head', *Med Biol Eng*, 5(5), pp. 445-53.
Hansen, E.N., Adeli, B., Kenyon, R. and Parvizi, J. (2014) 'Routine use of antibiotic laden bone cement for primary total knee arthroplasty: Impact on infecting microbial patterns and resistance profiles', *J Arthroplasty*, 29(6), pp. 1123-1127.
Harmsen, M., Lappann, M., Knochel, S. and Molin, S. (2010) 'Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*', *Appl Environ Microbiol*, 76(7), pp. 2271-2279.
Harris, L.G., El-Bouri, K., Johnston, S., Rees, E., Frommelt, L., Siemssen, N., Christner, M., Davies, A.P., Rohde, H. and Mack, D. (2010) 'Rapid identification of staphylococci from prosthetic joint infections using MALDI-TOF mass-spectrometry', *Int J Artif Organs*, 33(9), pp. 568-74.
Hartmann, A., Hannemann, F., Lutzner, J., Seidler, A., Drexler, H., Gunther, K.P. and Schmitt, J. (2013) 'Metal ion concentrations in body fluids after implantation of hip replacements with metal-on-metal bearing--systematic review of clinical and epidemiological studies', *PLoS One*, 8(8), p. e70359.
Head, N.E. and Yu, H. (2004) 'Cross-Sectional Analysis of Clinical and Environmental Isolates of *Pseudomonas aeruginosa*: Biofilm Formation, Virulence, and Genome Diversity', *Infect Immun*, 72(1), pp. 133-144.
Head, W.C., Bauk, D.J. and Emerson, R.H., Jr. (1995) 'Titanium as the material of choice for cementless femoral components in total hip arthroplasty', *Clin Orthop Relat Res*, (311), pp. 85-90.
Heilmann, C., Gerke, C., Perdreau-Remington, F. and Gotz, F. (1996) 'Characterization of Tn917 insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation', *Infect Immun*, 64(1), pp. 277-82.
Heinrich, P.C., Castell, J.V. and Andus, T. (1990) 'Interleukin-6 and the acute phase response', *Biochem J*, 265(3), pp. 621-36.
Henrici, A.T. (1933) 'Studies of freshwater bacteria: A direct microscopic technique', *J Bacteriol Res*, 25(3), pp. 277-87.
Herrmann, M., Vaudaux, P.E., Pittet, D., Auckenthaler, R., Lew, P.D., Schumacher-Perdreau, F., Peters, G. and Waldvogel, F.A. (1988) 'Fibronectin, fibrinogen, and laminin act as mediators of adherence of clinical staphylococcal isolates to foreign material', *J Infect Dis*, 158(4), pp. 693-701.

Hexter, A.T., Hislop, S.M., Blunn, G.W. and Liddle, A.D. (2018) 'The effect of bearing surface on risk of periprosthetic joint infection in total hip arthroplasty: a systematic review and meta-analysis', *Bone Joint J*, 100-b(2), pp. 134-142.
Heydorn, A., Nielsen, A.T., Hentzer, M., Sternberg, C., Givskov, M., Ersboll, B.K. and Molin, S. (2000) 'Quantification of biofilm structures by the novel computer program COMSTAT', *Microbiology*, 146 (Pt 10), pp. 2395-2407.
Hickson, C.J., Metcalfe, D., Elgohari, S., Oswald, T., Masters, J.P., Rymaszewska, M., Reed, M.R. and Sprowson, A.P. (2015) 'Prophylactic antibiotics in elective hip and knee arthroplasty: an analysis of organisms reported to cause infections and National survey of clinical practice', *Bone Joint Res*, 4(11), pp. 181-9.
Hiltunen, A.K., Savijoki, K., Nyman, T.A., Miettinen, I., Ihalainen, P., Peltonen, J. and Fallarero, A. (2019) 'Structural and functional dynamics of *Staphylococcus aureus* biofilms and biofilm matrix proteins on different clinical materials', *Microorganisms*, 7(12), p. 584.
Hobley, L., Harkins, C., MacPhee, C.E. and Stanley-Wall, N.R. (2015) 'Giving structure to the biofilm matrix: an overview of individual strategies and emerging common themes', *FEMS Microbiol Rev*, 39(5), pp. 649-669.
Hoiby, N., Bjarnsholt, T., Givskov, M., Molin, S. and Ciofu, O. (2010) 'Antibiotic resistance of bacterial biofilms', *Int J Antimicrob Agents*, 35(4), pp. 322-32.
Hoiby, N., Ciofu, O., Johansen, H.K., Song, Z.J., Moser, C., Jensen, P.O., Molin, S., Givskov, M., Tolker-Nielsen, T. and Bjarnsholt, T. (2011) 'The clinical impact of bacterial biofilms', *Int J Oral Sci*, 3(2), pp. 55-65.
Hooper, G.J., Rothwell, A.G., Frampton, C. and Wyatt, M.C. (2011) 'Does the use of laminar flow and space suits reduce early deep infection after total hip and knee replacement?: the ten-year results of the New Zealand Joint Registry', *J Bone Joint Surg Br*, 93(1), pp. 85-90.
Houston, P., Rowe, S.E., Pozzi, C., Waters, E.M. and O'Gara, J.P. (2011) 'Essential role for the major autolysin in the fibronectin-binding protein-mediated *Staphylococcus aureus* biofilm phenotype', *Infect Immun*, 79(3), pp. 1153-65.
Hsu, Y.M., Liao, C.H., Wei, Y.H., Fang, H.W., Hou, H.H., Chen, C.C. and Chang, C.H. (2013) 'Daptomycin-loaded polymethylmethacrylate bone cement for joint arthroplasty surgery', *Artif Organs*, 29(10), p. 12197.
Hu, C.Y. and Yoon, T.-R. (2018) 'Recent updates for biomaterials used in total hip arthroplasty', *Biomater Res*, 22, pp. 33-33.
Hugle, T., Schuetz, P., Mueller, B., Laifer, G., Tyndall, A., Regenass, S. and Daikeler, T. (2008) 'Serum procalcitonin for discrimination between septic and non-septic arthritis', *Clin Exp Rheumatol*, 26(3), pp. 453-6.
Hymes, S.R., Randis, T.M., Sun, T.Y. and Ratner, A.J. (2013) 'DNase inhibits *Gardnerella vaginalis* biofilms in vitro and in vivo', *J Infect*, 207(10), pp. 1491-1497.
Ibberson, C.B., Parlet, C.P., Kwiecinski, J., Crosby, H.A., Meyerholz, D.K. and Horswill, A.R. (2016) 'Hyaluronan modulation impacts biofilm infection', *Infect Immun*, 84(6), p. 1917.
Ibrahim, M.S., Raja, S., Khan, M.A. and Haddad, F.S. (2014) 'A multidisciplinary team approach to two-stage revision for the infected hip replacement: a minimum five-year follow-up study', *Bone Joint J*, 96-b(10), pp. 1312-8.
Ionescu, A., Wutscher, E., Brambilla, E., Schneider-Feyrer, S., Giessibl, F.J. and Hahnel, S. (2012) 'Influence of surface properties of resin-based composites on in vitro *Streptococcus mutans* biofilm development', *Eur J Oral Sci*, 120(5), pp. 458-65.
Izano, E.A., Amarante, M.A., Kher, W.B. and Kaplan, J.B. (2008) 'Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus*

aureus and *Staphylococcus epidermidis* biofilms', *Appl Environ Microbiol*, 74(2), pp. 470-6.
Jacovides, C.L., Parvizi, J., Adeli, B. and Jung, K.A. (2011) 'Molecular markers for diagnosis of periprosthetic joint infection', *J Arthroplasty*, 26(6 Suppl), pp. 99-103.e1.
Jain, S. and Reed, M. (2019) 'Laminar air flow handling systems in the operating room', *Surg Infect (Larchmt)*, 20(2), pp. 151-158.
Jakubovics, N.S., Shields, R.C., Rajarajan, N. and Burgess, J.G. (2013) 'Life after death: The critical role of extracellular DNA in microbial biofilms', *Lett Appl Microbiol*, 57(6), pp. 467-475.
James, M., Khan, W.S., Nannaparaju, M.R., Bhamra, J.S. and Morgan-Jones, R. (2015) 'Current evidence for the use of laminar flow in reducing infection rates in total joint arthroplasty', *Open Orthop J*, 9, pp. 495-8.
Jeans, E., Holleyman, R., Tate, D., Reed, M. and Malviya, A. (2018) 'Methicillin sensitive *Staphylococcus aureus* screening and decolonisation in elective hip and knee arthroplasty', *J Infect*, 77(5), pp. 405-409.
Jefferson, K.K., Goldmann, D.A. and Pier, G.B. (2005) 'Use of confocal microscopy to analyze the rate of vancomycin penetration through *Staphylococcus aureus* biofilms', *Antimicrob Agents Chemother*, 49(6), pp. 2467-2473.
Jenkins, C.L. and Bean, H.D. (2019) 'Influence of media on the differentiation of Staphylococcus spp. by volatile compounds', *J Breath Res*, 14(1), p. 016007.
Jennings, J.A., Carpenter, D.P., Troxel, K.S., Beenken, K.E., Smeltzer, M.S., Courtney, H.S. and Haggard, W.O. (2015) 'Novel antibiotic-loaded point-of-care implant coating inhibits biofilm', *Clin Orthop Relat Res*, 473(7), pp. 2270-82.
Ji, B., Zhang, X., Xu, B., Guo, W., Mu, W. and Cao, L. (2017) 'Single-stage revision for chronic fungal periprosthetic joint infection: An average of 5 years of follow-up', *J Arthroplasty*, 32(8), pp. 2523-2530.
Jolivet-Gougeon, A. and Bonnaure-Mallet, M. (2014) 'Biofilms as a mechanism of bacterial resistance', *Drug Discov Today Technol*, 11, pp. 49-56.
Kakwani, R.G., Yohannan, D. and Wahab, K.H. (2007) 'The effect of laminar air-flow on the results of Austin-Moore hemiarthroplasty', *Injury*, 38(7), pp. 820-3.
Kapadia, B.H., Banerjee, S., Issa, K., McElroy, M.J., Harwin, S.F. and Mont, M.A. (2013) 'Preoperative blood management strategies for total knee arthroplasty', *J Knee Surg*, 26(6), pp. 373-7.
Kaplan, J.B. (2009) 'Therapeutic potential of biofilm-dispersing enzymes', *Int J Artif Organs*, 32(9), pp. 545-554.
Kaplan, J.B., LoVetri, K., Cardona, S.T., Madhyastha, S., Sadovskaya, I., Jabbouri, S. and Izano, E.A. (2012) 'Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in *Staphylococci*', *J Antibiot (Tokyo)*, 65(2), pp. 73-7.
Kaplan, J.B., Ragunath, C., Ramasubbu, N. and Fine, D.H. (2003) 'Detachment of *Actinobacillus actinomycetemcomitans* biofilm cells by an endogenous beta-hexosaminidase activity', *J Bacteriol*, 185(16), pp. 4693-8.
Kaplan, J.B., Ragunath, C., Velliyagounder, K., Fine, D.H. and Ramasubbu, N. (2004) 'Enzymatic detachment of *Staphylococcus epidermidis* biofilms', *Antimicrob Agents Chemother*, 48(7), pp. 2633-6.
Karlsson, A. and Arvidson, S. (2002) 'Variation in extracellular protease production among clinical isolates of *Staphylococcus aureus* due to different levels of expression of the protease repressor sarA', *Infect Immun*, 70(8), pp. 4239-4246.
Katsikogianni, M. and Missirlis, Y.F. (2004) 'Concise review of mechanisms of bacterial adhesion to biomaterials and of techniques used in estimating bacteria-material interactions', *Eur Cell Mater*, 8, pp. 37-57.

Kavanaugh, J.S. and Horswill, A.R. (2016) 'Impact of environmental cues on staphylococcal quorum sensing and biofilm development', *J Biol Chem*, 291(24), pp. 12556-64.
Kazi, H.A., Whitehouse, S.L., Howell, J.R. and Timperley, A.J. (2019) 'Not all cemented hips are the same: a register-based (NJR) comparison of taper-slip and composite beam femoral stems', *Acta Orthop*, 90(3), pp. 214-219.
Kheir, M.M., Tan, T.L., Higuera, C., George, J., Della Valle, C.J., Shen, M. and Parvizi, J. (2017) 'Periprosthetic joint Infections caused by *Enterococci* have poor outcomes', *J Arthroplasty*, 32(3), pp. 933-947.
Kierzkowska, M., Pedzisz, P., Babiak, I., Janowicz, J., Kulig, M., Majewska, A., Sawicka-Grzelak, A. and Mlynarczyk, G. (2017) 'Orthopedic infections caused by obligatory anaerobic Gram-negative rods: report of two cases', *Med Microbiol Immunol*.
Kim, J.H., Chun, S.K., Yoon, Y.C., Lakhotia, D. and Shon, W.Y. (2014) 'Efficacy of debridement for early periprosthetic joint infection after hip arthroplasty', *Hip Pelvis*, 26(4), pp. 227-34.
Kim, K., Zhu, M., Cavadino, A., Munro, J.T. and Young, S.W. (2019) 'Failed debridement and implant retention does not compromise the success of subsequent staged revision in infected total knee arthroplasty', *J Arthroplasty*, 34(6), pp. 1214-1220.e1.
Kim, S.A., Yoo, S.M., Hyun, S.H., Choi, C.H., Yang, S.Y., Kim, H.J., Jang, B.C., Suh, S.I. and Lee, J.C. (2008) 'Global gene expression patterns and induction of innate immune response in human laryngeal epithelial cells in response to *Acinetobacter baumannii* outer membrane protein A', *FEMS Immunol Med Microbiol*, 54(1), pp. 45-52.
Klouche, S., Sariali, E. and Mamoudy, P. (2010) 'Total hip arthroplasty revision due to infection: a cost analysis approach', *Orthop Traumatol Surg Res*, 96(2), pp. 124-32.
Knight, S.R., Aujla, R. and Biswas, S.P. (2011) 'Total Hip Arthroplasty - over 100 years of operative history', *Orthop Rev (Pavia)*, 3(2), p. e16.
Koseki, H., Yonekura, A., Shida, T., Yoda, I., Horiuchi, H., Morinaga, Y., Yanagihara, K., Sakoda, H., Osaki, M. and Tomita, M. (2014) 'Early staphylococcal biofilm formation on solid orthopaedic implant materials: in vitro study', *PLoS One*, 9(10), p. e107588.
Kruszewski, K.M., Nistico, L., Longwell, M.J., Hynes, M.J., Maurer, J.A., Hall-Stoodley, L. and Gawalt, E.S. (2013) 'Reducing *Staphylococcus aureus* biofilm formation on stainless steel 316L using functionalized self-assembled monolayers', *Mater Sci Eng C Mater Biol Appl*, 33(4), pp. 2059-69.
Kuiper, J.W., Vos, S.J., Saouti, R., Vergroesen, D.A., Graat, H.C., Debets-Ossenkopp, Y.J., Peters, E.J. and Nolte, P.A. (2013) 'Prosthetic joint-associated infections treated with DAIR (debridement, antibiotics, irrigation, and retention): analysis of risk factors and local antibiotic carriers in 91 patients', *Acta Orthop*, 84(4), pp. 380-6.
Kuiper, J.W., Willink, R.T., Moojen, D.J., van den Bekerom, M.P. and Colen, S. (2014) 'Treatment of acute periprosthetic infections with prosthesis retention: Review of current concepts', *World J Orthop*, 5(5), pp. 667-76.
Kumar Shukla, S. and Rao, T.S. (2013) 'Dispersal of Bap-mediated *Staphylococcus aureus* biofilm by proteinase K', *J Antibiot (Tokyo)*, 66(2), pp. 55-60.
Kuo, F.C., Lu, Y.D., Wu, C.T., You, H.L., Lee, G.B. and Lee, M.S. (2018) 'Comparison of molecular diagnosis with serum markers and synovial fluid analysis in patients with prosthetic joint infection', *Bone Joint J*, 100-b(10), pp. 1345-1351.
Kurtz, S.M., Lau, E., Watson, H., Schmier, J.K. and Parvizi, J. (2012) 'Economic burden of periprosthetic joint infection in the United States', *J Arthroplasty*, 27(8 Suppl), pp. 61-5.e1.
Kurtz, S.M. and Ong, K. (2009) 'Contemporary total hip arthroplasty: hard-on-hard bearings and highly crosslinked UHMWPE', *UHMWPE Biomaterials Handbook*, pp. 55-79.
Lamagni, T. (2014) 'Epidemiology and burden of prosthetic joint infections', *J Antimicrob Chemother*, 69 Suppl 1, pp. i5-10.

Lauderdale, K.J., Malone, C.L., Boles, B.R., Morcuende, J. and Horswill, A.R. (2010) 'Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material', *J Orthop Res*, 28(1), pp. 55-61.

Laupland, K.B. and Church, D.L. (2014) 'Population-based epidemiology and microbiology of community-onset bloodstream infections', *Clin Microbiol Rev*, 27(4), pp. 647-64.

Lawrence, J.R., Swerhone, G.D., Kuhlicke, U. and Neu, T.R. (2007) 'In situ evidence for microdomains in the polymer matrix of bacterial microcolonies', *Can J Microbiol*, 53(3), pp. 450-8.

Lebeaux D, Chauhan A, Rendueles O, Beloin C. (2013) 'From in vitro to in vivo models of bacterial biofilm-related infections'. *Pathogens*. 13; 2(2): pp 288-356.

Lee, P.Y., Costumbrado, J., Hsu, C.Y. and Kim, Y.H. (2012) 'Agarose gel electrophoresis for the separation of DNA fragments', *J Vis Exp*, (62).

Lee, Y.H. and Pratley, R.E. (2005) 'The evolving role of inflammation in obesity and the metabolic syndrome', *Curr Diab Rep*, 5(1), pp. 70-5.

Lemire, J.A., Kalan, L., Gugala, N., Bradu, A. and Turner, R.J. (2017) 'Silver oxynitrate - an efficacious compound for the prevention and eradication of dual-species biofilms', *Biofouling*, 33(6), pp. 460-469.

Leonard, H.A.C., Liddle, A.D., Burke, O., Murray, D.W. and Pandit, H. (2014) 'Single- or two-stage revision for infected total hip arthroplasty? A systematic review of the literature', *Clin Orthop Relat Res*, 472(3), pp. 1036-42.

Lewis, K. (2012) 'Persister cells: molecular mechanisms related to antibiotic tolerance', *Handb Exp Pharmacol*, (211), pp. 121-33.

Lewis, S.S., Dicks, K.V., Chen, L.F., Bolognesi, M.P., Anderson, D.J., Sexton, D.J. and Moehring, R.W. (2015) 'Delay in diagnosis of invasive surgical site infections following knee arthroplasty versus hip arthroplasty', *Clin Infect Dis*, 60(7), pp. 990-6.

Li, B., Chen, F., Liu, Y. and Xu, G. (2017a) 'Synovial fluid alpha-defensin as a biomarker for peri-prosthetic joint infection: A systematic review and meta-analysis', *Surg Infect (Larchmt)*, 18(6), pp. 702-710.

Li, J., Koh, J.J., Liu, S., Lakshminarayanan, R., Verma, C.S. and Beuerman, R.W. (2017b) 'Membrane active antimicrobial peptides: translating mechanistic insights to design', *Front Neurosci*, 11, p. 73.

Lichstein, P., Gehrke, T., Lombardi, A., Romano, C., Stockley, I., Babis, G., Bialecki, J., Bucsi, L., Cai, X., Cao, L., de Beaubien, B., Erhardt, J., Goodman, S., Jiranek, W., Keogh, P., Lewallen, D., Manner, P., Marczynski, W., Mason, J.B., Mulhall, K., Paprosky, W., Patel, P., Piccaluga, F., Polkowski, G., Pulido, L., Stockley, I., Suarez, J., Thorey, F., Tikhilov, R., Velazquez, J.D. and Winkler, H. (2014) 'One-stage versus two-stage exchange', *J Orthop Res*, 32 Suppl 1, pp. S141-6.

Lidwell, O.M., Elson, R.A., Lowbury, E.J., Whyte, W., Blowers, R., Stanley, S.J. and Lowe, D. (1987) 'Ultraclean air and antibiotics for prevention of postoperative infection. A multicenter study of 8,052 joint replacement operations', *Acta Orthop Scand*, 58(1), pp. 4-13.

Lidwell, O.M., Lowbury, E.J., Whyte, W., Blowers, R., Stanley, S.J. and Lowe, D. (1982) 'Effect of ultraclean air in operating rooms on deep sepsis in the joint after total hip or knee replacement: a randomised study', *Br Med J (Clin Res Ed)*, 285(6334), pp. 10-4.

Lidwell, O.M., Lowbury, E.J., Whyte, W., Blowers, R., Stanley, S.J. and Lowe, D. (1984) 'Infection and sepsis after operations for total hip or knee-joint replacement: influence of ultraclean air, prophylactic antibiotics and other factors', *J Hyg (Lond)*, 93(3), pp. 505-29.

Lin, D.M., Koskella, B. and Lin, H.C. (2017) 'Phage therapy: An alternative to antibiotics in the age of multi-drug resistance', *World J Gastrointest Pharmacol Ther*, 8(3), pp. 162-173.

Lister, J.L. and Horswill, A.R. (2014) '*Staphylococcus aureus* biofilms: recent developments in biofilm dispersal', *Front Cell Infect Microbiol*, 4, p. 178.

Liu, J.Z., Saleh, A., Klika, A.K., Barsoum, W.K. and Higuera, C.A. (2014) 'Serum inflammatory markers for periprosthetic knee infection in obese versus non-obese patients', *J Arthroplasty*, 29(10), pp. 1880-3.

Lora-Tamayo, J., Murillo, O., Iribarren, J.A., Soriano, A., Sanchez-Somolinos, M., Baraia-Etxaburu, J.M., Rico, A., Palomino, J., Rodriguez-Pardo, D., Horcajada, J.P., Benito, N., Bahamonde, A., Granados, A., del Toro, M.D., Cobo, J., Riera, M., Ramos, A., Jover-Saenz, A. and Ariza, J. (2013) 'A large multicenter study of methicillin-susceptible and methicillin-resistant *Staphylococcus aureus* prosthetic joint infections managed with implant retention', *Clin Infect Dis*, 56(2), pp. 182-94.

Lora-Tamayo, J., Senneville, E., Ribera, A., Bernard, L., Dupon, M., Zeller, V., Li, H.K., Arvieux, C., Clauss, M., Uckay, I., Vigante, D., Ferry, T., Iribarren, J.A., Peel, T.N., Sendi, P., Miksic, N.G., Rodriguez-Pardo, D., Del Toro, M.D., Fernandez-Sampedro, M., Dapunt, U., Huotari, K., Davis, J.S., Palomino, J., Neut, D., Clark, B.M., Gottlieb, T., Trebse, R., Soriano, A., Bahamonde, A., Guio, L., Rico, A., Salles, M.J.C., Pais, M.J.G., Benito, N., Riera, M., Gomez, L., Aboltins, C.A., Esteban, J., Horcajada, J.P., O'Connell, K., Ferrari, M., Skaliczki, G., Juan, R.S., Cobo, J., Sanchez-Somolinos, M., Ramos, A., Giannitsioti, E., Jover-Saenz, A., Baraia-Etxaburu, J.M., Barbero, J.M., Choong, P.F.M., Asseray, N., Ansart, S., Moal, G.L., Zimmerli, W. and Ariza, J. (2017) 'The not-so-good prognosis of streptococcal periprosthetic joint infection managed by implant retention: the results of a large multicenter study', *Clin Infect Dis*, 64(12), pp. 1742-1752.

Lorenzetti, M., Dogsa, I., Stosicki, T., Stopar, D., Kalin, M., Kobe, S. and Novak, S. (2015) 'The influence of surface modification on bacterial adhesion to titanium-based substrates', *ACS Appl Mater Interfaces*, 7(3), pp. 1644-51.

Lovati, A.B., Bottagisio, M., de Vecchi, E., Gallazzi, E. and Drago, L. (2017) 'Animal models of implant-related low-grade infections. A twenty-year review', *Adv Exp Med Biol*, 971, pp. 29-50.

Lowik, C.A.M., Parvizi, J., Jutte, P.C., Zijlstra, W.P., Knobben, B.A.S., Xu, C., Goswami, K., Belden, K.A., Sousa, R., Carvalho, A., Martinez-Pastor, J.C., Soriano, A. and Wouthuyzen-Bakker, M. (2019) 'Debridement, antibiotics and implant retention is a viable treatment option for early periprosthetic joint infection presenting more than four weeks after index arthroplasty', *Clin Infect Dis*.

Lübbeke, A., Zingg, M., Vu, D., Miozzari, H.H., Christofilopoulos, P., Uçkay, I., Harbarth, S. and Hoffmeyer, P. (2016) 'Body mass and weight thresholds for increased prosthetic joint infection rates after primary total joint arthroplasty', *Acta Orthop*, 87(2), pp. 132-138.

Macdonald, N. and Bankes, M. (2014) 'Ceramic on ceramic hip prostheses: a review of past and modern materials', *Arch Orthop Trauma Surg*, 134(9), pp. 1325-33.

Mack, D., Fischer, W., Krokotsch, A., Leopold, K., Hartmann, R., Egge, H. and Laufs, R. (1996) 'The intercellular adhesin involved in biofilm accumulation of *Staphylococcus epidermidis* is a linear beta-1,6-linked glucosaminoglycan: purification and structural analysis', *J Bacteriol*, 178(1), pp. 175-83.

Madanat, R., Laaksonen, I., Graves, S.E., Lorimer, M., Muratoglu, O. and Malchau, H. (2018) 'Ceramic bearings for total hip arthroplasty are associated with a reduced risk of revision for infection', *Hip Int*, 28(3), pp. 222-226.

Maempel, J.F., Wickramasinghe, N.R., Clement, N.D., Brenkel, I.J. and Walmsley, P.J. (2016) 'The pre-operative levels of haemoglobin in the blood can be used to predict the risk of allogenic blood transfusion after total knee arthroplasty', *Bone Joint J*, 98-b(4), pp. 490-7.

Mah, T.F. (2012) 'Biofilm-specific antibiotic resistance', *Future Microbiol*, 7(9), pp. 1061-72.
Mah, T.F. and O'Toole, G.A. (2001) 'Mechanisms of biofilm resistance to antimicrobial agents', *Trends Microbiol*, 9(1), pp. 34-9.
Maiello, A., Leo, G., De Blasi, T., Cariti, G. and Di Ferri, G. (2005) *Orthopaedic Proceedings*. The British Editorial Society of Bone & Joint Surgery.
Makki, D., Elgamal, T., Evans, P., Harvey, D., Jackson, G. and Platt, S. (2017) 'The orthopaedic manifestation and outcomes of methicillin-sensitive *Staphylococcus aureus* septicaemia', *Bone Joint J*, 99-b(11), pp. 1545-1551.
Malekzadeh, D., Osmon, D.R., Lahr, B.D., Hanssen, A.D. and Berbari, E.F. (2010) 'Prior use of antimicrobial therapy is a risk factor for culture-negative prosthetic joint infection', *Clin Orthop Relat Res*, 468(8), pp. 2039-45.
Malhotra, R., Dhawan, B., Garg, B., Shankar, V. and Nag, T.C. (2019) 'A comparison of bacterial adhesion and biofilm formation on commonly used orthopaedic metal implant materials: An in vitro study', *Indian J Orthop*, 53(1), pp. 148-153.
Manandhar, S., Singh, A., Varma, A., Pandey, S. and Shrivastava, N. (2018) 'Biofilm producing clinical *Staphylococcus aureus* isolates augmented prevalence of antibiotic resistant cases in tertiary care hospitals of Nepal', *Front Microbiol*, 9, p. 2749.
Manger, I.D. and Relman, D.A. (2000) 'How the host 'sees' pathogens: global gene expression responses to infection', *Curr Opin Immunol*, 12(2), pp. 215-8.
Mann, E.E., Rice, K.C., Boles, B.R., Endres, J.L., Ranjit, D., Chandramohan, L., Tsang, L.H., Smeltzer, M.S., Horswill, A.R. and Bayles, K.W. (2009) 'Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation', *PLoS One*, 4(6), p. e5822.
Manuel, S.G., Ragunath, C., Sait, H.B., Izano, E.A., Kaplan, J.B. and Ramasubbu, N. (2007) 'Role of active-site residues of dispersin B, a biofilm-releasing beta-hexosaminidase from a periodontal pathogen, in substrate hydrolysis', *Febs j*, 274(22), pp. 5987-99.
Manzenreiter, R., Kienberger, F., Marcos, V., Schilcher, K., Krautgartner, W.D., Obermayer, A., Huml, M., Stoiber, W., Hector, A., Griese, M., Hannig, M., Studnicka, M., Vitkov, L. and Hartl, D. (2012) 'Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy', *J Cyst Fibros*, 11(2), pp. 84-92.
Marculescu, C.E., Berbari, E.F., Hanssen, A.D., Steckelberg, J.M., Harmsen, S.W., Mandrekar, J.N. and Osmon, D.R. (2006) 'Outcome of prosthetic joint infections treated with debridement and retention of components', *Clin Infect Dis*, 42(4), pp. 471-8.
Marculescu, C.E. and Cantey, J.R. (2008) 'Polymicrobial prosthetic joint infections: risk factors and outcome', *Clin Orthop Relat Res*, 466(6), pp. 1397-404.
Maric, S. and Vranes, J. (2007) 'Characteristics and significance of microbial biofilm formation', *Period Bilogor*, (109), pp. 115-121.
Markatos, K., Kaseta, M. and Nikolaou, V.S. (2015) 'Perioperative skin preparation and draping in modern total joint arthroplasty: current evidence', *Surg Infect (Larchmt)*, 16(3), pp. 221-5.
Marks, K.E., Nelson, C.L. and Lautenschlager, E.P. (1976) 'Antibiotic-impregnated acrylic bone cement', *J Bone Joint Surg Am*, 58(3), pp. 358-64.
Masters, J.P., Smith, N.A., Foguet, P., Reed, M., Parsons, H. and Sprowson, A.P. (2013) 'A systematic review of the evidence for single stage and two stage revision of infected knee replacement', *BMC Musculoskelet Disord*, 14, p. 222.
Mathur, T., Singhal, S., Khan, S., Upadhyay, D., Fatma, T. and Rattan, A. (2005) 'Adverse effect of staphylococci slime on in vitro activity of glycopeptides', *Jpn J Infect Dis*, 58(6), p. 353.
Matos, A.C., Goncalves, L.M., Rijo, P., Vaz, M.A., Almeida, A.J. and Bettencourt, A.F. (2014) 'A novel modified acrylic bone cement matrix. A step forward on antibiotic

delivery against multiresistant bacteria responsible for prosthetic joint infections', *Mater Sci Eng C Mater Biol Appl*, 38, pp. 218-26.
Matussek, A., Strindhall, J., Stark, L., Rohde, M., Geffers, R., Buer, J., Kihlstrom, E., Lindgren, P.E. and Lofgren, S. (2005) 'Infection of human endothelial cells with *Staphylococcus aureus* induces transcription of genes encoding an innate immunity response', *Scand J Immunol*, 61(6), pp. 536-44.
McGavin, M.J., Zahradka, C., Rice, K. and Scott, J.E. (1997) 'Modification of the *Staphylococcus aureus* fibronectin binding phenotype by V8 protease', *Infect Immun*, 65(7), pp. 2621-8.
Melville, S. and Craig, L. (2013) 'Type IV pili in Gram-positive bacteria', *Microbiol Mol Biol Rev*, 77(3), pp. 323-41.
Merghni, A., Bekir, K., Kadmi, Y., Dallel, I., Janel, S., Bovio, S., Barois, N., Lafont, F. and Mastouri, M. (2017) 'Adhesiveness of opportunistic *Staphylococcus aureus* to materials used in dental office: In vitro study', *Microb Pathog*, 103, pp. 129-134.
Merino, N., Toledo-Arana, A., Vergara-Irigaray, M., Valle, J., Solano, C., Calvo, E., Lopez, J.A., Foster, T.J., Penades, J.R. and Lasa, I. (2009) 'Protein A-mediated multicellular behavior in *Staphylococcus aureus*', *J Bacteriol*, 191(3), pp. 832-43.
Merola, M. and Affatato, S. (2019) 'Materials for hip prostheses: A review of wear and loading considerations', *Materials (Basel)*, 12(3).
Merritt, J.H., Kadouri, D.E. and O'Toole, G.A. (2005) 'Growing and analyzing static biofilms', *Curr Protoc Microbiol*, Chapter 1, pp. Unit 1B.1-Unit 1B.1.
Mikkelsen, H., Sivaneson, M. and Filloux, A. (2011) 'Key two-component regulatory systems that control biofilm formation in *Pseudomonas aeruginosa*', *Environ Microbiol*, 13(7), pp. 1666-81.
Encyclopedia and Dictionary of Medicine, Nursing, and Allied Health (2012) Elsevier.
Mirza, Y.H., Tansey, R., Sukeik, M., Shaath, M. and Haddad, F.S. (2016) 'Biofilm and the role of antibiotics in the treatment of periprosthetic hip and knee joint infections', *Open Orthop J*, 10, pp. 636-645.
Missiakas, D.M. and Schneewind, O. (2013) 'Growth and laboratory maintenance of *Staphylococcus aureus*', *Curr Protoc Microbiol*, Chapter 9, pp. Unit-9C.1.
Moley, J.P., McGrath, M.S., Granger, J.F., Stoodley, P. and Dusane, D.H. (2018) 'Reduction in *Pseudomonas aeruginosa* and *Staphylococcus aureus* biofilms from implant materials in a diffusion dominated environment', *J Orthop Res*, 36(11), pp. 3081-3085.
Molina-Manso, D., del Prado, G., Ortiz-Perez, A., Manrubia-Cobo, M., Gomez-Barrena, E., Cordero-Ampuero, J. and Esteban, J. (2013) 'In vitro susceptibility to antibiotics of staphylococci in biofilms isolated from orthopaedic infections', *Int J Antimicrob Agents*, 41(6), pp. 521-3.
Moormeier, D.E. and Bayles, K.W. (2017) '*Staphylococcus aureus* biofilm: a complex developmental organism', *Mol Microbiol*, 104(3), pp. 365-376.
Moormeier, D.E., Bose, J.L., Horswill, A.R. and Bayles, K.W. (2014) 'Temporal and stochastic control of *Staphylococcus aureus* biofilm development', *MBio*, 5(5), pp. e01341-14.
Mootz, J.M., Malone, C.L., Shaw, L.N. and Horswill, A.R. (2013) 'Staphopains modulate *Staphylococcus aureus* biofilm integrity', *Infect Immun*, 81(9), pp. 3227-38.
Moran, E., Masters, S., Berendt, A.R., McLardy-Smith, P., Byren, I. and Atkins, B.L. (2007) 'Guiding empirical antibiotic therapy in orthopaedics: The microbiology of prosthetic joint infection managed by debridement, irrigation and prosthesis retention', *J Infect*, 55(1), pp. 1-7.
Moroski, N.M., Woolwine, S. and Schwarzkopf, R. (2015) 'Is preoperative staphylococcal decolonization efficient in total joint arthroplasty', *The Journal of Arthroplasty*, 30(3), pp. 444-446.

Morris, J.L., Letson, H.L., Elliott, L., Grant, A.L., Wilkinson, M., Hazratwala, K. and McEwen, P. (2019) 'Evaluation of bacteriophage as an adjunct therapy for treatment of peri-prosthetic joint infection caused by *Staphylococcus aureus*', *PloS one*, 14(12), pp. e0226574-e0226574.

Mortazavi, S.M.J., Schwartzenberger, J., Austin, M.S., Purtill, J.J. and Parvizi, J. (2010) 'Revision total knee arthroplasty infection: incidence and predictors', *Clin Orthop Relat Res*, 468(8), pp. 2052-9.

Mu, H., Tang, J., Liu, Q., Sun, C., Wang, T. and Duan, J. (2016) 'Potent antibacterial nanoparticles against biofilm and intracellular bacteria', *Sci Rep*, 6, p. 18877.

Muhlhofer, H.M.L., Knebel, C., Pohlig, F., Feihl, S., Harrasser, N., Schauwecker, J. and von Eisenhart-Rothe, R. (2018) 'Synovial aspiration and serological testing in two-stage revision arthroplasty for prosthetic joint infection: evaluation before reconstruction with a mean follow-up of twenty seven months', *Int Orthop*, 42(2), pp. 265-271.

Mulcahy, H., Charron-Mazenod, L. and Lewenza, S. (2008) 'Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms', *Plos Pathog*, 4(11).

Murgier, J., Laffosse, J.M., Cailliez, J., Cavaignac, E., Murgier, P., Bayle-Iniguez, X., Chiron, P. and Bonnevialle, P. (2016) 'Is the prognosis the same for periprosthetic joint infections due to *Staphylococcus aureus* versus coagulase-negative staphylococci? A retrospective study of 101 patients with 2-year minimum follow-up', *Arch Orthop Trauma Surg*, 136(10), pp. 1357-61.

Nair, S., Desai, S., Poonacha, N., Vipra, A. and Sharma, U. (2016) 'Antibiofilm activity and synergistic inhibition of *S. aureu*s biofilms by bactericidal protein P128 in combination with antibiotics', *Antimicrob Agents Chemother*.

Narayanan, R., Anoushiravani, A.A., Elbuluk, A.M., Chen, K.K., Adler, E.M. and Schwarzkopf, R. (2018) 'Irrigation and debridement for early periprosthetic knee infection: Is it effective?', *J Arthroplasty*.

National Joint Registry (2015) 12th annual report 2015. National Joint Registry for England, Wales and Northern Ireland.

National Joint Registry (2019) '16th annual report 2019. National Joint Registry for England, Wales and Northern Ireland.'.

Nguyen, D., Joshi-Datar, A., Lepine, F., Bauerle, E., Olakanmi, O., Beer, K., McKay, G., Siehnel, R., Schafhauser, J., Wang, Y., Britigan, B.E. and Singh, P.K. (2011) 'Active starvation responses mediate antibiotic tolerance in biofilms and nutrient-limited bacteria', *Science*, 334(6058), p. 982.

Nguyen, T., Ghebrehiwet, B. and Peerschke, E.I. (2000) '*Staphylococcus aureus* protein A recognizes platelet gC1qR/p33: a novel mechanism for staphylococcal interactions with platelets', *Infect Immun*, 68(4), pp. 2061-8.

Nguyen, U.T. and Burrows, L.L. (2014) 'DNase I and proteinase K impair *Listeria monocytogenes* biofilm formation and induce dispersal of pre-existing biofilms', *Int J Food Microbiol*, 187, pp. 26-32.

Nielsen, C.K., Subbiahdoss, G., Zeng, G., Salmi, Z., Kjems, J., Mygind, T., Snabe, T. and Meyer, R.L. (2018) 'Antibacterial isoeugenol coating on stainless steel and polyethylene surfaces prevents biofilm growth', *J Appl Microbiol*, 124(1), pp. 179-187.

Nijland, R., Hall, M.J. and Burgess, J.G. (2010) 'Dispersal of biofilms by secreted, matrix degrading, bacterial DNase', *PLoS One*, 5(12), p. e15668.

Norambuena, G.A., Patel, R., Karau, M., Wyles, C.C., Jannetto, P.J., Bennet, K.E., Hanssen, A.D. and Sierra, R.J. (2017) 'Antibacterial and Bbocompatible titanium-copper oxide coating may be a potential strategy to reduce periprosthetic infection: An in vitro study', *Clin Orthop Relat Res*, 475(3), pp. 722-732.

Novotny, L.A., Clements, J.D. and Bakaletz, L.O. (2011) 'Transcutaneous immunization as preventative and therapeutic regimens to protect against experimental otitis media due to nontypeable *Haemophilus influenzae*', *Mucosal Immunol*, 4(4), pp. 456-67.
Nur, A., Hirota, K., Yumoto, H., Hirao, K., Liu, D., Takahashi, K., Murakami, K., Matsuo, T., Shu, R. and Miyake, Y. (2013) 'Effects of extracellular DNA and DNA-binding protein on the development of a *Streptococcus intermedius* biofilm', *J Appl Microbiol*, 115(1), pp. 260-270.
O'Gara, J.P. (2007) 'ica and beyond: biofilm mechanisms and regulation in *Staphylococcus epidermidis* and *Staphylococcus aureus*', *FEMS Microbiol Lett*, 270(2), pp. 179-88.
O'Neill, E., Pozzi, C., Houston, P., Humphreys, H., Robinson, D.A., Loughman, A., Foster, T.J. and O'Gara, J.P. (2008) 'A novel *Staphylococcus aureus* biofilm phenotype mediated by the fibronectin-binding proteins, FnBPA and FnBPB', *J Bacteriol*, 190(11), pp. 3835-50.
O'Toole, G., Kaplan, H.B. and Kolter, R. (2000) 'Biofilm formation as microbial development', *Annl Rev Microbiol*, 54, pp. 49-79.
Okshevsky, M., Regina, V.R. and Meyer, R.L. (2015) 'Extracellular DNA as a target for biofilm control', *Curr Opin Biotech*, 33, pp. 73-80.
Oliveira, M., Nunes, S.F., Carneiro, C., Bexiga, R., Bernardo, F. and Vilela, C.L. (2007) 'Time course of biofilm formation by *Staphylococcus aureus* and *Staphylococcus epidermidis* mastitis isolates', *Vet Microbiol*, 124(1-2), pp. 187-91.
Oliveira, W.F., Silva, P.M.S., Silva, R.C.S., Silva, G.M.M., Machado, G., Coelho, L. and Correia, M.T.S. (2018) '*Staphylococcus aureus* and *Staphylococcus epidermidis* infections on implants', *J Hosp Infect*, 98(2), pp. 111-117.
Olofsson, A.C., Hermansson, M. and Elwing, H. (2003) 'N-acetyl-L-cysteine affects growth, extracellular polysaccharide production, and bacterial biofilm formation on solid surfaces', *Appl Environ Microbiol*, 69(8), pp. 4814-22.
Olson, M.E., Ceri, H., Morck, D.W., Buret, A.G. and Read, R.R. (2002) 'Biofilm bacteria: formation and comparative susceptibility to antibiotics', *Can J Vet Res*, 66(2), pp. 86-92.
Opperman, T.J., Kwasny, S.M., Williams, J.D., Khan, A.R., Peet, N.P., Moir, D.T. and Bowlin, T.L. (2009) 'Aryl rhodanines specifically inhibit staphylococcal and enterococcal biofilm formation', *Antimicrob Agents Chemother*, 53(10), pp. 4357-67.
Orapiriyakul, W., Young, P.S., Damiati, L. and Tsimbouri, P.M. (2018) 'Antibacterial surface modification of titanium implants in orthopaedics', *J Tissue Eng*, 9, p. 2041731418789838.
Osmon, D.R., Berbari, E.F., Berendt, A.R., Lew, D., Zimmerli, W., Steckelberg, J.M., Rao, N., Hanssen, A. and Wilson, W.R. (2013) 'Diagnosis and management of prosthetic joint infection: Clinical practice guidelines by the infectious diseases Society of America', *Clin Infec Dis*, 56(1), pp. e1-e25.
Otto, M. (2008) 'Staphylococcal biofilms', *Curr Top Microbiol Immunol*, 322, pp. 207-28.
Otto, M. (2009) '*Staphylococcus epidermidis*--the 'accidental' pathogen', *Nat Rev Microbiol*, 7(8), pp. 555-67.
Oussedik, S., Gould, K., Stockley, I. and Haddad, F.S. (2012) 'Defining peri-prosthetic infection: do we have a workable gold standard?', *J Bone Joint Sur Br*, 94(11), pp. 1455-6.
Oxford English Dictionary 'Oxford English Dictionary', *Oxford English Dictionary*.
Parikh, M., Antony, S. (2016) 'A comprehensive review of the diagnosis and management of prosthetic joint infections in the absence of positive cultures', J. Infect. Public Health, 9 (5), pp. 545-556.
Parkinson, R.W., Kay, P.R. and Rawal, A. (2011) 'A case for one-stage revision in infected total knee arthroplasty?', *Knee*, 18(1), pp. 1-4.

Parvizi, J., Azzam, K., Ghanem, E., Austin, M.S. and Rothman, R.H. (2009) 'Periprosthetic infection due to resistant staphylococci: serious problems on the horizon', *Clin Orthop Relat Res*, 467(7), pp. 1732-9.
Parvizi, J. and Della Valle, C.J. (2010) 'AAOS Clinical Practice Guideline: diagnosis and treatment of periprosthetic joint infections of the hip and knee', *J Am Acad Orthop Surg*, 18(12), pp. 771-2.
Parvizi, J., Fassihi, S.C. and Enayatollahi, M.A. (2016) 'Diagnosis of periprosthetic joint infection following hip and knee arthroplasty', *Orthop Clin North Am*, 47(3), pp. 505-15.
Parvizi, J., Gehrke, T. and Chen, A.F. (2013) 'Proceedings of the international consensus on periprosthetic joint infection', *Bone Joint J*, 95-b(11), pp. 1450-2.
Parvizi, J., Tan, T.L., Goswami, K., Higuera, C., Della Valle, C., Chen, A.F. and Shohat, N. (2018) 'The 2018 definition of periprosthetic hip and knee infection: An evidence-based and validated criteria', *J Arthroplasty*, 33(5), pp. 1309-1314.e2.
Patel, S.S., Aruni, W., Inceoglu, S., Akpolat, Y.T., Botimer, G.D., Cheng, W.K. and Danisa, O.A. (2016) 'A comparison of *Staphylococcus aureus* biofilm formation on cobalt-chrome and titanium-alloy spinal implants', *J Clin Neurosci*, 31, pp. 219-223.
Patenge, N., Arndt, K., Eggert, T., Zietz, C., Kreikemeyer, B., Bader, R., Nebe, B., Stranak, V., Hippler, R. and Podbielski, A. (2012) 'Evaluation of antimicrobial effects of novel implant materials by testing the prevention of biofilm formation using a simple small scale medium-throughput growth inhibition assay', *Biofouling*, 28(3), pp. 267-77.
Pavithra, D. and Doble, M. (2008) 'Biofilm formation, bacterial adhesion and host response on polymeric implants--issues and prevention', *Biomed Mater*, 3(3), p. 034003.
Peel, T.N., Cheng, A.C., Buising, K.L. and Choong, P.F. (2012) 'Microbiological aetiology, epidemiology, and clinical profile of prosthetic joint infections: are current antibiotic prophylaxis guidelines effective?', *Antimicrob Agents Chemother*, 56(5), pp. 2386-91.
Peel, T.N., Cheng, A.C., Lorenzo, Y.P., Kong, D.C., Buising, K.L. and Choong, P.F. (2013) 'Factors influencing the cost of prosthetic joint infection treatment', *J Hosp Infect*, 85(3), pp. 213-9.
Peeters, O., Ferry, T., Ader, F., Boibieux, A., Braun, E., Bouaziz, A., Karsenty, J., Forestier, E., Laurent, F., Lustig, S., Chidiac, C., Valour, F. and Lyon, B.J.I.s.g. (2016) 'Teicoplanin-based antimicrobial therapy in *Staphylococcus aureus* bone and joint infection: tolerance, efficacy and experience with subcutaneous administration', *BMC Infect Dis*, 16(1), pp. 622-622.
Perez-Jorge, C., Gomez-Barrena, E., Horcajada, J.P., Puig-Verdie, L. and Esteban, J. (2016) 'Drug treatments for prosthetic joint infections in the era of multidrug resistance', *Expert Opin Pharmacother*, 17(9), pp. 1233-46.
Periasamy, S., Joo, H.S., Duong, A.C., Bach, T.H., Tan, V.Y., Chatterjee, S.S., Cheung, G.Y. and Otto, M. (2012) 'How *Staphylococcus aureus* biofilms develop their characteristic structure', *Proc Natl Acad Sci U S A*, 109(4), pp. 1281-6.
Peterson, B.W., He, Y., Ren, Y., Zerdoum, A., Libera, M.R., Sharma, P.K., van Winkelhoff, A.J., Neut, D., Stoodley, P., van der Mei, H.C. and Busscher, H.J. (2015) 'Viscoelasticity of biofilms and their recalcitrance to mechanical and chemical challenges', *FEMS Microbiol Rev*, 39(2), pp. 234-45.
Petheram, T.G., Bone, M., Joyce, T.J., Serrano-Pedraza, I., Reed, M.R. and Partington, P.F. (2013) 'Surface finish of the Exeter Trauma Stem: a cause for concern?', *Bone Joint J*, 95-B(2), pp. 173-176.
Petretta, R., Phillips, J. and Toms, A. (2016) 'Management of acute periprosthetic joint infection of the knee - Algorithms for the on call surgeon', *Surgeon*.
Pierce, C.G., Uppuluri, P., Tristan, A.R., Wormley, F.L., Jr., Mowat, E., Ramage, G. and Lopez-Ribot, J.L. (2008) 'A simple and reproducible 96-well plate-based method for

the formation of fungal biofilms and its application to antifungal susceptibility testing', *Nat Protoc*, 3(9), pp. 1494-500.
Pietrocola, G., Nobile, G., Rindi, S. and Speziale, P. (2017) '*Staphylococcus aureus* manipulates innate immunity through own and host-expressed proteases', *Front Microbiol*, 7, pp. 166-166.
Pijls, B.G., Meessen, J.M., Schoones, J.W., Fiocco, M., van der Heide, H.J., Sedrakyan, A. and Nelissen, R.G. (2016) 'Increased mortality in metal-on-metal versus non-metal-on-metal primary total hip arthroplasty at 10 years and longer follow-up: A systematic review and meta-analysis', *PLoS One*, 11(6), p. e0156051.
Pilz, M., Staats, K., Tobudic, S., Assadian, O., Presterl, E., Windhager, R. and Holinka, J. (2019) 'Zirconium nitride coating reduced *Staphylococcus epidermidis* biofilm formation on orthopaedic implant surfaces: An in vitro study', *Clin Orthop Relat Res*, 477(2), pp. 461-466.
Pitt, D. and Aubin, J.M. (2012) 'Joseph Lister: father of modern surgery', *Can J Surg*, 55(5), pp. E8-9.
Pitto, R.P. and Sedel, L. (2016) 'Periprosthetic joint infection in hip arthroplasty: Is there an association between infection and bearing surface type?', *Clin Orthop Relat Res*, 474(10), pp. 2213-8.
Pitts, B., Hamilton, M.A., Zelver, N. and Stewart, P.S. (2003) 'A microtiter-plate screening method for biofilm disinfection and removal', *J Microbiol Methods*, 54(2), pp. 269-76.
Poole, K. (2007) 'Efflux pumps as antimicrobial resistance mechanisms', *Ann Med*, 39(3), pp. 162-76.
Popat, K.C., Eltgroth, M., Latempa, T.J., Grimes, C.A. and Desai, T.A. (2007) 'Decreased *Staphylococcus epidermis* adhesion and increased osteoblast functionality on antibiotic-loaded titania nanotubes', *Biomaterials*, 28(32), pp. 4880-8.
Prokesová, L., Potuzníková, B., Potempa, J., Zikán, J., Radl, J., Hachová, L., Baran, K., Porwit-Bobr, Z. and John, C. (1992) 'Cleavage of human immunoglobulins by serine proteinase from *Staphylococcus aureus*', *Immunol Lett*, 31(3), pp. 259-65.
Prokuski, L. (2008) 'Prophylactic antibiotics in orthopaedic surgery', *J Am Acad Orthop Surg*, 16(5), pp. 283-93.
Pulido, L., Ghanem, E., Joshi, A., Purtill, J.J. and Parvizi, J. (2008) 'Periprosthetic joint infection: the incidence, timing, and predisposing factors', *Clin Orthop Relat Res*, 466(7), pp. 1710-5.
Qasim, S.N., Swann, A. and Ashford, R. (2017) 'The DAIR (debridement, antibiotics and implant retention) procedure for infected total knee replacement - a literature review', *Sicot j*, 3, p. 2.
Qin, Z., Ou, Y., Yang, L., Zhu, Y., Tolker-Nielsen, T., Molin, S. and Qu, D. (2007) 'Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*', *Microbiology*, 153(Pt 7), pp. 2083-92.
Qin, Z., Zhang, J., Hu, Y., Chi, Q., Mortensen, N.P., Qu, D., Molin, S. and Ulstrup, J. (2009) 'Organic compounds inhibiting S. epidermidis adhesion and biofilm formation', *Ultramicroscopy*, 109(8), pp. 881-8.
Rajarajan, N., Ward, A.C., Burgess, J.G. and Glassey, J. (2013) 'Use of physiological information and process optimisation enhances production of extracellular nuclease by a marine strain of *Bacillus licheniformis*', *Bioresour Technol*, 130, pp. 552-558.
Rajgopal, A., Panda, I., Rao, A., Dahiya, V. and Gupta, H. (2018) 'Does prior failed debridement compromise the outcome of subsequent two-stage revision done for periprosthetic joint infection following total knee arthroplasty?', *J Arthroplasty*.
Ramasubbu, N., Thomas, L.M., Ragunath, C. and Kaplan, J.B. (2005) 'Structural analysis of dispersin B, a biofilm-releasing glycoside hydrolase from the periodontopathogen Actinobacillus actinomycetemcomitans', *J Mol Biol*, 349(3), pp. 475-86.

Rand BC, Penn-Barwell JG, Wenke JC. (2015). 'Combined local and systemic antibiotic delivery improves eradication of wound contamination: An animal experimental model of contaminated fracture'. Bone Joint J. 2015 Oct;97-B(10), pp.1423-7.

Randelli, P., Evola, F.R., Cabitza, P., Polli, L., Denti, M. and Vaienti, L. (2010) 'Prophylactic use of antibiotic-loaded bone cement in primary total knee replacement', *Knee Surg Sports Traumatol Arthrosc*, 18(2), pp. 181-186.

Rang, M. (1966) *Anthology of Orthopaedics.* Edinburgh, London, New York: Churchill Livingstone.

Rao, N., Cannella, B., Crossett, L.S., Yates, A.J., Jr. and McGough, R., 3rd (2008) 'A preoperative decolonization protocol for *Staphylococcus aureus* prevents orthopaedic infections', *Clin Orthop Relat Res*, 466(6), pp. 1343-8.

Rao, N., Cannella, B.A., Crossett, L.S., Yates, A.J., Jr., McGough, R.L., 3rd and Hamilton, C.W. (2011) 'Preoperative screening/decolonization for *Staphylococcus aureus* to prevent orthopedic surgical site infection: prospective cohort study with 2-year follow-up', *J Arthroplasty*, 26(8), pp. 1501-7.

Reffuveille, F., de la Fuente-Nunez, C., Mansour, S. and Hancock, R.E. (2014) 'A broad-spectrum antibiofilm peptide enhances antibiotic action against bacterial biofilms', *Antimicrob Agents Chemother*, 58(9), pp. 5363-71.

Rendueles, O., Kaplan, J.B. and Ghigo, J.M. (2013) 'Antibiofilm polysaccharides', *Environ Microbiol*, 15(2), pp. 334-46.

Renner, L.D. and Weibel, D.B. (2011) 'Physicochemical regulation of biofilm formation', *MRS Bull*, 36(5), pp. 347-355.

Roach, D.R. and Donovan, D.M. (2015) 'Antimicrobial bacteriophage-derived proteins and therapeutic applications', *Bacteriophage*, 5(3), p. e1062590.

Roberts AE, Kragh KN, Bjarnsholt T, Diggle SP (2015). 'The limitations of in vitro experimentation in understanding biofilms and chronic infection'. J Mol Biol, 427(23), pp 3646-61.

Roche, F.M., Meehan, M. and Foster, T.J. (2003) 'The *Staphylococcus aureus* surface protein SasG and its homologues promote bacterial adherence to human desquamated nasal epithelial cells', *Microbiology*, 149(Pt 10), pp. 2759-67.

Rodríguez-Martínez, J.M., Ballesta, S. and Pascual, Á. (2007) 'Activity and penetration of fosfomycin, ciprofloxacin, amoxicillin/clavulanic acid and co-trimoxazole in *Escherichia coli* and *Pseudomonas aeruginosa* biofilms', *Int J Antimicrob Agents*, 30(4), pp. 366-368.

Romano, C.L., Manzi, G., Logoluso, N. and Romano, D. (2012) 'Value of debridement and irrigation for the treatment of peri-prosthetic infections. A systematic review', *Hip Int*, 22(8).

Routh, M.D., Zalucki, Y., Su, C.C., Zhang, Q., Shafer, W.M. and Yu, E.W. (2011) 'Efflux pumps of the resistance-nodulation-division family: a perspective of their structure, function, and regulation in gram-negative bacteria', *Adv Enzymol Relat Areas Mol Biol*, 77, pp. 109-46.

Salgado, C.D., Dash, S., Cantey, J.R. and Marculescu, C.E. (2007) 'Higher risk of failure of methicillin-resistant *Staphylococcus aureus* prosthetic joint infections', *Clin Orthop Relat Res*, 461, pp. 48-53.

Sambri, A., Pignatti, G., Romagnoli, M., Donati, D., Marcacci, M. and Cadossi, M. (2017) 'Intraoperative diagnosis of *Staphylococcus aureus* and coagulase-negative Staphylococcus using Xpert MRSA/SA SSTI assay in prosthetic joint infection', *New Microbiol*, 40(2), pp. 130-134.

Sanders, E.R. (2012) 'Aseptic laboratory techniques: plating methods', *JoVE J Vis Exp*, (63), pp. e3064-e3064.

Savage, V.J., Chopra, I. and O'Neill, A.J. (2013) '*Staphylococcus aureus* biofilms promote horizontal transfer of antibiotic resistance', *Antimicrob Agents Chemother*, 57(4), pp. 1968-1970.

Schiavone Panni, A., Corona, K., Giulianelli, M., Mazzitelli, G., Del Regno, C. and Vasso, M. (2016) 'Antibiotic-loaded bone cement reduces risk of infections in primary total knee arthroplasty? A systematic review', *Knee Surg Sports Traumatol Arthrosc*, 24(10), pp. 3168-3174.

Schmidt, C. (2019) 'Phage therapy's latest makeover', *Nat Biotechnol*, 37(6), pp. 581-586.

Schooling, S.R., Hubley, A. and Beveridge, T.J. (2009) 'Interactions of DNA with biofilm-derived membrane vesicles', *J Bacteriol*, 191(13), pp. 4097-4102.

Schroeder, K., Jularic, M., Horsburgh, S.M., Hirschhausen, N., Neumann, C., Bertling, A., Schulte, A., Foster, S., Kehrel, B.E., Peters, G. and Heilmann, C. (2009) 'Molecular characterization of a novel *Staphylococcus aureus* surface protein (SasC) involved in cell aggregation and biofilm accumulation', *PLoS One*, 4(10), p. e7567.

Sedrakyan, A., Normand, S.L., Dabic, S., Jacobs, S., Graves, S. and Marinac-Dabic, D. (2011) 'Comparative assessment of implantable hip devices with different bearing surfaces: systematic appraisal of evidence', *Bmj*, 343, p. d7434.

Seidl, K., Goerke, C., Wolz, C., Mack, D., Berger-Bachi, B. and Bischoff, M. (2008) '*Staphylococcus aureus* CcpA affects biofilm formation', *Infect Immun*, 76(5), pp. 2044-50.

Senneville, E., Joulie, D., Legout, L., Valette, M., Dezeque, H., Beltrand, E., Rosele, B., d'Escrivan, T., Loiez, C., Caillaux, M., Yazdanpanah, Y., Maynou, C. and Migaud, H. (2011) 'Outcome and predictors of treatment failure in total hip/knee prosthetic joint infections due to *Staphylococcus aureus*', *Clin Infect Dis*, 53(4), pp. 334-40.

Sentuerk, U., von Roth, P. and Perka, C. (2016) 'Ceramic on ceramic arthroplasty of the hip: new materials confirm appropriate use in young patients', *Bone Joint J*, 98-b(1 Suppl A), pp. 14-7.

Shah, K., Mohammed, A., Patil, S., McFadyen, A. and Meek, R.M. (2009) 'Circulating cytokines after hip and knee arthroplasty: a preliminary study', *Clin Orthop Relat Res*, 467(4), pp. 946-51.

Shahi, A. and Parvizi, J. (2015) 'Prevention of periprosthetic joint infection', *Arch Bone Jt Surg*, 3(2), pp. 72-81.

Shahi, A. and Parvizi, J. (2016) 'The role of biomarkers in the diagnosis of periprosthetic joint infection', *EFORT Open Rev*, 1(7), pp. 275-278.

Shahi, A., Parvizi, J., Kazarian, G.S., Higuera, C., Frangiamore, S., Bingham, J., Beauchamp, C., Valle, C.D. and Deirmengian, C. (2016) 'The alpha-defensin test for periprosthetic joint infections is not affected by prior antibiotic administration', *Clin Orthop Relat Res*, 474(7), pp. 1610-5.

Shakeri, S., Kermanshahi, R.K., Moghaddam, M.M. and Emtiazi, G. (2007) 'Assessment of biofilm cell removal and killing and biocide efficacy using the microtiter plate test', *Biofouling*, 23(1-2), pp. 79-86.

Shakir, A., Elbadawey, M.R., Shields, R.C., Jakubovics, N.S. and Burgess, J.G. (2012) 'Removal of biofilms from tracheoesophageal speech valves using a novel marine microbial deoxyribonuclease', *Otolaryngol Head Neck Surg*, 147(3), pp. 509-14.

Shanmugasundaram, S., Ricciardi, B.F., Briggs, T.W.R., Sussmann, P.S. and Bostrom, M.P. (2013) 'Evaluation and management of periprosthetic joint infection-an international, multicenter study', *HSS Journal*, pp. 1-9.

Sharma, D., Misba, L. and Khan, A.U. (2019) 'Antibiotics versus biofilm: an emerging battleground in microbial communities', *Antimicrob Resist Infect Control*, 8(1), p. 76.

Shen, C.J., Wu, M.S., Lin, K.H., Lin, W.L., Chen, H.C., Wu, J.Y., Lee, M.C. and Lee, C.C. (2013) 'The use of procalcitonin in the diagnosis of bone and joint infection: a systemic review and meta-analysis', *Eur J Clin Microbiol Infect Dis*, 32(6), pp. 807-14.

Shen, Y., Stojicic, S. and Haapasalo, M. (2010) 'Bacterial viability in starved and revitalized biofilms: comparison of viability staining and direct culture', *J Endod*, 36(11), pp. 1820-3.

Shida, T., Koseki, H., Yoda, I., Horiuchi, H., Sakoda, H. and Osaki, M. (2013) 'Adherence ability of *Staphylococcus epidermidis* on prosthetic biomaterials: an in vitro study', *Int J Nanomedicine*, 8, pp. 3955-3961.

Shields, R.C., Mokhtar, N., Ford, M., Hall, M.J., Burgess, J.G., ElBadawey, M.R. and Jakubovics, N.S. (2013) 'Efficacy of a marine bacterial nuclease against biofilm forming microorganisms isolated from chronic rhinosinusitis', *Plos One*, 8(2), p. 13.

Signore, A., Sconfienza, L.M., Borens, O., Glaudemans, A.W.J.M., Cassar-Pullicino, V., Trampuz, A., Winkler, H., Gheysens, O., Vanhoenacker, F.M.H.M., Petrosillo, N. and Jutte, P.C. (2019) 'Consensus document for the diagnosis of prosthetic joint infections: a joint paper by the EANM, EBJIS, and ESR (with ESCMID endorsement)', *Eur J Nucl Med Mol Imaging*, 46(4), pp. 971-988.

Silha, D., Hurdalkova, B., Papajova, M. and Silhova, L. (2019) 'In vitro activity of spiced extracts against *Arcobacter* spp. and influence on their biofilm formation', *J Microbiol Biotechnol Food Sci*, 9(3), pp. 552-556.

Singh, R., Ray, P., Das, A. and Sharma, M. (2010) 'Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms', *J Antimicrob Chemother*, 65(9), pp. 1955-1958.

Singh, R., Sahore, S., Kaur, P., Rani, A. and Ray, P. (2016) 'Penetration barrier contributes to bacterial biofilm-associated resistance against only select antibiotics, and exhibits genus-, strain- and antibiotic-specific differences', *Pathog Dis*, 74(6).

Smagur, J., Guzik, K., Bzowska, M., Kuzak, M., Zarebski, M., Kantyka, T., Walski, M., Gajkowska, B. and Potempa, J. (2009) 'Staphylococcal cysteine protease staphopain B (SspB) induces rapid engulfment of human neutrophils and monocytes by macrophages', *Biol Chem*, 390(4), pp. 361-71.

Smith, K. and Hunter, I.S. (2008) 'Efficacy of common hospital biocides with biofilms of multi-drug resistant clinical isolates', *J Med Microbiol*, 57(Pt 8), pp. 966-973.

Sofer, D., Regenbrecht, B. and Pfeil, J. (2005) 'Early results of one-stage septic revision arthroplasties with antibiotic-laden cement. A clinical and statistical analysis', *Orthopade*, 34(6), pp. 592-602.

Solarino, G., Abate, A., Vicenti, G., Spinarelli, A., Piazzolla, A. and Moretti, B. (2015) 'Reducing periprosthetic joint infection: what really counts?', *Joints*, 3(4), pp. 208-14.

Somma, S., Gastaldo, L. and Corti, A. (1984) 'Teicoplanin, a new antibiotic from *Actinoplanes teichomyceticus nov.* sp', *Antimicrob Agents Chemother*, 26(6), pp. 917-23.

Song, F., Koo, H. and Ren, D. (2015) 'Effects of material properties on bacterial adhesion and biofilm formation', *J Dent Res*, 94(8), pp. 1027-34.

Song, M. and Kellum, J.A. (2005) 'Interleukin-6', *Crit Care Med*, 33(12 Suppl), pp. S463-5.

Soriano, A., Popescu, D., Garcia, S., Bori, G., Martinez, J.A., Balasso, V., Marco, F., Almela, M. and Mensa, J. (2006) 'Usefulness of teicoplanin for preventing methicillin-resistant *Staphylococcus aureus* infections in orthopedic surgery', *Eur J Clin Microbiol Infect Dis*, 25(1), pp. 35-8.

Speziale, P., Pietrocola, G., Foster, T.J. and Geoghegan, J.A. (2014) 'Protein-based biofilm matrices in Staphylococci', *Front Cell Infect Microbiol*, 4, p. 171.

Steichen, C.T., Cho, C., Shao, J.Q. and Apicella, M.A. (2011) 'The Neisseria gonorrhoeae biofilm matrix contains DNA, and an endogenous nuclease controls its incorporation', *Infect Immun*, 79(4), pp. 1504-11.

Stewart, E.J., Payne, D.E., Ma, T.M., VanEpps, J.S., Boles, B.R., Younger, J.G. and Solomon, M.J. (2017) 'Effect of antimicrobial and physical treatments on growth of multispecies staphylococcal biofilms', *Appl Environ Microbiol*, 83(12).

Stewart, P., Zhang, T., Xu, R., Pitts, B., Walters III, M., Roe, F., Kikhney, J. and Moter, A. (2016) 'Reaction-diffusion theory explains hypoxia and heterogeneous growth within microbial biofilms associated with chronic infections. NPJ Biofilms Microbiomes 2: 16012'.

Stewart, P.S. (2002) 'Mechanisms of antibiotic resistance in bacterial biofilms', *International J Med Microbiol* 292(2), pp. 107-113.

Stewart, P.S. (2015) 'Antimicrobial tolerance in biofilms', *Microbiol Spectr*, 3(3).

Stewart, P.S. and Costerton, J.W. (2001) 'Antibiotic resistance of bacteria in biofilms', *Lancet*, 358(9276), pp. 135-8.

Stewart, P.S., Davison, W.M. and Steenbergen, J.N. (2009) 'Daptomycin rapidly penetrates a *Staphylococcus epidermidis* biofilm', *Antimicrob Agents Chemother*, 53(8), pp. 3505-3507.

Stewart, P.S. and Franklin, M.J. (2008) 'Physiological heterogeneity in biofilms', *Nat Rev Microbiol*, 6(3), pp. 199-210.

Stone, G., Wood, P., Dixon, L., Keyhan, M. and Matin, A. (2002) 'Tetracycline rapidly reaches all the constituent cells of uropathogenic *Escherichia coli* biofilms', *Antimicrob Agents Chemother*, 46(8), pp. 2458-2461.

Stoodley, P., Sauer, K., Davies, D.G. and Costerton, J.W. (2002) 'Biofilms as complex differentiated communities', *Ann Rev Microbiol*, 56, pp. 187-209.

Stoodley, P., Wilson, S., Hall-Stoodley, L., Boyle, J.D., Lappin-Scott, H.M. and Costerton, J.W. (2001) 'Growth and detachment of cell clusters from mature mixed-species biofilms', *Appl Environ Microbiol*, 67(12), pp. 5608-13.

Sugimoto, S., Iwamoto, T., Takada, K., Okuda, K., Tajima, A., Iwase, T. and Mizunoe, Y. (2013) '*Staphylococcus epidermidis* Esp degrades specific proteins associated with *Staphylococcus aureus* biofilm formation and host-pathogen interaction', *J Bacteriol*, 195(8), pp. 1645-55.

Sugimoto, S., Sato, F., Miyakawa, R., Chiba, A., Onodera, S., Hori, S. and Mizunoe, Y. (2018a) 'Broad impact of extracellular DNA on biofilm formation by clinically isolated Methicillin-resistant and -sensitive strains of *Staphylococcus aureus*', *Sci Rep*, 8(1), p. 2254.

Sugimoto, S., Sato, F., Miyakawa, R., Chiba, A., Onodera, S., Hori, S. and Mizunoe, Y. (2018b) 'Broad impact of extracellular DNA on biofilm formation by clinically isolated Methicillin-resistant and -sensitive strains of *Staphylococcus aureus*', *Sci Rep*, 8(1), p. 2254.

Sukeik, M., Patel, S. and Haddad, F.S. (2012) 'Aggressive early debridement for treatment of acutely infected cemented total hip arthroplasty', *Clin Orthop Relat Res*, 470(11), pp. 3164-70.

Suresh, M.K., Biswas, R. and Biswas, L. (2019) 'An update on recent developments in the prevention and treatment of *Staphylococcus aureus* biofilms', *Int J Med Microbiol*, 309(1), pp. 1-12.

Swartjes, J., Das, T., Sharifi, S., Subbiahdoss, G., Sharma, P.K., Krom, B.P., Busscher, H.J. and van der Mei, H.C. (2013) 'A functional DNase I coating to prevent adhesion of bacteria and the formation of biofilm', *Adv Funct Mater*, 23(22), pp. 2843-2849.

Tan, T.L., Kheir, M.M., Tan, D.D. and Parvizi, J. (2016a) 'Polymicrobial periprosthetic joint infections: Outcome of treatment and identification of risk factors', *J Bone Joint Surg Am*, 98(24), pp. 2082-2088.

Tan, T.L., Springer, B.D., Ruder, J.A., Ruffolo, M.R. and Chen, A.F. (2016b) 'Is vancomycin-only prophylaxis for patients with penicillin allergy associated with increased risk of infection after arthroplasty?', *Clin Orthop Relat Res*, 474(7), pp. 1601-6.

Tande, A.J. and Patel, R. (2014) 'Prosthetic joint infection', *Clin Microbiol Rev*, 27(2), pp. 302-45.

Tang, J.N., Kang, M.S., Chen, H.C., Shi, X.M., Zhou, R., Chen, J. and Du, Y.W. (2011) 'The staphylococcal nuclease prevents biofilm formation in *Staphylococcus aureus* and other biofilm-forming bacteria', *Sci China-Life Sci*, 54(9), pp. 863-869.

Tang, L., Schramm, A., Neu, T.R., Revsbech, N.P. and Meyer, R.L. (2013) 'Extracellular DNA in adhesion and biofilm formation of four environmental isolates: a quantitative study', *Fems Microbiol Ecol*, 86(3), pp. 394-403.

Tetz, G.V., Artemenko, N.K. and Tetz, V.V. (2009) 'Effect of DNase and antibiotics on biofilm characteristics', *Antimicrob Agents Chemother*, 53(3), pp. 1204-9.

Teughels, W., Van Assche, N., Sliepen, I. and Quirynen, M. (2006) 'Effect of material characteristics and/or surface topography on biofilm development', *Clin Oral Implants Res*, 17 Suppl 2, pp. 68-81.

Tolker-Nielsen, T. (2015) 'Biofilm Development', *Microbiol Spectr*, 3(2), pp. Mb-0001-2014.

Tornero, E., Garcia-Oltra, E., Garcia-Ramiro, S., Martinez-Pastor, J.C., Bosch, J., Climent, C., Morata, L., Camacho, P., Mensa, J. and Soriano, A. (2012) 'Prosthetic joint infections due to *Staphylococcus aureus* and coagulase-negative staphylococci', *Int J Artif Organs*, 35(10), pp. 884-92.

Tornero, E., Garcia-Ramiro, S., Martinez-Pastor, J.C., Bori, G., Bosch, J., Morata, L., Sala, M., Basora, M., Mensa, J. and Soriano, A. (2015) 'Prophylaxis with teicoplanin and cefuroxime reduces the rate of prosthetic joint infection after primary arthroplasty', *Antimicrob Agents Chemother*, 59(2), pp. 831-7.

Tornero, E., Senneville, E., Euba, G., Petersdorf, S., Rodriguez-Pardo, D., Lakatos, B., Ferrari, M.C., Pilares, M., Bahamonde, A., Trebse, R., Benito, N., Sorli, L., del Toro, M.D., Baraiaetxaburu, J.M., Ramos, A., Riera, M., Jover-Saenz, A., Palomino, J., Ariza, J. and Soriano, A. (2014) 'Characteristics of prosthetic joint infections due to *Enterococcus* sp. and predictors of failure: a multi-national study', *Clin Microbiol Infect*, 20(11), pp. 1219-24.

Tsang, S.J., Ting, J., Simpson, A. and Gaston, P. (2017) 'Outcomes following debridement, antibiotics and implant retention in the management of periprosthetic infections of the hip: a review of cohort studies', *Bone Joint J*, 99-b(11), pp. 1458-1466.

Tsukayama, D.T., Estrada, R. and Gustilo, R.B. (1996) 'Infection after total hip arthroplasty. A study of the treatment of one hundred and six infections', *J Bone Joint Surg Am*, 78(4), pp. 512-23.

Urban, J.A., Garvin, K.L., Boese, C.K., Bryson, L., Pedersen, D.R., Callaghan, J.J. and Miller, R.K. (2001) 'Ceramic-on-polyethylene bearing surfaces in total hip arthroplasty. Seventeen to twenty-one-year results', *J Bone Joint Surg Am*, 83(11), pp. 1688-94.

Uribe-Alvarez, C., Chiquete-Félix, N., Contreras-Zentella, M., Guerrero-Castillo, S., Peña, A. and Uribe-Carvajal, S. (2015) '*Staphylococcus epidermidis*: metabolic adaptation and biofilm formation in response to different oxygen concentrations', *Pathog Dis*, 74(1).

Urish, K.L., Bullock, A.G., Kreger, A.M., Shah, N.B., Jeong, K. and Rothenberger, S.D. (2017) 'A multicenter study of irrigation and debridement in total knee arthroplasty periprosthetic joint infection: Treatment failure is high', *J Arthroplasty*.

Urish, K.L., Bullock, A.G., Kreger, A.M., Shah, N.B., Jeong, K. and Rothenberger, S.D. (2018) 'A Multicenter Study of Irrigation and Debridement in Total Knee Arthroplasty Periprosthetic Joint Infection: Treatment Failure Is High', *J Arthroplasty*, 33(4), pp. 1154-1159.

Urish, K.L., DeMuth, P.W., Craft, D.W., Haider, H. and Davis, C.M., 3rd (2014) 'Pulse lavage is inadequate at removal of biofilm from the surface of total knee arthroplasty materials', *J Arthroplasty*, 29(6), pp. 1128-32.

Vacheethasanee, K., Temenoff, J.S., Higashi, J.M., Gary, A., Anderson, J.M., Bayston, R. and Marchant, R.E. (1998) 'Bacterial surface properties of clinically isolated *Staphylococcus epidermidis* strains determine adhesion on polyethylene', *J Biomed Mater Res*, 42(3), pp. 425-32.

Vanhegan, I.S., Malik, A.K., Jayakumar, P., Ul Islam, S. and Haddad, F.S. (2012a) 'A financial analysis of revision hip arthroplasty: the economic burden in relation to the national tariff', *J Bone Joint Surg Br*, 94(5), pp. 619-23.

Vanhegan, I.S., Morgan-Jones, R., Barrett, D.S. and Haddad, F.S. (2012b) 'Developing a strategy to treat established infection in total knee replacement: a review of the latest evidence and clinical practice', *J Bone Joint Surg Br*, 94(7), pp. 875-81.

Vaudaux, P., Ferry, T., Uckay, I., Francois, P., Schrenzel, J., Harbarth, S. and Renzoni, A. (2012) 'Prevalence of isolates with reduced glycopeptide susceptibility in orthopedic device-related infections due to methicillin-resistant *Staphylococcus aureus*', *Eur J Clin Microbiol Infect Dis*, 31(12), pp. 3367-74.

Vaudaux, P., Suzuki, R., Waldvogel, F.A., Morgenthaler, J.J. and Nydegger, U.E. (1984) 'Foreign body infection: role of fibronectin as a ligand for the adherence of *Staphylococcus aureus*', *J Infect Dis*, 150(4), pp. 546-53.

Verdonschot, N. (2005) 'Implant choice: Stem design philosophies', in Steffen B, H.M. (ed.) *The well-cemented total hip arthroplasty: Theory and practice*. USA: Spirnger, pp. 168-79.

Viganor, L., Howe, O., McCarron, P., McCann, M. and Devereux, M. (2016) 'The antibacterial activity of metal complexes containing 1,10-phenanthroline: Potential as alternative therapeutics in the era of antibiotic resistance', *Curr Top Med Chem*.

Vipra, A.A., Desai, S.N., Roy, P., Patil, R., Raj, J.M., Narasimhaswamy, N., Paul, V.D., Chikkamadaiah, R. and Sriram, B. (2012) 'Antistaphylococcal activity of bacteriophage derived chimeric protein P128', *BMC Microbiol*, 12, p. 41.

von Foerster, G., Kluber, D. and Kabler, U. (1991) 'Mid- to long-term results after treatment of 118 cases of periprosthetic infections after knee joint replacement using one-stage exchange surgery', *Orthopade*, 20(3), pp. 244-52.

Vorregaard, M. (2008) Comstat2 - a modern 3D image analysis environment for biofilms, in Informatics and Mathematical Modelling. Academic Thesis thesis. Technical University of Denmark: Kongens Lyngby, Denmark

Waldrop, R., McLaren, A., Calara, F. and McLemore, R. (2014) 'Biofilm growth has a threshold response to glucose in vitro', *Clin Orthop Relat Res*, 472(11), pp. 3305-10.

Wang, J., Wang, Q., Shen, H. and Zhang, X. (2018) 'Comparable outcome of culture-negative and culture-positive periprosthetic hip joint infection for patients undergoing two-stage revision', *Int Orthop*, 42(3), pp. 469-477.

Wang, R., Braughton, K.R., Kretschmer, D., Bach, T.H., Queck, S.Y., Li, M., Kennedy, A.D., Dorward, D.W., Klebanoff, S.J., Peschel, A., DeLeo, F.R. and Otto, M. (2007) 'Identification of novel cytolytic peptides as key virulence determinants for community-associated MRSA', *Nat Med*, 13(12), pp. 1510-4.

Wang, R., Khan, B.A., Cheung, G.Y., Bach, T.H., Jameson-Lee, M., Kong, K.F., Queck, S.Y. and Otto, M. (2011) '*Staphylococcus epidermidis* surfactant peptides promote biofilm

maturation and dissemination of biofilm-associated infection in mice', *J Clin Invest*, 121(1), pp. 238-48.
Watanakunakorn, C., & Bakie, C. (1973). ' Synergism of vancomycin-gentamicin and vancomycin-streptomycin against enterococci '. *Antimicrobial agents and chemotherapy*, *4*(2), pp. 120–124.
Welliver, R.C., Jr., Hanerhoff, B.L., Henry, G.D. and Kohler, T.S. (2014) 'Significance of biofilm for the prosthetic surgeon', *Curr Urol Rep*, 15(6), pp. 411-411.
Wessinghage, D. (1991) 'Themistocles Gluck. 100 years artificial joint replacement', *Z Orthop Ihre Grenzgeb*, 129(5), pp. 383-8.
Whitchurch, C.B., Tolker-Nielsen, T., Ragas, P.C. and Mattick, J.S. (2002) 'Extracellular DNA required for bacterial biofilm formation', *Sci*, 295(5559), p. 1487.
Whiteside, L.A., Peppers, M., Nayfeh, T.A. and Roy, M.E. (2011) 'Methicillin-resistant *Staphylococcus aureus* in TKA treated with revision and direct intra-articular antibiotic infusion', *Clin Orthop Relat Res*, 469(1), pp. 26-33.
Wiegand, I., Hilpert, K. and Hancock, R.E. (2008) 'Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances', *Nat Protoc*, 3(2), pp. 163-75.
Wijesinghe, G., Dilhari, A., Gayani, B., Kottegoda, N., Samaranayake, L. and Weerasekera, M. (2019) 'Influence of laboratory culture media on in vitro growth, adhesion, and biofilm formation of *Pseudomonas aeruginosa* and *Staphylococcus aureus*', *Med Princ Pract*, 28(1), pp. 28-35.
Wimmer, M.D., Friedrich, M.J., Randau, T.M., Ploeger, M.M., Schmolders, J., Strauss, A.A., Hischebeth, G.T., Pennekamp, P.H., Vavken, P. and Gravius, S. (2016) 'Polymicrobial infections reduce the cure rate in prosthetic joint infections: outcome analysis with two-stage exchange and follow-up two years', *Int Orthop*, 40(7), pp. 1367-73.
Wirtz, D.C., Heller, K.D., Miltner, O., Zilkens, K.W. and Wolff, J.M. (2000) 'Interleukin-6: a potential inflammatory marker after total joint replacement', *Int Orthop*, 24(4), pp. 194-6.
Workgroup Convened by the Musculoskeletal Infection, S. (2011) 'New definition for periprosthetic joint infection', *J Arthroplasty*, 26(8), pp. 1136-8.
World Health Organization (2014) 'Antimicrobial resistance: Global report on surveillance', *World Health Organization*
Worthington, T., Dunlop, D., Casey, A., Lambert, R., Luscombe, J. and Elliott, T. (2010) 'Serum procalcitonin, interleukin-6, soluble intercellular adhesin molecule-1 and IgG to short-chain exocellular lipoteichoic acid as predictors of infection in total joint prosthesis revision', *Br J Biomed Sci*, 67(2), pp. 71-6.
Wouthuyzen-Bakker, M., Ploegmakers, J.J.W., Kampinga, G.A., Wagenmakers-Huizenga, L., Jutte, P.C. and Muller Kobold, A.C. (2017) 'Synovial calprotectin: a potential biomarker to exclude a prosthetic joint infection', *Bone Joint J*, 99-b(5), pp. 660-665.
Wouthuyzen-Bakker, M., Ploegmakers, J.J.W., Ottink, K., Kampinga, G.A., Wagenmakers-Huizenga, L., Jutte, P.C. and Kobold, A.C.M. (2018) 'Synovial calprotectin: An inexpensive biomarker to exclude a chronic prosthetic joint infection', *J Arthroplasty*, 33(4), pp. 1149-1153.
Wyatt, M.C., Beswick, A.D., Kunutsor, S.K., Wilson, M.J., Whitehouse, M.R. and Blom, A.W. (2016) 'The Alpha-Defensin Immunoassay and Leukocyte Esterase Colorimetric Strip Test for the Diagnosis of Periprosthetic Infection: A Systematic Review and Meta-Analysis', *J Bone Joint Surg Am*, 98(12), pp. 992-1000.
Xie, K., Dai, K., Qu, X. and Yan, M. (2017) 'Serum and synovial fluid interleukin-6 for the diagnosis of periprosthetic joint infection', *Sci Rep*, 7(1), p. 1496.

Xu, Z., Liang, Y., Lin, S., Chen, D., Li, B., Li, L. and Deng, Y. (2016) 'Crystal violet and XTT assays on *Staphylococcus aureus* biofilm quantification', *Curr Microbiol*, 73(4), pp. 474-82.

Yarwood, J.M., Bartels, D.J., Volper, E.M. and Greenberg, E.P. (2004) 'Quorum sensing in *Staphylococcus aureus* biofilms', *J Bacteriol*, 186(6), pp. 1838-1850.

Yilmaz, M., Mete, B., Ozaras, R., Kaynak, G., Tabak, F., Tenekecioglu, Y. and Ozturk, R. (2011) '*Aspergillus fumigatus* infection as a delayed manifestation of prosthetic knee arthroplasty and a review of the literature', *Scand J Infect Dis*, 43(8), pp. 573-8.

Yoon, H.K., Cho, S.H., Lee, D.Y., Kang, B.H., Lee, S.H., Moon, D.G., Kim, D.H., Nam, D.C. and Hwang, S.C. (2017) 'A review of the literature on culture-negative periprosthetic joint infection: Epidemiology, diagnosis and treatment', *Knee Surg Relat Res*, 29(3), pp. 155-164.

Zappe, B., Graf, S., Ochsner, P.E., Zimmerli, W. and Sendi, P. (2008) '*Propionibacterium* spp. in prosthetic joint infections: a diagnostic challenge', *Arch Orthop Trauma Surg*, 128(10), pp. 1039-46.

Zatorska B, Groger M, Moser D, Diab-Elschahawi M, Lusignani LS, Presterl E.(2017) 'Does extracellular DNA production vary in staphylococcal biofilms isolated from infected implants versus controls? ' Clin Orthop Relat Res.475(8), pp 2105-2113

Zeller, V., Ghorbani, A., Strady, C., Leonard, P., Mamoudy, P. and Desplaces, N. (2007) '*Propionibacterium acnes*: an agent of prosthetic joint infection and colonization', *J Infect*, 55(2), pp. 119-24.

Zhang, J., Liu, H., Zhu, K., Gong, S., Dramsi, S., Wang, Y.-T., Li, J., Chen, F., Zhang, R., Zhou, L., Lan, L., Jiang, H., Schneewind, O., Luo, C. and Yang, C.-G. (2014) 'Antiinfective therapy with a small molecule inhibitor of *Staphylococcus aureus* sortase', *Proc Natl Acad Sci USA*, 111(37), pp. 13517-13522.

Zhao, L., Chu, P.K., Zhang, Y. and Wu, Z. (2009) 'Antibacterial coatings on titanium implants', *J Biomed Mater Res B Appl Biomater*, 91(1), pp. 470-80.

Zheng, Z. and Stewart, P.S. (2002) 'Penetration of rifampin through *Staphylococcus epidermidis* biofilms', *Antimicrob Agents Chemother*, 46(3), pp. 900-903.

Zheng, Z. and Stewart, P.S. (2004) 'Growth limitation of *Staphylococcus epidermidis* in biofilms contributes to rifampin tolerance', *Biofilms*, 1(1), pp. 31-35.

Zhou, Y., Zhao, R., Ma, B., Gao, H., Xue, X., Qu, D., Li, M., Meng, J., Luo, X. and Hou, Z. (2016) 'Oligomerization of RNAIII-Inhibiting peptide inhibits adherence and biofilm formation of methicillin-resistant *Staphylococcus aureus* in vitro and in vivo', *Microb Drug Resist*, 22(3), pp. 193-201.

Zimmerli, W., Lew, P.D. and Waldvogel, F.A. (1984) 'Pathogenesis of foreign body infection. Evidence for a local granulocyte defect', *J Clin Invest*, 73(4), pp. 1191-200.

Zimmerli, W., Trampuz, A. and Ochsner, P.E. (2004) 'Prosthetic-joint infections', *N Engl J Med*, 351(16), pp. 1645-54.

Zmantar, T., Bettaieb, F., Chaieb, K., Ezzili, B., Mora-Ponsonnet, L., Othmane, A., Jaffrézic, N. and Bakhrouf, A. (2011) 'Atomic force microscopy and hydrodynamic characterization of the adhesion of S*taphylococcus aureus* to hydrophilic and hydrophobic substrata at different pH values', *World J Microbiol Biotechnol*, 27(4), pp. 887-896.

Zmistowski, B.M., Manrique, J., Patel, R. and Chen, A.F. (2016) 'Recurrent periprosthetic joint infection after irrigation and debridement with component retention is most often due to identical organisms', *J Arthroplasty*.