Extracellular DNA as a Component of Dental Plaque

Nasrin Abduljalil

Thesis submitted in partial fulfilment of the requirements of the regulations for the degree of Doctor of Philosophy

Institute for Cell and Molecular Biosciences

Centre of Oral Health Science

School of Dental Sciences

Faculty of Medical Sciences

Newcastle University

Wordcount: 34,590

January 2021

Declaration

I certify that this thesis contains my own work, except where acknowledged, and that no part of this material has been previously submitted for a degree or any other qualification at this or any other university.

Abstract

Therapeutics that can control microbial biofilms while preserving the natural microbiota represent a promising strategy in managing biofilm-related diseases such as periodontitis. Extracellular DNA (eDNA) is an important structural component of many biofilms, including dental plaque. Bacteria within dental plaque produce deoxyribonuclease (DNase) enzymes that could digest eDNA. This project aimed to investigate whether a DNase produced by an oral bacterium can degrade eDNA in oral biofilms, thereby inhibiting the biofilm growth or disrupting mature biofilms, and compare its activity to a DNase from a non-oral bacterium. Preliminary investigations were also made into the microbial composition and immunostimulatory properties of eDNA. NucB, a DNase from a marine isolate of *Bacillus licheniformis,* inhibited biofilm growth and dispersed preformed biofilms of *Fusobacterium nucleatum*. It also inhibited the growth of model plaque biofilms, but did not affect preformed biofilms, implying that eDNA is more important or more accessible during the initial phases of the plaque biofilm formation. Furthermore, NucB inhibited the growth of anaerobic model plaque biofilms, but not those cultured in aerobic sucrose-rich conditions indicating that it may be particularly useful for reducing subgingival plaque, which is essentially anaerobic. Conversely, SsnA, a DNase from the oral bacterium *Streptococcus gordonii*, inhibited the growth of *Fusobacterium nucleatum* biofilms but did not disrupt preformed biofilms. SsnA Also lacked any antibiofilm effect with model plaque biofilms. NucB efficiently degraded various DNA substrates, whereas SsnA was only effective against single stranded and low molecular weight DNA, suggesting that eDNA in the plaque biofilm is predominantly double stranded DNA of high molecular weight. Overall, this work provided key insights into the activity of a DNase from an oral bacterium and showed that there is a scope to enhance subgingival biofilm control using exogenous DNases from non-oral bacteria.

Acknowledgements

First, I would like to express my sincere gratitude to my supervisor Dr Nick Jakubovics, who kindly guided this work and helped whenever I was in need. I also extend my thanks to my co-supervisors Dr John Taylor and Prof Philip Preshaw. Although they were not involved directly in the project, they offered support and advice along the way. Special thanks to John for offering to help with carrying out the cell culture experiments. This thesis could not have been completed without the kindness and support of all of you. I would also like to thank our lab technician Nicola for performing the cell culture experiments. Thanks also to Dr Gavin Wee at Monash University in Malaysia for doing the sequencing Data analysis. Thanks to people at the Bioimaging Unit and to the super friendly electron microscopy team at Newcastle University. Massive thanks to Waleed for his help and support, his advice was always valuable. I may not mention everyone by name here, but to all those students/ staff who were friendly, kind and willing to help, thank you.

I would also like to convey my deepest thankfulness to my loving parents for their continuous support and prayers, and to my sister and brothers for their support and encouragement. Finally, the biggest thank you goes to my daughter and sunshine Masa who was my buddy throughout those four years. Thank you, Masa, for listening to my frustrating lab stories patiently, cheering me up and telling me not to give up. Thank you for your help with punctuation and for telling me if a sentence sounds good when I was not sure.

Contents

List of Tables

List of Figures

Chapter 1: Introduction

1.1 Background

Microorganisms naturally prefer to attach to surfaces and form polymicrobial biofilms rather than to live as dispersed single cells. The accumulation of such mixed-species biofilms (plaque) on surfaces of teeth contributes to the development of many oral diseases such as dental caries and periodontitis (Mira et al., 2017, Sanz et al., 2017). Oral diseases affect about 3.5 billion people around the world of which 796 million have severe periodontitis (Bernabe et al., 2020).

Periodontitis is a chronic inflammatory condition that is caused by unfavourable interaction between subgingival biofilm and host inflammatory immune response and could eventually result in tissues destruction and teeth loss (Thurnheer et al., 2018, Könönen et al., 2019). It also appears that patients with severe periodontitis have high risk of developing systemic diseases such as diabetes, atherosclerosis and rheumatoid arthritis (Liljestrand et al., 2018, Bui et al., 2019). Periodontitis is primarily treated by mechanical removal of supra- and subgingival bacterial biofilms from exposed tooth surfaces. While periodontal treatment can reduce the risk of tooth loss and improve the quality of life, successful treatment requires patients to keep regular teeth cleaning regimen and recurrent professional maintenance care as well as control lifestyle risk factors such as smoking (Tonetti et al., 2015, Graziani et al., 2017). Additionally, non-surgical periodontal therapy does not achieve the treatment goals in some patients, necessitating periodontal surgery, which could be associated with post-operative pain and swelling (Mei et al., 2016, Deas et al., 2016). The administration of systemic antibiotics are also required in the treatment of severe cases increasing the risk of antibiotic resistance (Pretzl et al., 2019). As periodontal disease is mostly driven by the exposure of the periodontal tissues

to bacteria living within microbial biofilms, prevention and treatment are based mainly on removing and preventing the formation of the biofilm.

Biofilm bacteria are surrounded and held by an extracellular matrix which consists of polysaccharides, lipids, proteins, and extracellular DNA (eDNA) (Flemming and Wingender, 2010, Dragoš and Kovács, 2017). Biofilms are more resistant to treatment by antibiotics (Ceri et al., 1999, Stewart and Costerton, 2001, Hall and Mah, 2017, Yan and Bassler, 2019) and defences of the host immune system (Campoccia et al., 2019) when compared to planktonic bacteria. Many mechanisms were found to be contributing to this biofilm resistance, including components of the biofilm matrix such as polysaccharides and eDNA (Wilton et al., 2016, Hall and Mah, 2017). It has also been shown that the transfer of exogenously added oral health substances such as fluoride and triclosan is hindered by the dental biofilm matrix (Robinson, 2011). Recently, research has focused on the disruption of the biofilm matrix as this can reverse the bacteria to the planktonic state which is weaker and therefore easier to treat or control.

For years, exopolysaccharides were regarded as the main and the most critical constituent of biofilm matrix. However, it has subsequently become clear that extracellular DNA is another important component that has an active role in the development and stability of many biofilms (Okshevsky et al., 2015, Jakubovics and Burgess, 2015, Okshevsky and Meyer, 2015, Ibáñez de Aldecoa et al., 2017). There is also evidence that extracellular DNA plays a vital role in the development of the dental plaque biofilm (Rostami et al., 2017, Schlafer et al., 2017). According to these observations, targeting eDNA with DNases can be a potentially promising approach for controlling dental plaque. Several oral bacteria produce DNase enzymes but whether these can degrade eDNA in the biofilm matrix thereby affecting biofilm development or stability is unclear. Understanding the functions of eDNA and activity of bacterial DNases in dental plaque is critical for the development of novel strategies to combat dental plaque based on targeting eDNA.

1.2 Dental plaque as a microbial biofilm

Oral bacteria must attach to surfaces in order to be protected from being removed by shear forces and lost by swallowing. Several mechanisms are implicated in the formation of the plaque biofilm among which, the adherence of bacteria to one another in suspension (co-aggregation) (Cisar et al., 1979) and the adherence of unattached bacteria to bacteria already attached to the tooth surface (co-adhesion) (Kolenbrander et al., 2000). These are critical processes for dental plaque formation as they allow the attachment of late colonizers to a preformed biofilm of predecessor bacteria (Kolenbrander et al., 2010). Epithelial cells sloughed from the oral mucosa might also aid in the transfer and adherence of bacterial cells onto teeth surfaces in the initial stage of dental plaque formation (Tinanoff and Gross, 1976, Brecx et al., 1981).

1.2.1 Stages of dental plaque formation

As soon as a tooth is cleaned and within seconds of its exposure to the oral environment, certain molecules are adsorbed to the tooth surface forming a conditioning film known as the acquired enamel pellicle (AEP). The enamel pellicle contains saliva- as well as bacteria-derived components, however, the major components of the AEP are salivary proteins and glycoproteins (Siqueira et al., 2012). Glucans, which have an important role in bacterial attachment, can also be seen in the pellicle. Furthermore, the pellicle includes several enzymes such as amylase, lysozyme, fructosyltransferases and glucosyltransferases (Hannig et al., 2005). Salivary proteins and glycoproteins present within the pellicle provide specific receptors for bacteria to attach, and consequently, the composition of the pellicle can determine the early microbial colonizers. For instance, *S. gordonii* binds to α-amylase in the acquired pellicle (Rogers et al., 2001) and *Actinomyces* spp. use their type 1 fimbriae to adhere to proline-rich proteins and to statherin (Li et al., 2001). Salivary proline-rich proteins are also known to interact with streptococci (Ruhl et al., 2004).

Only a few oral species are motile, therefore, bacteria are usually carried passively to the tooth surface by the salivary flow (Marsh et al., 2011). As microorganisms approach the pellicle coated surface, weak physicochemical

interactions are generated between the charge of the proteins present in pellicle and that of bacterial cell surface (Busscher and Van Der Mei, 1997, Bos et al., 1999, Busscher et al., 2008) resulting in a reversible attachment phase. At this stage, epithelial cells could also be attracted to the tooth surface carrying bacteria. Due to the preferential adsorption of certain bacterial species to epithelial cells (Hoffman and Frank, 1966), species with higher affinity are likely to be more prevalent in this reversible adhesion model. However, only bacteria that that can participate in specific interactions with receptors in the pellicle will achieve permanent attachment to the tooth surface.

Bacterial attachment becomes more permanent if bacterial cells can come closer to the pellicle surface as strong interactions occur between molecules on the bacterial cell surface (adhesins) and complementary molecules (receptors) in the acquired pellicle (Whittaker et al., 1996, Jenkinson and Lamont, 1997, Hojo et al., 2009). These interactions are highly specific, and therefore, certain species are frequently associated with a particular surface or site. Oral streptococci are considered the main early microbial colonisers and can be observed on the tooth surface within minutes. This is mainly because they express various cell surface adhesins that can recognize receptors in the acquired enamel pellicle (Wright et al., 2013). Moreover, within two to four hours, streptococci have been found to make up about 60-80% of the dental plaque bacteria (Nyvad and Kilian, 1987, Nyvad and Kilian, 1990, Diaz et al., 2006, Dige et al., 2009). *Actinomyces* species (Palmer et al., 2003) as well as *Haemophilus, Veillonella* and *Neisseria* (Nyvad and Kilian, 1990) are also among the pioneer organisms and they are frequently isolated after two hours. At this stage, no or low numbers of obligate anaerobes are usually observed.

Due to the growth and metabolism of the early colonizers, the local environment is altered, and conditions become suitable for colonization by fastidious species. Streptococci and other initial colonizers present unique receptor sites for the attachment of later colonizers such as *Fusobacterium nucleatum* (He et al., 2012), *Tannerella forsythia, Treponema denticola* and *Porphyromonas gingivalis* (Kuboniwa and Lamont, 2010, Periasamy and Kolenbrander, 2010), which are more pathogenic and linked to the development of periodontitis (Socransky et al., 1998, Van Winkelhoff et al., 2002, Byrne et al., 2009, Haffajee and

Socransky, 1994). Secondary and late colonizers adhere to already attached bacteria through an interaction between adhesins and receptors on the bacterial cell surface (co-adhesion) (Kolenbrander, 2000). A well-known bacterial interaction between early and late colonizers occurs between *S. gordonii* and *P. gingivalis* and can aid the attachment of *P. gingivalis* to pre-formed biofilms of *S. gordonii* (Lamont et al., 2002, Park et al., 2005, Wright et al., 2013). Coaggregation among oral bacteria has been studied *in vitro*, and *Fusobacterium nucleatum* was found to coaggregate with nearly all oral bacterial species (Kolenbrander et al., 2006). Some late colonizers such as *Prevotella* or *Eubacterium* species cannot coaggregate with early colonizers. However, *F. nucleatum* coaggregates with these late colonizers as well as early colonizers. Therefore, *F. nucleatum* seems to act as a bridge between early and late colonizers (Bradshaw et al., 1998, Kolenbrander et al., 2010). The absence of *F. nucleatum* in a supragingival biofilm model was found to result in a significant decrease in total bacterial numbers in the biofilm (Thurnheer and Belibasakis, 2018).

If left undisturbed, the microbial diversity of the dental biofilm gradually increases leading to the development of multispecies community (microbial succession)(Kolenbrander et al., 2006) and maturation. A sample of mature dental plaque might consist of 100 or more different bacterial species (Jakubovics and Kolenbrander, 2010). The maturation of a biofilm is associated with a slow growth rate of the individual bacteria and the formation of an extracellular matrix of polymers by these bacteria (Marsh et al., 2016)

1.2.2 Bacterial interactions in dental plaque biofilm

Bacteria exist in close physical proximity in biofilms, consequently, several synergistic and antagonistic interspecies interactions can take place (Miller et al., 2019). One example of synergism between oral bacteria is the ability of some obligate anaerobes to survive aerobic conditions when they coaggregate with oxygen consuming or tolerating bacteria(Bradshaw et al., 1997, Diaz et al., 2002, Marsh and Zaura, 2017). Furthermore, some organisms can catabolize complex host proteins and glycoproteins if they partner with different species(Bradshaw et al., 1994, Wickström et al., 2009, Takahashi, 2015, Zhou et al., 2016), while

others are able to use the metabolic product of a second organism as a source of nutrition (Hojo et al., 2009, Tan et al., 2014). An example of metabolic cooperation happens between *P. gingivalis* and *T. denticola*, both of which have been associated with severity and progression of periodontitis. Glycine and isobutyric acid generated by *P. gingivalis* promote the growth of *T. denticola* which in turn produces succinic acid which is essential for the growth *P. gingivalis* (Grenier, 1992, Tan et al., 2014, Kin et al., 2020). Oral bacteria also engage in antagonistic interactions most commonly between commensal streptococci and oral pathogens. For instance, some oral streptococci secrete bacteriocins, peptides that have bactericidal effects on some other oral strains (Rogers et al., 1979, van der Ploeg, 2005, Hossain and Biswas, 2011, Merritt and Qi, 2012, Zhu et al., 2018). The production of bacteriocins can be beneficial to the producing organisms as it is associated with the development of competence, a condition where bacteria can take up eDNA and use it as an energy source or to obtain new genetic information (van der Ploeg, 2005, Kreth et al., 2006, Kreth et al., 2008b, Perry et al., 2009b). Oral streptococci also produce hydrogen peroxide (H_2O_2) which can cause bacterial cell stress or death due its ability to cross cell membranes and oxidize intracellular macromolecules including lipids, DNA and proteins (Keke et al., 2017). Streptococcal H_2O_2 can kill other oral bacteria or inhibit their growth, whereas the producing bacteria are usually resistant to its inhibitory effects (Holmberg and Hallander, 1973, Jakubovics et al., 2008, Herrero et al., 2016, Redanz et al., 2018). However, the release of H_2O_2 might also enable non-producing species to benefit. For instance, *Streptococcus gordonii*-derived hydrogen peroxide could assist *Porphyromonas gingivalis* in heme acquisition (Brown et al., 2018).

1.2.3 Dental plaque from periodontal health to disease

Periodontitis has long been attributed to specific organisms mainly the `red complex` bacteria which are *Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia (Socransky et al., 1998)*. However, subsequent metagenomic and mechanistic studies indicated that periodontitis is instigated

by polymicrobial synergy and dysbiosis rather than by specific periodontal pathogens (Hajishengallis and Lamont, 2012).

When the dental biofilm is formed, a degree of stability or balance is usually present among the constituent species (Marsh, 1989). This stability, known as microbial homeostasis, is attributed to the dynamic balance created by the various synergistic and antagonistic bacterial interactions taking place within the biofilm. However, changes in local environmental conditions could result in disrupting the microbial homeostasis and re-organization of the biofilm structure and composition (Valm, 2019). Under the new conditions, the species that previously were only making a minority could become more competitive and consequently more dominant (Marsh, 2003). Therefore,the transition from periodontal health to disease is usually associated with a shift in the composition and the activity of the subgingival biofilm community as well as changes in the host-microbe interaction which can cause destructive inflammation and bone loss (Lamont et al., 2018, Curtis et al., 2020).

While the microbial community as a whole is involved in challenging the host periodontal tissues, keystone pathogens, microbial species that have relatively large effects on their environment relative to their abundance, are thought to orchestrate dysbiosis by disrupting the host-microbe homeostasis. Keystone pathogens such as *P. gingivalis* are, even in small numbers, able to interact with other species and elevate the virulence of the entire community (Hajishengallis et al., 2011, Hajishengallis et al., 2012). As the dysbiotic community grows, it stimulates inflammatory responses that may be poorly controlled, particularly in susceptible hosts, leading eventually to periodontal tissue destruction. The periodontal community is eventually remodeled into an inflammophilic microbiota which not only tolerates the inflammatory environment, but also utilizes nutrients derived from inflammatory tissue breakdown (Hajishengallis, 2014).

1.3 The biofilm matrix

A significant part of any bacterial biofilm is composed of material other than bacterial cells. In some biofilms, microorganisms might constitute less than 10%

of the dry weight of biofilm, whereas more than 90% is made up by an extracellular matrix (Flemming and Wingender, 2010). The structural integrity of the biofilm and their tolerance to environmental stresses and antimicrobial agents is significantly dependent on the biofilm matrix (Flemming et al., 2016, Dragoš and Kovács, 2017, Costa et al., 2020). The matrix preserves water, nutrients and enzymes within the biofilm and limits the penetration of other particles particularly charged antimicrobials (Tatevossian, 1985, Hata and Mayanagi, 2003, Flemming and Wingender, 2010, Flemming et al., 2016).This extracellular matrix is developed due to the release of large molecules by the biofilm bacteria either via cell lysis or by active secretion. Polysaccharides, lipids, proteins and eDNA are the main structural molecules of the biofilm matrix (Flemming and Wingender, 2010). The matrix formation is dependent on the availability of nutrients, the synthesis and secretion of extracellular substances, shear and other stresses. The oral biofilm matrix is thought to provide binding sites for oral organisms and prevent normal oral hygiene procedures from removing the biofilm (Bowen et al., 2018). At present, our knowledge of biofilm matrix components is based mostly on studying single- species biofilm models. However, it is still not clear if bacteria produce these extracellular materials in the same way when they live in natural multispecies biofilm communities such as dental plaque.

1.3.1 Supragingival biofilm matrix

Exopolysaccharides particularly insoluble glucans produced by *S. mutans* represent the major matrix component in supragingival cariogenic biofilms (Yamashita et al., 1993, Mattos-Graner et al., 2000, Bowen and Koo, 2011, Koo et al., 2013, Klein et al., 2015). Other oral bacteria including *S. gordonii*, *S. sanguinis* and *Actinomyces* spp. use dietary sucrose and starch to synthesize soluble extracellular glucans and fructans (Bowen et al., 2018). Glucans are produced by glucosyltransferase (GTF) enzymes, which are released by bacteria and incorporated into the pellicle. Most oral species including *Actinomyces* spp., *Lactobacillus casei*, and *Candida albicans* cannot synthesize glucans until they bind to GTFs of *S. mutans* which enable them to contribute to the biofilm matrix of multispecies biofilms (Koo et al., 2013, Cugini et al., 2019). Oral bacteria

release GTFs extracellularly where they bind to teeth surfaces and produce glucans in situ. These glucans can act as specific bacterial binding sites (Schilling and Bowen, 1992). Therefore, glucans formed on surfaces facilitate the accumulation of microbial cells on teeth, while enhancing cell to cell adhesion and interspecies interactions (Bowen et al., 2018).

The production of exopolysaccharides in situ promotes the local colonization and clustering of microorganisms, and as the biofilm develops, the microbes are enmeshed and surrounded by these polymers. This leads to the formation of an insoluble matrix that assists the assembly of cohesive but spatially heterogeneous 3-dimensional multicellular scaffold (Koo et al., 2013, Klein et al., 2015, Castillo Pedraza et al., 2017). There is evidence that the insoluble polysaccharide-rich matrix could restrict diffusion into and out of the dental biofilm, which might promote acid accumulation and restrict access by saliva around the teeth inhibiting its neutralizing effect (Hata and Mayanagi, 2003, Bowen and Koo, 2011, Koo et al., 2013). Although enamel dissolution and dental caries result primarily from acid production, it is clear that the biofilm matrix provides a sheltering effect which is likely to increase the demineralization capacity of acids when saliva is present. The exact mechanisms involved in restricting diffusion are not yet clear, however, the uneven distribution of polysaccharides within plaque biofilms, and their higher density at the tooth interface (Reese and Guggenheim, 2007) may influence diffusion properties and mass transport throughout the biofilm (Thurnheer et al., 2003, Robinson et al., 2006).

Proteins including amyloid forming proteins, host proteins and glycoproteins are also present in the cariogenic biofilm matrix. Amyloids were detected in supragingival dental plaque and were found to be produced by the cariogenic species *S. mutans* (Oli et al., 2012, Besingi et al., 2017, Chen et al., 2019a). Further, known inhibitors of amyloid fibrillization decreased *S. mutans* biofilm formation indicating that these amyloids contribute to the structure of the matrix (Oli et al., 2012, Chen et al., 2019a). Exposure to sucrose has been shown to increase the protein content of dental plaque fluid by 50% (Gao et al., 2001) and cause changes in the protein composition of the extracellular matrix of plaque-like biofilms (Paes Leme et al., 2008). However, the roles of these changes in cariogenic dental plaque are not yet well understood.

The extracellular matrix of cariogenic biofilms also appears to be rich in lipoteichoic acids (LTA) (Rølla et al., 1980, Klein et al., 2015). *S. mutans* produces high amounts of extracellular LTA that could enhance glucan synthesis and improve the microbial adhesion to surfaces (Ciardi et al., 1977, Rølla et al., 1980) thereby participating in the building of the cariogenic biofilms. Recently, LTA were shown to be particularly abundant in the late stages of cariogenic biofilm formation (Castillo Pedraza et al., 2017). Furthermore, compounds that target the metabolism of LTA in *S. mutans* inhibited the formation of *S. mutans* biofilms. However, this effect was more prominent when glucan synthesis inhibitors were added emphasizing that both LTA and glucans cooperate to build the biofilm matrix (Castillo Pedraza et al., 2020a).

The presence of eDNA in cariogenic biofilms has been mainly studied using biofilms of *S. mutans* (Perry et al., 2009a, Das et al., 2010, Das et al., 2011, Liao et al., 2014, Nagasawa et al., 2020b). These studies demonstrated that eDNA enhances *S. mutans* adhesion and surface aggregation which are important steps in biofilm formation (Das et al., 2010, Das et al., 2011). However, Liao et al. (2014) found that the ability of eDNA to enhance adhesion is mediated by glucans since this effect for eDNA was not seen in the absence of glucans. It is worth noting that Liao et al. (2014) used *S. mutans* UA159, while strain LT11 was used in the two aforementioned studies emphasizing that different strains could use eDNA differently. DNase treatment reduced the biomass of *S. mutans* biofilms suggesting that eDNA participates in the establishment of the biofilm matrix (Petersen et al., 2005, Perry et al., 2009a, Liao et al., 2014). Yet, a recent study reported conflicting results as the presence of DNase I during *S. mutans* biofilm formation did not reduce the biofilm biomass (Chen et al., 2019a). In this study, eDNA in *S. mutans* biofilms was demonstrated to form a complex with amyloid fibres. Combining amyloid fibres inhibitors with DNase I reduced both the amount of amyloid fibres and biofilm biomass (Chen et al., 2019a). The interaction of eDNA with amyloid fibrils in *S.mutans* biofilms was also detected in a previous study (Liao et al., 2014). eDNA in *S. mutans* biofilms also appeared to be interacting with glucans as the DNase sensitive nanofibers observed in the matrix of *S. mutans* biofilms were also sensitive to dextranase. This was confirmed by the appearance of integrating glucans and eDNA in SEM images of *S. mutans*

biofilms grown in the presence of sucrose (Liao et al., 2014). Castillo Pedraza et al. (2017) found that eDNA content in single and mixed species cariogenic biofilms was negatively corelated with the biofilm biomass. This observation might imply that eDNA is not a major component of mature cariogenic biofilms and that it only plays a role during the early phases of biofilm formation. Recently, the density and the spatial distribution of the cells and extracellular matrix of a mixed species biofilm containing *S. mutans* was substantially reduced by including DNase I and proteinase K during the biofilm growth, however, the biofilm was grown in anaerobic conditions and therefore might not fully represent supragingival biofilms (Karygianni et al., 2020). Almost all the studies investigating the matrix composition of supragingival biofilms use cariogenic biofilm models. One recent study has investigated whether eDNA is a structural component of non-cariogenic supragingival biofilms by treating biofilms grown *in situ*, without exposure to dietary carbohydrates, by DNase I. The amount of the biofilm was strongly diminished in the very early phases of biofilm growth, but the antibiofilm effect of DNase I decreased as the biofilm aged beyond 7.5 hours (Schlafer et al., 2017).

1.3.2 Subgingival biofilm matrix

The literature investigating the matrix composition of subgingival biofilms is scarce. Glucans and fructans are not likely to be major components of the subgingival matrix as gingival crevicular fluid, an exudate derived from serum, is the main source of nutrition for bacteria growing below the gum line, and there is very low concentration of simple sugars available for these bacteria (Jakubovics and Kolenbrander, 2010). Thurnheer et al. (2016) followed changes in the structure of an *in vitro* mixed species biofilm as the culturing conditions were shifted from supragingival (aerobic) to subgingival (anaerobic). The authors found that exopolysaccharides were gradually reduced as the biofilm was converted from aerobic to microaerophilic stage and were undetectable by the end of the anaerobic phase. However, it is known that *Aggregatibacter actinomycetemcomitans,* one of the species that make up the framework of subgingival biofilm, produces poly-N-acetylglucosamine (PNAG) which is a well-studied matrix exopolysaccharide. PNAG is synthesized independently of dietary

sugars and was found to support biofilm cohesion and facilitate intercellular adhesion (Izano et al., 2008b). Nonetheless, type IV pili (known as Flp-pili) are likely to be the key structural matrix component in *A. actinomycetemcomitans* biofilms, since PNAG mutants were still able to form tenacious biofilms, while FIp-pili mutants were deficient in making biofilms. Biofilms produced by PNAG or FIp-pili mutants, unlike wild type biofilms, were sensitive to detachment by DNase I enzyme suggesting that eDNA also contributes to the cohesion of *A. actinomycetemcomitans* biofilms (Izano et al., 2008b). Other exopolysaccharide-producing subgingival bacteria are *Prevotella nigrescens* and *Prevotella intermedia* (Yamanaka et al., 2011), but the contribution of their polysaccharides to biofilm development and stability is undefined.

Ali Mohammed et al. (2013) measured the concentrations of carbohydrate, proteins and eDNA in the matrix of mono- and dual species biofilms of *P. gingivalis* and *F. nucleatum*, two important species in subgingival biofilm. The authors found that proteins and carbohydrates are the major constituents of the matrix, whereas eDNA constituted much lower amount and was of low molecular weight. Additionally, DNase I added during or after the formation of the biofilms did not result in any noticeable effect on the growth or removal of biofilms. Although the biofilms were rich in proteins as evidenced by microscopy, treatment with Proteinase K was also not sufficient to disperse the biofilms suggesting that the matrix was stabilized by the carbohydrates (Ali Mohammed et al., 2013).

DNA-binding proteins, which are known to stabilise the structure of eDNA, have been detected in monospecies biofilms of *P. gingivalis* and several oral streptococci implying that this type of matrix proteins could exist in supra as well as subgingival biofilms. The biofilm development and stability in these species appear to be highly dependent on the presence of DNA binding proteins in the matrix as antibodies derived against those proteins disrupted the biofilm formation and integrity (Rocco et al., 2017, Rocco et al., 2018).

1.4 Extracellular DNA as a component of plaque biofilms

The structure of supragingival and subgingival dental plaque has been examined in a series of studies using transmission electron microscopy (TEM) and scanning electron microscopy (SEM). The use of TEM revealed for the first time the presence of an extracellular material between microbial cells (Frank, 1970). SEM images further confirmed that cells are covered in an amorphous layer of extracellular matrix, though these images had limited resolution (Eastcott and Stallard, 1973). Samples must undergo dehydration in order to be examined by SEM and TEM, which affects the structural integrity of the extracellular matrix. Confocal laser scanning microscopy, which does not involve any dehydration, shows dental biofilm as more open and heterogeneous structure of cells surrounded by a matrix that is permeated by channels and voids (Wood et al., 2000). These channels and voids seem to be filled by a fluid which could contain a small quantity of unknown extracellular polymer.

1.4.1 Extracellular DNA is present in oral biofilms.

Holliday et al. (2015) employed field emission SEM to examine ex vivo specimens of subgingival dental plaque and observed an extensive web of extracellular strands which were proposed to be eDNA. However, the authors were unable to to label eDNA using antibodies to confirm that eDNA is a constitute of the biofilm matrix. More recently, eDNA was clearly visualized in ex vivo specimens of subgingival dental plaque on extracted teeth and dental implants (Rostami et al., 2017). In addition, membrane vesicles which are often rich in DNA (Sahu et al., 2012, Pérez-Cruz et al., 2015) were found to be abundant in dental plaque according to electron microscopy images (Frank, 1970). DNA containing outer membrane vesicles were also detected in *P. gingivalis* (Ho et al., 2015) and *S. mutans* (Liao et al., 2014). The presence of eDNA in dental plaque was also evident when PCR technique was performed with the application of propidium monoazide and bright light which cross-links the DNA present outside the cells or in dead cells and inhibits its amplification (Yasunaga et al., 2013). The authors found that this technique inhibited the amplification of two thirds of *S. mutans* DNA indicating that this DNA was not present inside viable cells. Furthermore,

oral bacteria such as *Streptococcus* spp. and *P. gingivalis* seem to be exposed to free DNA in their natural environment as systems for the incorporation and uptake of DNA exist in these bacteria (Tribble et al., 2012). The genomic analysis of several oral bacteria provides an indirect indication that eDNA is present in oral biofilms as it shows extensive evidence of recombination which is likely to be due to the uptake and incorporation of eDNA (Do et al., 2011).

1.4.2 Origins of extracellular DNA in oral biofilms

The accumulation of eDNA in the biofilm matrix might occur through several mechanisms among which death and lysis of microbial cell within the biofilm seem to be the most obvious. Cell lysis could be a passive process that results from aging or external factors such as antimicrobial agents, but some oral bacteria are also able to actively kill adjacent cells of similar or different species via the production of hydrogen peroxide and lactic acid (Jakubovics and Burgess, 2015, Alakomi et al., 2000, Keke et al., 2017). Early dental plaque colonizers such as *S. gordonii* and *S. sanguinis* have been found to release DNA when produce hydrogen peroxide (Kreth et al., 2009a, Itzek et al., 2011a).

Autolysis has been also recognized as a potential mechanism of releasing eDNA and modulating biofilm development. Autolysis, in some cases, is mediated by a process known as fratricide where a proportion of cell population is stimulated and release a factor that initiate lysis in their siblings. *Enterococcus faecalis*, bacteria commonly associated with endodontic infections, experience fratricide at high cell density due to a quorum-sensing signal (Thomas et al., 2009). Enterococcal fratricide is mediated by GelE, an extracellular protease that can trigger the activation of the primary autolysin AtlA. Only a proportion of the *E. faecalis* population produce GelE and cells that produce it are immune to its cell death effect by secreting the protease SprE. However, the rest of the population is not a GelE or SprE producer, and therefore, susceptible to cell lysis which results in the release of eDNA and the formation of the biofilm. However, the biofilm formation in *E. faecalis* might not be completely dependent on autolysis and eDNA release as robust biofilms were formed even when autolysis and DNA release decreased following the disruption of a gene expression modulator, the alternate sigma factor (Iyer and Hancock, 2012).

In response to environmental stress, some oral bacteria secrete peptides that can cause death of target cells. Competence-stimulating peptide (CSP)-induced death has been found to cause the release of chromosomal DNA into the extracellular matrix of *S. mutans* biofilms (Perry et al., 2009a, Nagasawa et al., 2020b). Furthermore, there is evidence that the major autolysin AtlS and a murein hydrolase, LytF, play a role in cell lysis and DNA release from a subpopulation of *S. gordonii* (Liu and Burne, 2011). Nonetheless, LytF and AtlS were still important for DNA release in *S. gordonii* when there was no evident cell lysis (Xu and Kreth, 2013a). It has also been shown that eDNA in *E. faecalis* and S. *mutans* biofilms was released substantially in the early stages of biofilm formation before cell lysis becomes significant (Barnes et al., 2012, Liao et al., 2014). Therefore, whether cell lysis is essential for eDNA release from such oral bacterial species is still unclear.

In addition to cell lysis, bacteria may also have the ability to actively release DNA via membrane vesicles (Dorward and Garon, 1990a, Schooling et al., 2009). The presence of membrane vesicles that contain eDNA has recently been reported in *S. mutans* (Liao et al., 2014) and *P. gingivalis* (Ho et al., 2015). Furthermore, the release of DNA into the biofilm matrix through type IV secretion system has been described in *Neisseria gonorrhoeae* (Hamilton et al., 2005). Similar systems could be present in oral bacteria as DNA release is reduced following the disruption of genes encoding the secretion system of sortase A in *S. mutans* (Liao et al., 2014).

eDNA of microbial biofilms could also be derived from DNA of host cells. eDNA isolated from patients with cystic fibrosis has been found to be nearly all of human origin(Lethem et al., 1990). Similarly, DNA released because of epithelial cell lysis could contribute to eDNA of oral biofilms considering that epithelial cells are located nearby bacteria on oral soft tissues (Jakubovics and Burgess, 2015). Epithelial cells were detected in the early supragingival dental plaque, but not in mature plaque (Tinanoff and Gross, 1976). Neutrophil extracellular traps which comprise DNA and associated histones released by neutrophils, have been detected on periodontal pockets epithelium and in gingival crevicular fluid (Vitkov et al., 2009, Vitkov et al., 2010). Currently, it is not clear what proportion of eDNA originates from bacteria and how much eDNA is host derived. It is also undefined if certain oral species contribute more than others to eDNA present in

the dental biofilm and whether this has implications for the pathogenesis of periodontal disease.

1.4.3 Potential roles for eDNA in oral biofilms

Whitchurch et al. (2002) were the first to discover the adhesive role of eDNA as *Pseudomonas aeruginosa* biofilms were dissolved and cells were almost completely dispersed following treatment with DNase I. Several subsequent studies have studied eDNA in various mono-species and sometimes mixed-species biofilms, but only a few of these have focused on oral bacteria. However, there is growing evidence that eDNA is important for adhesion and structure of oral biofilms. In *A. actinomycetemcomitans*, the adhesion of a leukotoxin to cell surfaces was found to be mediated by eDNA which appears to interact with membrane vesicles (Ohta et al., 1991). Recently, leukotoxin A has been found important for biofilm formation and colonisation of *A. actinomycetemcomitans* (Velusamy et al., 2019). DNase I significantly reduced the adhesion of *E. faecalis* to dentin blocks and to 96-well plates indicating that eDNA promotes this adhesion (Li et al., 2012, Schlafer et al., 2018b). However, its effect on adhesion to dentin walls of extracted teeth was not significant (Schlafer et al., 2018b). The initial adhesion and surface aggregation of *S. mutans* cells were also decreased in the absence of eDNA (Das et al., 2010). Liao et al. (2014) found that DNA enhanced the adherence of *S. mutans* to saliva-coated hydroxyapatite substrate, but only when glucans were present. Nevertheless, different results were reported by a previous study where the presence of DNA alone increased the adhesion strength of *S. mutans* (Das et al., 2011).

A study by Shields et al. (2013) investigated the function of eDNA in chronic rhinosinusitis associated biofilms, which include biofilms formed by several oral streptococci. The results of this study emphasized the importance of eDNA as a structural support for oral biofilms as one-hour incubation with NucB enzyme reduced all biofilms except for one strain of *S. salivarius*. *E. faecalis* biofilms grown or treated with DNase I showed more porous and disintegrated features when compared to control biofilms. Additionally, the volume of exopolysaccharides in DNase biofilms was significantly lower than that of the

control biofilms implying that eDNA could act as a support for other components of the matrix (Yu et al., 2019).

Antibiotics and other antimicrobials are used as adjunctive treatment of oral diseases including periodontal and endodontic infections, and oral hygiene products often comprise antimicrobial agents. Nevertheless, the role of eDNA in the protection of oral bacteria against antimicrobials has not been clearly identified (Jakubovics and Burgess, 2015). In non-oral bacteria, eDNA has been shown to protect microbial cells in biofilms against the effect of various antimicrobial agents. For instance, DNase I promotes the efficacy of antibiotic treatment of non-typeable *Hemophilus influenzae* (NTHI) biofilms (Cavaliere et al., 2014), and increases the sensitivity of *Mycobacterium tuberculosis* biofilm cells to isoniazid (Ackart et al., 2014). eDNA is a negatively charged molecule and could limit the penetration of positively charged antibiotics such as tobramycin via ionic interactions (Tseng et al., 2013). Recently, DNase I treatment has been found to enhance the antimicrobial efficacy of tobramycin (Waryah et al., 2017) and povidone iodine (Kaplan et al., 2018) against *Staphylococcus aureus* biofilms. However, the protective role of eDNA against antimicrobials might not exist in all biofilms as the susceptibility of *Burkholderia cepacia* biofilms to tobramycin was not improved by DNase I treatment (Messiaen et al., 2014). This could be due to the presence of DNA binding proteins which might protect eDNA from being degraded by DNase I. The effect of eDNA on the sensitivity of *E. faecalis,* a leading cause of recurrent endodontic infections, to chlorhexidine has been investigated in several studies and it has been found that DNase I sensitized the *E. faecalis* biofilms to chlorohexidine (Li et al., 2012, Ganesh et al., 2015, Yu et al., 2019). Furthermore, Yu et al. (2018) found that antibiotic-induced biofilm formation in *E. faecalis* is associated with increased eDNA levels. This biofilm induction effect was only seen with drugs that affect cell wall integrity, and was reduced by DNase treatment indicating that cell lysis and eDNA release is the mechanism behind this protective response. eDNA-dependent biofilm formation has also been induced in *S. mutans* upon exposure to cell wall-targeting antibiotics (Nagasawa et al., 2020a).

eDNA might also provide resistance against host defense antimicrobials. DNase treatment of NTHI biofilms increased the antibiofilm efficacy of human beta

defensin 3 (hBD-3) highlighting a role for eDNA in protection from antimicrobial peptides mediated killing (Jones et al., 2013).The latter observations could be relevant to the function of eDNA in oral biofilms as hBDs are secreted by gingival epithelial cells and upregulated in periodontitis(Liu et al., 2014). Additionally, hBD-3 is able to neutralize the oral pathogen *P. gingivalis* by intervening with its binding to host cell receptors thereby reducing inflammation (Pingel et al., 2008). By counteracting the action of hBDs, eDNA might have a role in the progression of periodontal disease.

Extracellular DNA has been found to act as source of nitrogen, phosphate and carbon for bacteria in various biofilms (Palchevskiy and Finkel, 2006, Pinchuk et al., 2008, Mulcahy et al., 2010, Lewenza et al., 2020). In *Pseudomonas aeruginosa* biofilms, a secreted DNase is required to degrade eDNA and utilize DNA fragments or nucleotides as nutrients (Mulcahy et al., 2010). Similarly, oral bacteria might be able to use eDNA as a nutritional source particularly since many of them produce DNase enzymes that can digest DNA (Palmer et al., 2012). Moreover, eDNA binds cationic metal ions and it could consequently aid to keep trace metals within biofilms. The oral environment seems to have low content of some metal ions such as manganese, which is essential for the growth of bacteria. In *S. gordonii,* Genes encoding manganese transport system that is repressed by high Mn^{2+} were found to be upregulated in the presence of saliva (Jakubovics et al., 2000). Further research is required in order to understand the role of eDNA as a nutrient resource for bacteria within oral biofilms.

Extracellular DNA released into biofilm matrices might be taken up by biofilm bacteria and utilized for genetic transformation. The analysis of the nucleotide sequence of genomic DNA from oral streptococci emphasizes the contribution of eDNA uptake to the genetic evolution of these species as it shows extensive evidence of recombination (Do et al., 2011, Hoshino et al., 2005, Roberts and Kreth, 2014). Furthermore, DNA uptake and natural transformation have been reported in *P. gingivalis* (Tribble et al., 2012) and *Tannerella forsythia* (Nishikawa and Tanaka, 2013). Gene transfer between different species can contribute to transfer of antibiotic resistance to pathogenic species. Using an experimental model, genomic DNA from *Veillonella dispar* was able to transform *Streptococcus mitis* to tetracycline resistance (Hannan et al., 2010) . Besides

that, gene transfer from *S. mitis* and *S. oralis* is likely to be responsible for the increasing penicillin resistance among *Streptococcus pneumoniae* (Chi et al., 2007).

1.5 The activity of bacterial DNases in dental plaque

While many bacterial species produce extracellular nucleases, the effect of these native DNases on eDNA in biofilms is still unclear. The activity of bacterial nucleases was shown to regulate the amount of biofilm formation by digesting eDNA and enhancing biofilm dispersal in several species including *Neisseria gonorrhoeae, Vibrio cholerae, Haemophilus influenzae*, *Shewanella oneidensis* and *Staphylococcus aureus*. In these organisms, increased eDNA accumulation and thicker biofilms were reported in nuclease mutants when compared to the wild-type strains (Steichen et al., 2011, Seper et al., 2011, Cho et al., 2015, Gödeke et al., 2011). However, different nucleases could have different functions within the biofilms as only two of the three extracellular nucleases produced by *S. oneidensis* MR-1 affected biofilm formation (Heun et al., 2012). Additionally, one of these nucleases exhibited its effect on biofilm formation under static but not hydrodynamic conditions (Gödeke et al., 2011). Nuclease mutants of *S. aureus* showed enhanced capacity to form a biofilm *in vitro,* whilst mutation of nuclease genes *in vivo* was associated with compromised biofilm formation (Kiedrowski et al., 2011, Beenken et al., 2012). Hence, extracellular nucleases appear to have different roles under *in vitro* versus *in vivo* conditions. Many oral bacteria including periodontal pathogens have been shown to produce DNase enzymes (Palmer et al., 2012, Doke et al., 2017). These DNases were primarily investigated for their capability to degrade NETs; however, it is unknown if and to what extent these enzymes are involved in eDNA degradation and plaque biofilm remodeling. Recently, a DNase produced by *S. mutans,* identified as DeoC, has been examined for a potential role in biofilm dispersal. Supernatant from the nuclease mutant biofilms, unlike the wild-type strain supernatant, showed no DNA degradation activity, implying that DeoC plays a role in eDNA degradation (Liu et al., 2017). Furthermore, DeoC added to preformed biofilms of *S. mutans* significantly reduced the biofilm biomass. However, the purified DeoC could not degrade DNA independently highlighting a

discrepancy between the antibiofilm and the DNA degradation activity of this DNase. It is also not unknown whether the dispersal effect of DeoC is restricted to *S. mutans* biofilms or if it extends to affect biofilms formed by other oral species and more importantly dental plaque. Since dental biofilms were found to be sensitive to exogenous DNases such as NucB (Rostami et al., 2017), DNases produced naturally by oral bacteria could be upregulated to reduce biofilm formation or disperse pre-established plaque biofilms.

1.6 eDNA-host interaction

Bacteria-host interactions are initiated by the recognition of various bacterial molecules and patterns by the immune system. Tokunaga et al. (1984) were the first to discover that bacterial DNA has the ability to stimulate the host immune cells. Several subsequent studies have shown that DNA from gram positive and gram-negative bacteria can efficiently activate host immune cells triggering the release of proinflammatory cytokines (Cowdery et al., 1996, Sparwasser et al., 1997, Stacey et al., 1996). In contrast to mammalian DNA, bacterial DNA contains unmethylated CpG motifs, hence, the immune system can distinguish it from host DNA and, consequently, sense the threat of infection (Lipford et al., 1997, Krieg, 2002). DNA isolated from cells of periodontopathogenic bacteria induced the production of tumor necrosis factor alpha and interleukin-6 by murine macrophages and human gingival fibroblasts (Nonnenmacher et al., 2003b, Soto-Barreras et al., 2017). DNA extracted from oral bacteria was also capable of stimulating gingival epithelial cells to produce interleukin-8 (Kim et al., 2012a). Since bacterial DNA is recognized by Toll-like receptor 9 on the surface of innate immune cells and B cells, both innate and adaptive immune response can be stimulated by bacterial DNA. Although DNA present in the biofilm matrix is likely to contain the same immunogenic patterns present in genomic DNA, it could also include DNA from host cell lysis and consequently it might be less antigenic than intracellular bacterial DNA (Watters et al., 2016). Treating *in vitro* biofilms of *P. aeruginosa* with DNase I significantly reduced the ability of the biofilms to stimulate the release of cytokines IL-8 and IL-1b from neutrophils (Fuxman Bass et al., 2010). These results suggest that eDNA is a major proinflammatory component of *P. aeruginosa* biofilms. Additionally, the use of DNase I to treat

cystic fibrosis patients led to a decrease in the markers of inflammation and neutrophil derived metalloproteinases reinforcing the potential of eDNA being a trigger for immune cell response (Konstan and Ratjen, 2012). Similarly, eDNA may have an important effect on sensing the dental biofilm by host immune cells. Ramirez et al. (2019) assessed the immunogenic potential of eDNA extracted from *E. faecalis* monospecies biofilms on macrophages. The authors found that eDNA has the ability to induce a low-grade inflammatory response which could be associated with chronic inflammation. Pathogenesis of periodontitis is likely to be affected by the interactions between host immune cells and eDNA of subgingival biofilm, however, little is known about these interactions.

1.7 Targeting eDNA for oral biofilm control

Since eDNA seems to play an important role in maintaining the structural integrity and increasing the antimicrobial resistance of numerous different biofilms, targeting with DNase enzymes represents a promising strategy for biofilm control. DNases have been shown to degrade several mono- and multispecies biofilms of gram-positive and gram-negative bacteria (Okshevsky and Meyer, 2015). The degradation of eDNA can release microorganisms from the biofilm matrix or weaken the biofilm and increase its sensitivity to antibiotics. Currently, the antibiotic treatment of patients with cystic fibrosis is combined with recombinant human DNase I to degrade eDNA thereby reducing mucus viscosity and facilitate its clearance in the lung (Konstan and Ratjen, 2012, Manzenreiter et al., 2012). Bovine DNase I also showed excellent antibiofilm effects against Gardnerella vaginalis, a predominant organism in bacterial vaginosis, *in vitro* as well as *in vivo* and enhanced the activity of metronidazole against these biofilms (Hymes et al., 2013).

Furthermore, there is evidence that DNases can be incorporated in and act synergistically with other biofilm targeting approaches such as nanoparticles. For instance, ciprofloxacin nanoparticles coated with DNase I prevented *P. auroginosa* biofilm formation and substantially reduced the mass, size and living cell density of established biofilms (Baelo et al., 2015). In a recent study, DNase I was found to potentiate the effect of nanoparticles activated with an antimicrobial enzyme against polymicrobial biofilms of *Candida albicans* and

Staphylococcus aureus in vivo (Tan et al., 2020). Nanoparticles have been successfully used to deliver antimicrobials such as doxycycline and chlorhexidine to oral biofilms (Zhou et al., 2018, Zhao et al., 2019). A combinatorial approach comprising nanoparticles and DNases could achieve enhanced outcomes in oral biofilm control.

Coating surfaces of oral prostheses such as implants or dentures with DNases is also a potential option that could help prevent or inhibit oral biofilm accumulation. Swartjes et al. (2013) have reported that applying DNase I enzyme coating to polymethylmethacrylate (PMMA) material significantly reduced the adhesion of *S. aureus* and *P. aeruginosa* and inhibited biofilm formation for up to 14 hrs. PMMA is a commonly used material in making dentures, hence, the DNase coating approach could be utilized to tackle the problem of denture stomatitis. However, the coating technique might need some optimization as the enzyme was found to lose activity between 8 and 24 h. Functional DNase coating of titanium, a common dental implant material, has also been investigated and shown to reduce adhesion of *S. mutans* and *S. aureus* and inhibit biofilm formation over 24 h although the activity of the enzyme only lasted for 16 hours (Ye et al., 2017).

The use of mammalian DNases is limited by their high cost as glycosylation is required for their full activity, and consequently, their production in cheap bacterial expression systems is not possible. Bacterial nucleases have recently been described and demonstrated to inhibit or disperse biofilms. One example is NucB from *B. licheniformis* which has been shown to be advantageous when used against clinically relevant biofilms (Shakir et al., 2012, Shields et al., 2013). (Rostami et al., 2017) have assessed the efficacy of NucB against oral biofilm models and demonstrated that biofilm formation is strongly inhibited by NucB treatment. The authors, however, found that preformed biofilms were relatively resistant to the treatment.

The age of the biofilm appears to be a limiting factor when using DNases against biofilms as several reports showed that DNase treatment becomes less effective once the biofilm has matured beyond a certain stage (Whitchurch et al., 2002, 2007, Barnes et al., 2012, Schlafer et al., 2017, Schlafer et al., 2018b, Yu et al.,

2019). It is still not clear why more aged biofilms are resistant to DNases, but a possible explanation is that other components of the biofilm matrix might substitute or bind eDNA and protect it from enzymatic degradation. It is, therefore, important to identify interactions between eDNA and other components of the matrix such as proteins and polysaccharides, and to develop strategies that can target these interactions.

DNases could have species-specific effects as some late colonizers of dental plaque including *P. gingivalis* were less abundant in biofilms grown in the presence of NucB than early colonizers such as streptococci (Rostami et al., 2017). Early colonizers express a variety of cell surface adhesins that can mediate adherence to receptors in the salivary pellicle (Nobbs et al., 2011), hence, they might be less dependent on eDNA for adhesion. If NucB is capable of excluding certain species from the biofilm, it might be due to an effect of eDNA on some synergistic interbacterial interactions that are important for dental plaque formation.

While adding DNases exogenously has already shown promising results as a way of targeting eDNA in biofilms, the induction of bacteria to degrade their own biofilms by upregulating endogenous DNases is another potential approach. Gnanadhas et al. (2015) found that treatment with L-Methionine induced the DNase production in *P. aeruginosa* rendering resistant biofilms susceptible to ciprofloxacin. These results were also reflected *in vivo,* in the murine chronic PA lung infection model, where mice treated with ciprofloxacin and L-Methionine showed enhanced survival in comparison to mice treated with ciprofloxacin alone. Several oral bacteria produce DNase enzymes, however, the capability of these enzymes to digest eDNA and disrupt plaque biofilm is not yet clear. Furthermore, the stimulation of endogenous nucleases production in bacteria might be complicated. Different organisms use different regulation systems for the expression of their nucleases, and it is, therefore, challenging to find a universal control approach for the wide range eDNA dependent bacteria in the biofilm (Okshevsky et al., 2015). The induction of nuclease production in one of many species in a mixed-species biofilm could be an easier strategy, but whether it would be effective at removing the whole biofilm remains to be investigated.

Clearly, eDNA seems to play an important role in oral biofilm formation and stability and it can, therefore, be a potential target for oral biofilm control. However, there are still many unanswered questions regarding the functions and sources of eDNA in oral biofilm. For example, do all bacterial species in dental plaque contribute to the release of eDNA to the same extent and does eDNA have a role in the immunopathogenesis of periodontitis? It would also be interesting to see if targeting eDNA can exclude pathogenic species from the dental biofilm and whether the dental biofilm is as sensitive to DNases produced by the biofilm species as it is to DNases from non-oral bacteria.

1.8 Aims and objectives:

This project aims to develop an enhanced understanding of the roles of eDNA in dental plaque by addressing the following objectives:

1. Establish if certain bacteria are dependent on eDNA during mixed-species biofilm formation.

2. Assess the antibiofilm activity of native DNases in dental plaque.

3. Characterize the bacterial composition of eDNA from dental plaque.

4. Determine the ability of eDNA to stimulate host cell responses

Chapter 2: Materials and Methods

2.1 Bacterial culture

2.1.1 Bacterial strains and routine culture

The bacterial strains used in this thesis are *Streptococcus gordonii* DL1, *Fusobacterium nucleatum* ATCC 25586 and *Porphyromonas gingivalis* 381 (obtained as a gift from Dr Graham Stafford, Sheffield). *S. gordonii* was grown in BHYE liquid medium [Brain heart infusion 37 g/L (Melford Laboratories, Ipswich, UK), and yeast extract 5 g/L (Melford)] and the culture was incubated at 37ºC aerobically (without shaking) overnight. *F. nucleatum* ATCC 25586 was cultured in BHYEG [37 g/L brain heart infusion (Melford), 5 g/L yeast extract (Melford), and 2.5 g/L L-glutamic acid (Sigma Aldrich, Gillingham, UK)] and incubated at 37ºC in anaerobic conditions (10% CO_2, 10% H_2, and 80% N_2) for 48 h. *Porphyromonas gingivalis* was grown anaerobically in trypticase soy broth (TSB) (Melfords) supplemented with 1 µg/ml menadione and 5 µg/ml hemin (TSBHM) at 37°C for 48h. After incubation, purity was checked using phase contrast microscopy and gram staining.

2.1.2 Glycerol stocks of bacteria

The relevant broths were inoculated from the laboratory's main 'culture collection' stocks (-80ºC) and incubated as appropriate for each strain. Purity was checked as mentioned above and an inoculum from the culture was streaked on BHI [37 g/L Brain Heart Infusion (Melford laboratories), 5 g/L Yeast Extract (Melford), and 15 g/L Agar (Melford)] for *S. gordonii* and Fastidious Anaerobic Agar [Melford laboratories]. *F. nucleatum* and *P. gingivalis*. The plates were incubated for 24 h in case of *S. gordonii,* 48 h for *F. nucleatum* and 5 days for *P. gingivalis*, and one colony was then used to inoculate 20 ml sterile broth. After checking purity, the broths were transferred to sterile centrifuge tubes and centrifuged at 3,800 xg for 10 min at 20ºC [Sigma 3K10 centrifuge, swing out rotor, Sigma Aldrich]. The supernatant was removed, and the cell pellets were resuspended in a mixture of 500 µL appropriate sterile broth and 500 µL sterile

50% glycerol (VWR). The glycerol stock was transferred to a sterile Eppendorf tube and stored at -80ºC to be used when needed.

2.2 Sample collection

2.1.2 Saliva samples collection

Ethical approval was gained by the National Research Ethics Service Committee, North East (ref. 14898/2018). Whole stimulated saliva was collected as described by Nance et al. (2013). Donors were healthy adults who had not taken antibiotics for at least 2 weeks and had not consumed food or drink for 2 h prior to collection. Saliva samples were collected on ice and used as an inoculum to grow model dental plaque biofilms within 15 min of collection.

2.3 Biofilm Models

2.3.1 Static monospecies and multispecies bacterial biofilm models

To culture mono-species biofilms of *F. nucleatum*, 1 ml of 48 h old culture (OD_{600}= 1.2) was added to triplicate wells of a sterile polystyrene 12 well plate [Greiner Bio One, Stonehouse, UK] containing 3 ml BHYEG. The plate was sealed with parafilm and incubated in anaerobic conditions for 4 days to allow biofilm formation. At the end of the incubation time, the growth medium was removed, and the biofilms were washed once with 1 ml phosphate buffered saline (PBS) to remove loosely attached cells. The biofilms were quantified by crystal violet staining and the absorbance A_{570} was read in a Synergy HT microplate reader (BioTek, Bedfordshire, UK). To culture mono-species biofilms of *P. gingivalis*, 2.5 µl of *P. gingivalis* stock or 500 µl of 48 h old culture of *P. gingivalis* was used to inoculate triplicate wells of sterile polystyrene 12 well microtiter plate containing 2 ml TSBHM. The plate was incubated in anaerobic conditions for 4 days. *Streptococcus gordonii* DL1 and *Porphyromonas gingivalis* 381 were used to establish a dual-species biofilm model. Concentrated stocks of both species were prepared as follows: *P. gingivalis* was grown on fastidious anaerobic agar (FAA) plates at 37 ˚C in anaerobic conditions (5% CO_2, 10% H_2, and 80% N_2) for 5 days. A few colonies were used to inoculate 45 ml of TSBHM, and the bacteria were grown anaerobically at 37˚C for 48 h. After checking purity, the broth cultures were transferred to sterile centrifuge tubes and centrifuged at 3,800 g, 4˚C for

10 minutes. The pellets were resuspended in 500 µL sterile broth media and 500 µl sterile glycerol. These concentrated glycerol stocks were transferred to sterile Eppendorf tubes and stored at -80ºC to be used as inocula for initiation of biofilms. *S. gordonii* was maintained on BHYE agar plates for 18 h and few colonies were used to inoculate 45 ml of BHYE broth, and the culture was then grown aerobically overnight at 37 ˚C. Cells were collected by centrifugation, and stocks were prepared, as described for *P. gingivalis*. Bacterial counts of the prepared stocks of both species were determined by the method of Miles et al. (1938) to be able to add a consistent number of cells to biofilm growth experiments. To initiate the dual species biofilms, 13 µl (1×10^8 CFU/ml) of *S. gordonii* previously prepared stocks were used to inoculate each well of a 12 well tissue culture plate containing 2 ml BHYE. Cultures were grown anaerobically at 37˚C for 24 h to allow biofilm formation. The culture medium was subsequently removed from streptococcal biofilms and 2.5 µl (1×10^8 CFU/ml) of *P. gingivalis* stock were added with 2 ml TSBHM to each biofilm. The biofilms were allowed to grow anaerobically for additional 20 h at 37˚C. The growth medium was then removed, and the developed biofilms were washed once with 1 ml PBS and harvested in 3 ml PBS using a cell scraper. Cells were harvested by centrifugation at 3,800 xg at 4˚C for 10 min and prepared for chromosomal DNA extraction.

2.3.2 Static *in vitro* dental plaque biofilms

Whole human saliva was used to inoculate artificial saliva 1:100 (Pratten, 2007). Biofilms were cultured on the bottoms of 12 well microtiter plates to be quantified by crystal violet staining or on sterile glass coverslips placed in 6 well plates to be visualized by microscopy. The biofilms were grown for 24 h at 37˚C either aerobically (without shaking) with 2% (w/v) sucrose to model supragingival dental plaque or anaerobically with no sucrose to mimic the growth conditions for subgingival dental plaque.

2.3.3 Biofilm formation in a Microfluidic biofilm system

A microfluidic biofilm system was used to grow *F. nucleatum biofilms* to study the effect of DNases enzymes on the biofilms under hydrodynamic conditions. The experiment was started by loading 200 µl of BHYEG in the inlet wells of a

Bioflux 48-well plate (Bioflux 1000, Fluxion Biosciences, USA). The growth medium was pumped towards the outlet wells at 2.0 dyne cm^{-2} for 2 min at room temperature. The flow was then stopped, and the plate channels were allowed to be primed for 1 h at room temperature to enhance cell adhesion and biofilm formation. At the end of the priming period, the excess growth medium was aspirated from the output wells and 200 µL of 48 h old culture of *F. nucleatum* were added. The bacterial suspensions were then pumped from the output wells to the input wells at a rate of 2.0 dynes cm^{-2} for 5–10 seconds to fill the microfluidic channels of the plate. The suspension was incubated statically in the channels for 1 h at 37°C to allow the cells to settle. 800 µl of fresh sterile BHYGE medium were then added to the outlet wells and flow was started and adjusted to a slower rate of 0.2 dyne cm^{-2}. To follow the growth of the biofilms, bright-field images were acquired once every 30 mins over 24 h at 20X magnification using an inverted microscope (Nikon) mounted with a CCD camera (1392 × 1040 pixels).

2.4 Molecular biology methods

2.4.1 Extraction of intracellular DNA

Intracellular DNA (iDNA) was extracted from planktonic cultures of *S. gordonii* and *P. gingivalis*, monospecies biofilms and mixed species biofilms. Bacterial cells (10 ml) were harvested from 48 h old broth culture of *P. gingivalis* and overnight culture of *S. gordonii* by centrifugation at 3,800 xg for 10 min at 4°C. Pellets were resuspended in 150 µl of Spheroplasting Buffer [20 mM Tris-HCl (Melford, UK), 10 mM $MgCl_2$ (Sigma, UK), and 26% (w/v) Raffinose.$5H_2O$ (Sigma, UK)]. 10 µl of 250 µg/mL lysozyme (Sigma) and 5 µl of 10,000 U/mL mutanolysin (Sigma) were then added and the mixture was incubated in a water bath at 37°C for 30 min.150 µl of 2X T&C lysis solution [(Epicentre kit) CamBio, Cambridge, UK] were added to each sample and the samples were placed with 25 mg of acid washed glass beads (0.1 mm) in a bead lysis machine [(TissueLyser LT), Qiagen, Manchester, UK] at 50 Hz for 5 min. Samples were immediately placed on ice and 1 µl of Proteinase K (20 mg/ml) [CamBio] was added to each sample. Samples were incubated at 65°C for 30 min, vortexing briefly every 5 min. The mixture was cooled at 37°C for 10 min,1 µl of RNase A (5 µg/µl) [CamBio] was added, and

the samples were incubated at 37°C for 30 min. Samples were then placed on ice for 3-5 min, and eDNA was purified as follows. 350 µl of Protein Precipitation Reagent [CamBio] was added and samples were vortexed vigorously for 10 sec and centrifuged at 10,000 xg for 10 min at 4°C in a microcentrifuge [PrismR refrigerated microcentrifuge, Labnet, Edison, NJ, USA]. Supernatant was collected in a clean microcentrifuge tube and 500 µl of isopropanol was added. Tubes were inverted gently to mix and placed on ice for 10 min. DNA was pelleted by centrifugation at 10,000 xg for 10 min at 4°C, isopropanol was removed, and the pellet was rinsed with 500 µl of 75% ethanol. The sample was centrifuged at 4°C for 5 min at 10,000 xg and the ethanol rinse was repeated. The ethanol was removed, and the precipitated eDNA pellet was air dried and resuspended in 25 µl of PCR grade water. The purity and concentration of each sample was determined using a NanoDrop ND-1000 Spectrophotometer [ThermoFisher Scientific, Winsford, UK]. DNA Samples were also visualized by agarose gel electrophoresis (Section 2.4.4) to assess quality. Samples were stored at -20°C to be used later in relevant experiments.

2.4.2 Extraction of extracellular DNA

Extracellular DNA (eDNA) was extracted from *F. nucleatum* biofilms and from model dental plaque biofilms. After growth medium removal, biofilms were washed once with 1 ml PBS to remove unattached and loosely attached cells, and the biofilms were harvested in 1 ml PBS using a plastic cell scraper (Greiner Bio One LTD, Kremsmünster, Austria). The collected biofilms were transferred to Eppendorf tubes, vortexed for 20 sec and centrifuged in a microcentrifuge [PrismR refrigerated microcentrifuge, Labnet, Edison, NJ, USA] at 12,000 xg for 30 min at 4°C. Supernatants were then carefully transferred to clean Eppendorf tubes. Supernatant and cell pellets were stored at -20 ºC ready for the purification process. eDNA was purified from supernatants according to a protocol used by (Wittenberger and Angelo, 1970) and Sullivan et al. (2000). Briefly, an equal volume of 25:24:1 phenol-chloroform isoamyl alcohol was added to the supernatant containing eDNA, mixed by 30-40 times inversion and centrifuged at 16,000 xg for 5 min at 4°C. This step was repeated, and the

supernatant (upper phase) was carefully collected. DNA was precipitated by adding 1/10 volume 3M Na acetate (pH 5.2) and 2/3 volume isopropanol. After mixing and centrifuging for 5 min, eDNA pellet was air dried and resuspended in 25 µl of 10 mM Tris-HCl (pH 8) and stored at -20 ºC. The purity and the concentration of eDNA was measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific-USA) and eDNA was subsequently visualized using agarose (1% w/v) gel electrophoresis.

2.4.3 Nanodrop spectrophotometry

The NanoDrop ND-1000 spectrophotometer was used to determine the concentration and purity of iDNA and eDNA samples. A blank value of light absorbance was first obtained by placing 2 µl of the elution buffer on the NanoDrop stage. 2 µl of DNA samples were then loaded onto the NanoDrop stage and the absorbance was measured. The ratio of 260/ 280 nm absorbance was used to assess DNA purity (a ratio of 1.8-2.0 was regarded as acceptable purity).

2.4.4 Agarose gel electrophoresis

To make agarose gels, agarose powder [Agarose, Melford] was added to 50X TAE buffer [242 g Tris, 57.1 ml acetic acid (Fisher Scientific), and 100 ml of EDTA 0.5 M pH 8 adjusted to 1 L by H_2O_2] to the appropriate percentage and heated until dissolved. Gel solution was either stained by adding GelRed 10,000x stain in DMSO [Cambridge Bioscience Ltd, UK] 5 µl/50ml or ethidium bromide [Sigma Aldrich] 20 µl/50ml. The gel was then poured into an appropriate gel tray and allowed to solidify. Samples were mixed with loading dye [Bioline, Nottingham, UK] 5:4 before loading into the gel wells. MassRuler DNA Ladder Mix [80-10,000 bp; (Bioline)] and HyperLadder 1 kb Plus [250-12,007 bp; (Bioline)] were used as DNA molecular weight markers. Gel electrophoresis was run at 85 V/cm2 for 60 or 90 min, using a Bio-Rad Power Pac 300 [BioRad]. Gels were subsequently viewed on the G: BOX Transilluminator [Syngene] and images were captured using GeneSnap software (Syngene, UK).

2.4.5 Plasmid extraction from E. coli strains for qPCR standards

Plasmids were isolated from a sample of 10 µl *E. coli* stocks containing pTOPO-Pg (Field et al., 2012) and pTOPO-Sg [constructed in the Oral Biology lab at Newcastle University as follows. Oligonucleotide primers Sg DNaseF2 and Sg DNaseR2 were used to amplify a fragment (approximately 96 bp) of the restriction endonuclease (Putative type I site specific DNase) from *S. gordonii*. The PCR reaction included template DNA (2 nM) and the forward and reverse primers each at 250 nM in Reddymix reaction mixture. The PCR amplification reaction was run in the following order: 94˚C for 2 min (separation of DNA strands), 94˚C for 10 s (35 cycles), 52˚C for 30 s, 68˚C for 1 min 20 s followed by further incubation at 68˚C for 7 min. The amplified fragment was cloned in plasmid vector pCR2.1-TOPO to generate plasmid pTOPO-Sg (4027 bp). *Escheichia coli* DH5α was transformed with the plasmid, and transformants were selected on LB agar supplemented with ampicillin (100 µg /mL)]. *E. coli* containing pTOPO-Sg and pTOPO-Pg were cultured in 5 ml of LB broth with ampicillin (1µl/ml), and incubated overnight in a shaker incubator (Orbital Shaker, Jencons, UK) at 250 rpm, 37˚C. Broth cultures were centrifuged at 3,800 xg for 10 min at 4˚C, and the supernatant was discarded. The plasmids were extracted from the pellet using QIAPrep Spin Miniprep plasmid kit (Qiagen, UK) and eluted in 50 µl of elution buffer (provided with the kit). Samples were visualized using agarose gel electrophoresis, and stored at -20˚C.

2.4.6 Quantification of double stranded DNA by PicoGreen assays

To obtain an accurate measurement of plasmid DNA concentrations, ThermoFisher Scientific's Quant-iT™ PicoGreen™ dsDNA Assay Kit was used according to manufacturer's instructions. Standard λ DNA solution (2 µg/ml) was diluted in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) to make a range of concentrations (200 ng/ml to 0 ng/ml). The PicoGreen reagent was also diluted 200-fold and samples were diluted 1:50 in TE buffer. 75 µl of either standard λ DNA solution, or DNA templates, were placed in triplicate wells of black 96 well plates [Griener Bio One]. An equal volume of 75 µl from the prepared PicoGreen was added to both the standard and DNA template wells to obtain a total volume of 150 µl in each well. Fluorescence was measured in a Synergy HT microplate

reader (BioTek, Bedfordshire, UK) at excitation 480 nm and emission 520 nm. The resulting standard curve derived from the λ DNA was used to calculate the unknown concentration of the DNA.

2.4.7 Quantitative PCR

qPCR reactions were run for each of *S. gordonii and P. gingivalis* separately using their applicable primers, probe [see Table 2.1] and thermocycle. A standard curve was generated using 10-fold dilution series of plasmid DNA. The qPCR reaction for *S. gordonii* contained the following reagents: 1 µl of forward and reverse primers (300 nm) and 0.5 µl of TaqMan probe (150 nm), 6.25 µl of MyTaq enzyme (Bioline, UK), 2.75 µl PCR grade water, and 1 µl of either DNA template or PCR grade water in a total volume of 12.5 µl. When the qPCR was run for *P. gingivalis*, each qPCR reaction contained: forward and reverse primer 1.2 µl each (2.5 µM), probe 0.6 µl (2.5 µM), 7.5 µl of MyTaq Enzyme , 3.3 µl PCR grade water, and 1 µl of either DNA template or PCR grade water in a total volume of 13.8 µl. Triplicate samples were used for each analysis. The qPCR reaction was run in a QuantStudio 3 thermal cycler (Thermo-Fisher Scientific, UK) with the following cycling program : 95°C for 10 min (initial denaturation), 95°C for 15 s, 60°C for 1 min, plate read. Cycle from step 2 was repeated for 39 cycles in the qPCR reaction of *S. gordonii* and for 40 times when the qPCR was run for *P. gingivalis*.

Primers and probes	Sequences (5'-3')	Target region	Reference
S. gordonii (F)	GGTGTTGTTTGACCCGTTCAG	Restriction endonuclease fragment	(Suzuki et al., 2004)
S. gordonii (R)	AGTCCATCCCACGAGCACAG		
S. gordonii (P)	FAM-AACCTTGACCCGCTCATTACCAGCTAGTATG-TAMRA		

P. gingivalis (F)	ACCTTACCCGGGATTGAAATG	16s rRNA gene	(Field et al., 2012)
*P. gingivalis (*R)	CAACCATGCAGCACCTACATAGAA		
P. gingivalis (P)	FAM-ATGACTGATGGTGAAAACCGTCTTCCCTTC-TAMRA		

Table 2.1 Quantitative PCR forward primers (F), reverse primers (R) and probes (P) sequences*. FAM is a fluorescent reporter dye, TAMRA is a fluorescent quencher.*

2.4.8 DNA sequencing

The microbial composition of iDNA and eDNA samples extracted from model dental plaque biofilms [see Section 2.3.1 and Section 2.3.2] was characterized using 16S rRNA gene sequencing. Prior to sequencing, sample concentrations were measured using a NanoDrop ND-1000 spectrophotometer and samples were analyzed by 1% agarose gel electrophoresis. Samples were sent to Source BioScience [UK Ltd] to perform the 16S rRNA gene amplification and Illumina MiSeq sequencing. The V1-V2 variable regions of 16S rRNA genes were amplified using primers 27F-YM and 338R-R (Hamady et al., 2008). After Illumina paired-end library preparation, cluster generation and 299-bp paired-end sequencing were performed for all 26 samples (12 eDNA samples, 12 iDNA samples, 2 negative controls) in one run. Data analysis was performed by Dr Gavin Wee [Monash University, Malaysia]. During the analysis, paired-end reads and low-quality reads were first removed from sequence adaptors using BBDuk of the BBTools package (https://sourceforge.net/projects/bbmap/). After this, the forward and reverse reads were merged using USEARCH v11.0.667 (https://www.drive5.com/usearch/). Sequences shorter than 150 bp or longer than 600 bp (sequenced on the MiSeq platform) were removed from the

downstream processing. Reads were then aligned with 16S rRNA (SILVA Release 132) or UNITE ITS database and inspected for chimeric errors using VSEARCH v2.6.2. After these quality assessment steps, reads were clustered de novo into OTUs at 97% similarity using UPARSE v11.0.667; rare OTUs with less than 2 reads (doubleton) which are often spurious, were deleted from downstream processing. A single representative sequence from each OTU was randomly chosen, and Pynast (https://www.ncbi.nlm.nih.gov/pubmed/19914921) was used to align and construct a phylogenetic tree against the SILVA 132 16S rRNA database. Taxonomic assignment of OTU was achieved using QIIME V1.9.1 against the Silva database 16S rRNA database (release 132).

2.5 Microscopy

2.5.1 Scanning electron microscopy (SEM)

Scanning electron microscopy was used to visualize the structure of model dental plaque biofilms grown on glass coverslips, and to assess the effect of the DNase enzymes NucB and SsnA on the formation of these biofilms and removal of preformed biofilms. Samples were fixed in 2% (v/v) glutaraldehyde for 24 h at 4°C and washed twice in PBS. The samples were then dehydrated in series of ethanol concentrations (25%, 50%, 75% 30 min each and 2 x 100% for 1 h) at room temperature. Samples were then handed to the electron microscopy team at Newcastle University where they were dried in a critical point dryer (Bal-tec), mounted on aluminium stubs and sputter coated with gold. Biofilms were subsequently visualized using TESCAN VEGA 3LMU scanning electron microscope (Tescan, Cambridge, UK). Digital images were obtained with TESCAN software (Vega3 control software, version 4.2.13.1). Triplicates samples were used in all experiments.

2.5.2 Confocal laser scanning microscopy (CLSM)

The effect of DNase enzymes NucB and SsnA on the biofilm architecture of *F. nucleatum* biofilms was visualized by confocal laser scanning microscopy (CLSM). The biofilms were grown on sterile 13 mm diameter circle glass coverslips placed in wells of a six-well tissue culture plate containing 4 mL growth medium. The plate was incubated statically in anaerobic conditions at 37°C for 4 days. Growth

medium was then removed, and coverslips were washed once with PBS, and incubated in 5 µM SYTO 9 stain (Thermo Fisher Scientific, UK) for 15 min. Coverslips were then washed with PBS to remove unbound stain and inverted onto a gene frame 25 µL (1.0 x 1.0 cm) that had been placed on a microscope slide and filled with PBS. Biofilms were examined using a Leica SP8-STED3X inverted confocal microscope and biofilm stacks were captured using 63x water immersion objective lens. Excitation of SYTO® 9 was achieved with a 483 nm laser and emission wavelength was 503 nm. Four image stacks were captured for each sample. Each Image stack was converted to .mat file and imported into BAIT (Luo et al., 2019) where thresholding of the image stack was performed using the biovolume elasticity method (BEM)(Luo et al., 2018). Biofilms were quantified by calculating three biofilm architecture parameters (biovolume, number of objects, surface area). The output of results was exported as a. csv file and analysed in Microsoft Excel.

2.6 Anti-biofilm Activity of NucB and SsnA

2.6.1 Crystal violet staining assay

Crystal violet staining was used to quantify biofilm extent when assessing the effect of the DNase enzymes NucB (Baslé et al., 2017) and SsnA (Robert Shields, PhD thesis) on biofilm growth and removal. Biofilms were grown on the bottoms of 12 well plates and stained by adding 300 µL of 0.1% (w/v) crystal violet (per well). Plates were incubated for 15 minutes at room temperature with gentle rocking (20 rpm). Wells were then washed 3 times with PBS and 600 µl of 7% (v/v) acetic acid was added to each well to dissolve the residual stain. A_{570} was read in a microplate reader (Synergy HT). To evaluate biofilm extent, the A_{570} values of blank (stained but un-inoculated) wells were subtracted from the absorbance values of the biofilm samples. All assays were performed in triplicates.

2.6.2 Testing NucB and SsnA effect against microbial biofilms models

To assess the effect of NucB and SsnA on the inhibition and dispersal of microbial biofilms, biofilms were cultured in 12 well microtiter plates and biofilm biomass was quantified with the crystal violet assay. To assess inhibition, NucB (0.5 µg/ml) [produced by Alastair Hawkins, the Protein Production Facility, Newcastle

University] or SsnA (5 µg/mL) [produced by Paul Thompson, the Protein Production Facility, Newcastle University] was included with media and inoculae during biofilm formation. To test biofilm dispersal, enzyme was added after biofilm formation and the plates were incubated at 37˚C for 1 hour. The crystal violet assay was then used to compare the biofilm extent in control versus enzyme treated biofilms.

2.7 Nuclease activity assays

2.7.1 Measurement of NucB specific activity

The specific activity of NucB was measured using a method developed by Kunitz (1950) with a slight modification and DNase I enzyme was included as a control. A range of concentrations of NucB or DNase I enzyme were incubated at 37˚C with 125 µg of calf thymus DNA (CT DNA) [Sigma Aldrich] in a reaction buffer of 25 mM HEPES (pH 8.0) and 5 mM $MnSO_4$ in a total reaction volume of 250 µl. The reaction was stopped after 60 min by adding an equal volume of cold 4% (v/v) perchloric acid [Sigma Aldrich, UK] to 200 µl of the reaction volume and the mixture was then placed on ice for 40 min. The high molecular weight DNA was then pelleted by centrifugation in a benchtop microfuge [Prism R, Labnet International Inc, New Jersey, USA] at 13,000 rpm, 4ºC for 3 min, and the amount of low molecular weight DNA recovered in the supernatant was measured in a Nanodrop spectrophotometer at 260 nm. A unit of activity is the amount required to generate an absorbance of 1 when incubated with a set amount of CT DNA for a fixed amount of time. One unit is the production of perchloric acid soluble DNA products that generate an absorbance of 1.0 (using 1 ml volume in a 1 cm pathlength cuvette) at 260 nm, per hour at 37ºC in 25 mM HEPES pH8.0, 5 mM MnSO4. Only the products of the nuclease reaction (typically less than 500 bp in length) are soluble in 4% perchloric acid while high molecular weight DNA is not acid soluble. The rest of the reaction volume was utilized to visualise the digestion products of NucB-treated calf thymus DNA using 1% (w/v) agarose gel electrophoresis. To perform this, an equal volume of phenol/chloroform/isoamyl alcohol (Sigma Aldrich, UK) was added to 50 µl of the reaction volume and the mixture was vortexed vigorously for 30 sec to form an emulsion. The sample was

centrifuged for 3 min at 13,000 rpm and 4˚C in a benchtop microcentrifuge forming two layers. 5 µl of the upper layer were loaded on the gel.

2.7.2 Testing NucB and SsnA activity against double stranded and plasmid DNA.

A range of concentrations of NucB or SsnA enzyme were incubated at 37˚C with 125 µg of calf thymus DNA (CT DNA), salmon sperm DNA [Sigma Aldrich] or 1 µg plasmid DNA from *E. coli*. The reaction buffer for NucB consisted of 25 mM HEPES (pH 8.0) and 5 mM MnSO4 in a total reaction volume of 250 µl. For SsnA, the reaction buffer contained 25 mM Tris (pH 8.0), 40 µM EDTA and 80 µM $MgCl_2$. The reaction was stopped after 60 min by adding an equal volume of cold 4% (v/v) perchloric acid to half the reaction volume and the mixture was then placed on ice for 40 min. The high molecular weight DNA was pelleted by centrifugation in a benchtop microfuge at 4ºC and the amount of low molecular weight DNA recovered in the supernatant was measured in a Nanodrop spectrophotometer at 260 nm. Agarose gel electrophoresis was also used to monitor the reaction as described in Section 2.7.1.

2.7.3 Förster resonance energy transfer assay (FRET)

The DNase activity of NucB and SsnA against single stranded DNA was measured quantitatively using a fluorescence-based assay in a method originally developed by Kiedrowski et al. (2011). The substrate for FRET assay was single stranded oligonucleotide of a short sequence (5' CCCCGGATCCACCCC 3'), modified at the 5' end with a Hex fluorophore and at the 3' end with a Black Hole Quencher 2. FRET is a distance-dependent energy transfer from a donor chromophore to an acceptor chromophore so that no photon is emitted. When the enzyme cleaves the phosphodiester bond between two nucleotides, the donor and acceptor chromophores will no longer be in close proximity, enabling the emission of fluorescence. A range of concentrations of NucB and SsnA were incubated with 2 µM of FRET substrate in a reaction buffer to a total reaction volume of 25 µl in a well of a 384-well microtiter plate (Greiner Bio-One), The reaction buffer for SsnA contained 25 mM Tris-HCl (pH 8), 80 µM EDTA and 40 µM $MgCl_2$. For NucB, the buffer consisted of 25 mM HEPES and 5 mM MnSO4. Fluorescence was

measured using the Synergy HT (BioTek) microplate reader (excitation 530 nm / emission 590 nm) over 30 min at 30°C. The background fluorescence measured for the control mix (FRET substrate incubated with the buffer only) was subtracted from the fluorescence value of the various enzyme concentrations.

2.8 Protein analysis

2.8.1 Bradford protein assay

The total extracellular protein concentration in samples of saliva derived model plaque biofilms was measured using Bradford assay. Bradford assay is based on the binding of the Coomassie brilliant dye to proteins in the samples which results in change in the colour of from brown to blue and consequently an immediate shift in absorption from 465nm to 595nm. After the growth medium removal, biofilms were scraped using a plastic cell scraper (Greiner Bio One LTD, Kremsmünster, Austria] and collected in 1 ml PBS. The collected biofilms were transferred to Eppendorf tubes, vortexed for 20 sec and centrifuged in a in a microcentrifuge [(PrismR refrigerated microcentrifuge), Labnet, Edison, NJ, USA] at 12,000 xg for 30 min at 4°C. Supernatants were then carefully transferred to clean Eppendorf tubes. The protein concentration in these supernatants was then determined. A standard curve was first generated using concentrations of bovine serum albumen ranging from 0.1 to 1.6 mg/ml. 250 µl of Pierce™ Coomassie Plus (Bradford) Assay Reagent [Thermofisher Scientific], was added to 5 µl of each standard or unknown sample in a 96 well microtiter plate [Greiner Bio One] and mixed on a plate shaker for 30 seconds. The plate was then incubated at room temperature for 10 minutes before the absorbance was measured at 595nm in a Synergy HT plate reader [BioTek, Swindon, UK]. The standard curve was used to determine the protein concentration in the biofilm samples.

2.8.2 Protein gel electrophoresis

SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis), was used to separate proteins in samples from model plaque biofilms. The first step was to prepare 10 ml of resolving gel solution containing 12% acrylamide (4 ml of 30% acrylamide) [Sigma Aldrich], 0.1 ml of 10 % SDS [Melford], 2.6 ml of 1.5M Tris pH8.8 [Melford], 3.2 ml dH2O, 100 µL of 10% w/v ammonium persulfate (AP)

[Sigma Aldrich], 10 µL of N,N,N′,N′-Tetramethylethylenediamine (TEMED), [Sigma Aldrich]. The resolving gel solution was then pipetted into a gel cast and a layer of isopropanol (2-propanol) [Sigma Aldrich] was added on the top. The resolving gel was left to set for 20-30 min before adding the stacking gel. The stacking gel solution (5 ml) was prepared as follows: 0.67 ml of 30% Acrylamide [Sigma Aldrich], 1.25 ml of 0.5 M Tris-HCl pH 6.8, 0.05 ml of 10% w/v SDS [Melford], 2.975 ml dH_2O, 50 µL of 10% w/**v** ammonium persulfate (AP) [Sigma Aldrich], 5 µL of N,N,N′,N′-Tetramethylethylenediamine (TEMED), [Sigma Aldrich]. The isopropanol was removed, the stacking gel was added, and a comp inserted. After the stacking gel was completely set, the comp was removed and the 1X SDS page running buffer was poured into the gel tank until the gel is covered. Protein samples were diluted with 5x sample buffer [0.25 M Tris-HCl pH6.8 [Melford], 50% glycerol [VWR], 10% SDS [Melford], 0.5 M diothiothreitol (DTT) (Sigma Aldrich), 0.25% (w/v) Bromophenol blue (BPB) (Sigma Aldrich)]. Diluted samples were heated at 100°C for 10 min to denature proteins. Samples were then centrifuged at 10,000 rpm for 1 minute and loaded into the gel alongside the protein molecular weight marker [PageRuler unstained Broad Range protein ladder (10-200 kDa), Thermo Scientific, UK]. The electrophoresis was run using a BioRad Power Pac 300 [BioRad, Watford, UK] at 120 V/cm^2 for 1 hour. Gel was then stained by immersion in Coomassie blue stain [InstantBlue, Sigma Aldrich] for 30 minutes with gentle shaking on a horizontal shaker. The gel was immersed in dH_2O on the same shaker overnight for destaining.

2.9 eDNA-macrophages interaction experiments

Macrophages differentiated from THP-1 promonocytes [ECAC public health England] using PMA were grown in RPMI-1640 (+glutamine) medium [Sigma Aldrich] supplemented with 10% Heat inactivated foetal bovine serum (FBS), Penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were counted and diluted to 0.5 x 10^6 cells/0.9 ml. About 1.8ml (10^6 cells) was added into each well of 12 well plate [Greiner Bio One, Stonehouse, UK] and incubated for 24 h at 37 °C, 5% CO2.The cells were exposed to 1 µg/ml eDNA [purified from monospecies biofilms of *F. nucleatum*, see Section 2.4.2] for 24 hours. LPS from *E. coli* (100 ng/m) [R & D Systems, UK] was used as a positive control. The DNase

enzyme NucB (1µg/ml), and NucB digested eDNA were also included as controls. The concentrations of tumour necrosis factor alpha (TNFα) released by the stimulated cells were measured using an enzyme-linked immunosorbent assay (ELISA) [performed by Nicola Griffins in our cell culture lab] with the Human TNF-α DuoSet® ELISA kit [R & D Systems, UK]. TNF-a concentration was expressed as picogram per millilitre.

2.10 Immunodot assay

An immune dot assay was employed to detect eDNA in monospecies biofilms of *F. nucleatum*. Biofilms were washed once with 1 ml PBS to remove unattached and loosely attached cells before being harvested in 1 ml PBS using a plastic cell scraper (Greiner Bio One LTD, Kremsmünster, Austria). The collected biofilms were transferred to Eppendorf tubes, vortexed for 20 sec and centrifuged in a in a microcentrifuge [PrismR refrigerated microcentrifuge, Labnet, Edison, NJ, USA] at 12,000 xg for 30 min at 4°C. Supernatants were then carefully transferred to clean Eppendorf tubes. A dilution series of these supernatants (1 µl) were dotted onto nitrocellulose membrane, 0.45 µm [Bio-Rad, UK] and left to dry at room temperature for 1 hour. iDNA purified from cell pellets extracted from the same biofilms was used as a control. The membrane was incubated in 5% (w/v) milk protein in TBST [Tris-buffered saline; 20 mM Tris-HCl, 140 mM NaCl, pH 7.5 plus 0.1% Tween-20, v/v] for 1 h to block all nonspecific protein-binding sites. The membrane was then Incubated overnight in primary antibody [Mouse monoclonal Anti-ds DNA antibody (Abcam, UK)] diluted at 1:1000 fold in TBST. After being washed extensively in TBST, the membrane was incubated for 1.5 h in secondary antibody [Goat Anti-Mouse IgG H &L conjugated to Horseradish peroxidase (HRP) (Abcam, UK)] diluted in the region of 2000 fold in TBST. The membrane was subsequently washed extensively in TBST and incubated in enzyme marker substrate solution [4-chloro-1-naphthol (Sigma Aldrich) + 30% H_2O_2 (Sigma Aldrich) added just before use to a final concentration of 0.01%] at room temperature for 15-30 min or until purple dots appeared at the antigen antibody complex sites. The nitrocellulose membrane was blotted on paper towel and washed briefly in 10 mM Tris-CL 150 mM NaCl. The membrane was washed in

two changes of water and a photo was taken. The membrane was left to dry overnight by blotting between filter papers.

2.11 Statistical analysis

Statistical analysis was performed using three independent experiments which were carried out in technical triplicates. For CLSM, three independent experiments were completed, but each experiment included only one sample for each treatment and control. However, four different areas of each sample were imaged to be used as technical replicates. Graphs and tables were produced using Microsoft Excel or SigmaPlot 14.0 [Systat Software, San Jose, CA, USA]. Statistical significance was analysed using IBM SPSS Statistics 26. The two-sample t-test was used to compare the mean values of two groups to determine the significant difference between the groups. Prior to using the two-sample test, the normality of the distribution was confirmed by a test of normality. When there were more than two groups to compare, one-way analysis of variance (ANOVA) and the Tukey post hoc test were employed. A p-value that is less that 0.05 was considered an acceptable indication of statistical significance.

Chapter 3: The effect of NucB on biofilms formed by periodontal bacteria

3.1 Outline

Previous research indicated that eDNA is important for the adherence and biofilm formation of several oral bacteria (Das et al., 2011, Li et al., 2012, Schlafer et al., 2018a, Chen et al., 2019a). Subgingival dental plaque on extracted teeth and dental implants has recently been shown to contain substantial amounts of eDNA (Rostami et al., 2017). Therefore, there might be a potential of using DNA degrading enzymes to reduce subgingival plaque and consequently prevent or control periodontal disease. The use of simple biofilm models to assess the importance of eDNA for bacteria in subgingival biofilm can help investigate this potential.

NucB has emerged as a potent bacterial DNase that could effectively disrupt various mono and mixed species microbial biofilms (Nijland et al., 2010).This enzyme has been purified from a marine isolate of *Bacillus licheniformis* after the supernatant of this bacteria was found to disperse biofilms. Baslé et al. (2017) recently investigated the structure and activity of NucB to enhance the understanding of its antibiofilm properties. The authors found that NucB is a non-specific endonuclease as both single- and double-stranded DNA can be effectively digested by the enzyme. NucB could also hydrolyze supercoiled plasmid DNA which is a topologically complex molecule. These findings might explain the apparent antibiofilm effect of NucB since biofilm formation usually involves both single- and double-stranded DNA (Zweig et al., 2014). NucB properties clearly make it a precise candidate for evolving a potential preventive or therapeutic system to combat biofilms.

F. nucleatum and *P. gingivalis* are two important bacteria in the subgingival biofilm. Both species are frequently isolated from sub-gingival dental plaque of patients with periodontitis (Socransky et al., 1998). *F. nucleatum* act as a bridge between early and late colonizing bacteria of the oral biofilm playing a vital role in the development of mature dental plaque (He et al., 2012, Brennan and Garrett, 2019b, Zhang et al., 2019). *P. gingivalis* has been designated as a

keystone pathogen which can disrupt the host-microbe homeostasis and initiate periodontal pathogenicity (Hajishengallis and Lamont, 2016). Currently, there are no therapeutic interventions that can specifically target such periodontal pathogens.

Shields et al. (2013) found that biofilms formed by several oral streptococci were sensitive to treatment by NucB. Recently, Rostami et al. (2017) have reported that *P. gingivalis*, among other species, were less abundant in *in vitro* dental plaque models grown in the presence of NucB when compared to control biofilms. *P. gingivalis* usually enter dental plaque by binding to an initial biofilm formed by early colonizers such as *S. gordonii*. Hence, by degrading eDNA, NucB might be interfering with certain synergistic interactions and thereby, excluding *P. gingivalis* from the biofilm.

Confocal laser scanning microscope (CLSM) has been widely used in conjunction with image analysis software tools to evaluate biofilms. To classify pixels of the image acquired by CLSM as foreground (biofilm) or background (noise), a cut-off value or threshold that represents the pixel's signal intensity is set either manually or using an automatic method. Recently, a new image analysis software program called BAIT (biofilm architecture inference tool) was used to quantify the architecture of *in vitro* multispecies oral biofilms following treatment with antibiofilm agents (Luo et al., 2019). BAIT utilizes a novel automatic thresholding technique developed for confocal fluorescent signal and named the biovolume elasticity method (BEM). When compared to other methods of thresholding, BEM was found to calculates lower thresholds thereby minimizing data loss and preserving low-intensity architecture. Furthermore, BEM has provided the highest visual and quantitative acuity of single cells (Luo et al., 2018).

This chapter aimed to determine the effect of NucB on *F. nucleatum* and *P. gingivalis*. The objectives to achieve this aim were:

1. To investigate the presence of eDNA in monospecies biofilms of *F. nucleatum*
2. To evaluate the effect of NucB on *F. nucleatum* biofilms.
3. To assess whether *P. gingivalis* entry into pre-existing biofilms of *S. gordonii* can be deterred by NucB.

3.2 Detection of eDNA in F. nucleatum biofilms using an immunodot assay.

The presence of eDNA in *F. nucleatum* biofilms was assessed in a simple immunodot assay, using 10-fold serial dilutions of supernatants from *F. nucleatum* biofilms. eDNA was detected in two independent biofilm extracts as indicated by a visible purple dot developing at the antigen antibody complex sites (Figure 3.1). Intracellular DNA extracted from cell pellets of the same biofilms produced dots of higher colour intensity than those seen with biofilm supernatant samples. Additionally, DNA was diluted in a dose-dependent manner in all samples since dots become lighter as the concentration decreased. Lower concentrations exhibited weakly positive reactions and sometimes no reaction at all. eDNA could be detected until 10^{-3} dilution of the biofilm supernatant. Overall, these data demonstrate that eDNA is present in *F. nucleatum* biofilms.

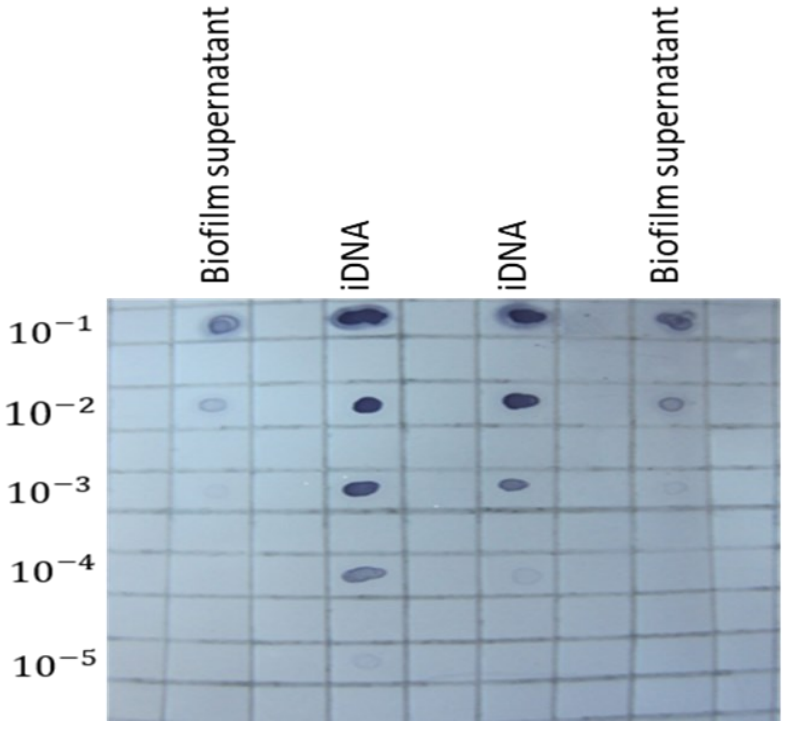

Figure 3.1 An immunodot assay for the detection of eDNA in F. nucleaum biofilms. *Supernatants from F. nucleatum biofilms were 10-fold serially diluted and dotted (1 µl) onto nitrocellulose paper. The immunodot test was carried out with anti-ds DNA monoclonal antibody. Positive reaction is indicated by a purple colour appeared at the*

antigen antibody complex sites. Intracellular DNA (iDNA) extracted from F. nucleatum biofilms was included a as positive control.

3.3 Purification of eDNA from F. nucleatum biofilms

To further characterize eDNA in *F. nucleatum* biofilms, eDNA and intracellular DNA (iDNA) were extracted from 4 days old *F. nucleatum* biofilms. The purified eDNA was measured using a Nanodrop 1000 spectrophotometer. The eDNA extracted was of relatively high purity as indicated by the sharp peak at 260 nm wavelength and the A_{260}/A_{280} ratio (Figure 3.2 A). Both eDNA and iDNA were visualized by 1% agarose gel electrophoresis which showed eDNA as a distinctive band of high molecular weight, similar in size to intracellular DNA extracted from cells within the same bacterial biofilm (Figure 3.2 B). eDNA samples varied in concentrations from 206 ng/µl to 1323 ng/µl.

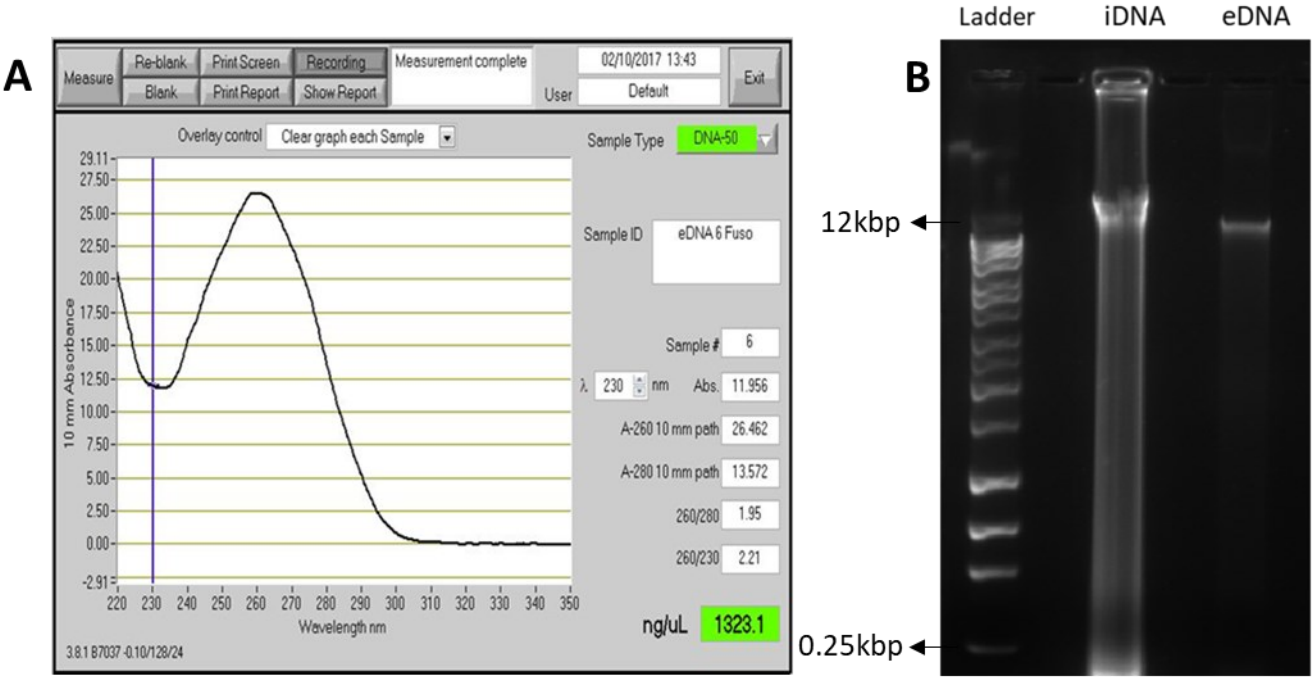

Figure 3.2 Extracellular DNA extraction from F. nucleatum biofilms. *(A) eDNA was extracted from F. nucleatum biofilms and measured by NanoDrop spectrophotometry.*

(B) iDNA and eDNA purified from F. nucleatum biofilms were analysed by agarose gel electrophoresis. Hyperladder 1kb plus was included as a molecular weight marker.

3.4 Measurement of NucB specific activity

Having shown that eDNA is present in *F. nucleatum* biofilms, the next step was to assess their sensitivity to NucB. In order to add consistent amounts of NucB to all experiments, it was important to measure the specific activity of the enzyme. An essay was devloped to standardize the activity of NucB against calf thymus DNA, and DNase I was used as as a control.

The specific activity of DNase I enzyme and NucB was measured using spectrophotometric measurements of calf thymus DNA (CTDNA) digestion products which are quantitatively solubilised by perchloric acid (Figure 3.3). One unit was defined as a production of perchloric acid soluble DNA products that generate an absorbance of 1.0 at 260 nm, per hour at 37˚C in 25 mM HEPES pH 8.0, 5mM $MnSO_4$. Since a sample of DNA with an absorbance of 1 at 260 nm (A260) contains 50 µg DNA ml^{-1}, one unit of activity is the production of 50 µg of perchloric acid soluble DNA products per hour at 37˚C in 25mM HEPES pH8.0, 5mM $MnSO_4$ using 125 µg of CTDNA. The digestion of 125 µg CTDNA was also monitored by agarose gel electrophoresis (Figure 3.4). For both enzymes, the units of activity were calculated using the highest concentration that achieved maximum degradation of high molecular weight CTDNA into low molecular weight DNA before the point of saturation (10 µg for DNaseI and 10 ng for NucB). For NucB,10 ng of the enzyme was capable of digesting 125 µg of calf thymus DNA almost completely in 60 min at 37˚C. As 10 ng achieved maximum degradation of CTDNA before saturation was reached and produced 2× 2.5 absorbance units of acid soluble product per hour, the mean specific activity of NucB calculated was 5×10^5 units mg^{-1}. The mean specific activity of DNase I enzyme calculated was 1.1×10^3 units mg^{-1}.

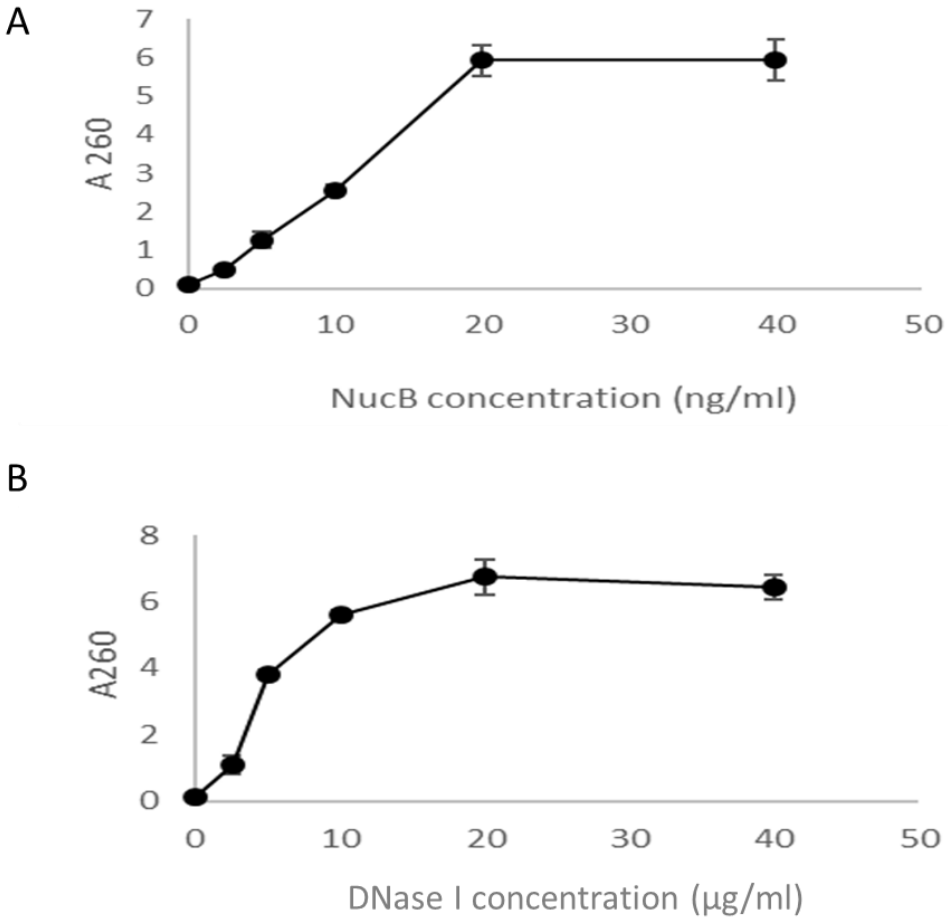

Figure 3.3 Measurement of specific activity of DNase I and NucB enzyme. *125 µg CTDNA was incubated with a range of concentrations of (A) NucB and (B) DNase I at 37C° for 1 h and the amount of low molecular weight DNA generated was measured in a spectrophotometer at 260 nm. Points represent mean values from three independent repeats and errors represent standard error.*

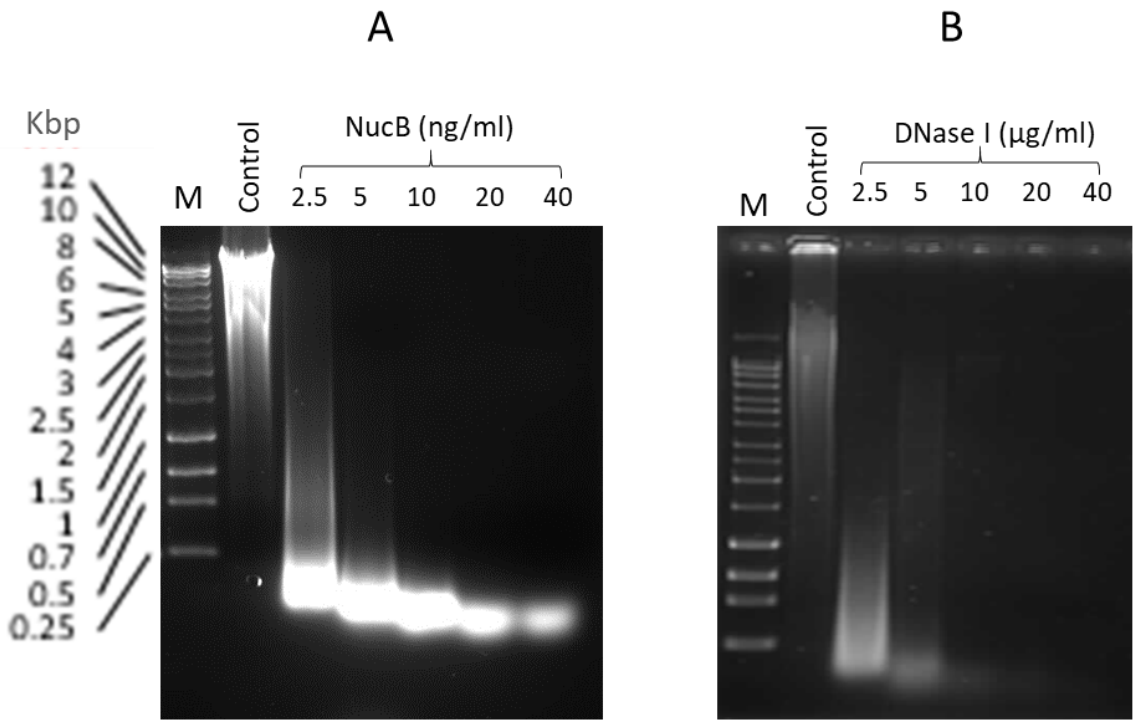

Figure 3.4 Concentration dependent degradation of Calf thymus DNA by NucB. *A range of concentrations of NucB (A) and DNase I (B) were incubated with CTDNA for 60 min at 37˚C for 1 h. Samples of the digestion products generated by the enzymes were separated by agarose (1% w/v) gel electrophoresis and the DNA made visible by staining with gel red. Hyperladder 1kb plus was included as a molecular weight marker (M)*

3.5 Testing the sensitivity of F. nucleatum biofilms to NucB

In order to assess the effect of NucB on biofilms of *F. nucleatum*, a range of NucB concentrations were incubated with pre-established biofilms. The biofilm biomass was reduced by more than 50% when biofilms were treated with 100 ng/ml (50 units), and by more than 70% when treated with 500 ng/ml (250 units) of NucB in comparison to buffer treated controls (*Figure 3.5*). A statistically significant reduction in biofilm biomass was observed with all concentrations used. As a result of these experiments, it was decided to use NucB at a concentration of 500 ng/ml (250 units) in subsequent experiments of both dispersal (removal of preformed biofilms) and inhibition (disruption of biofilm formation). Similar to its effect on the dispersal of preformed biofilms, when NucB was present during biofilm formation (4 days), the reduction in biofilm biomass was statistically significant ($P < 0.05$) (Figure 3.6).

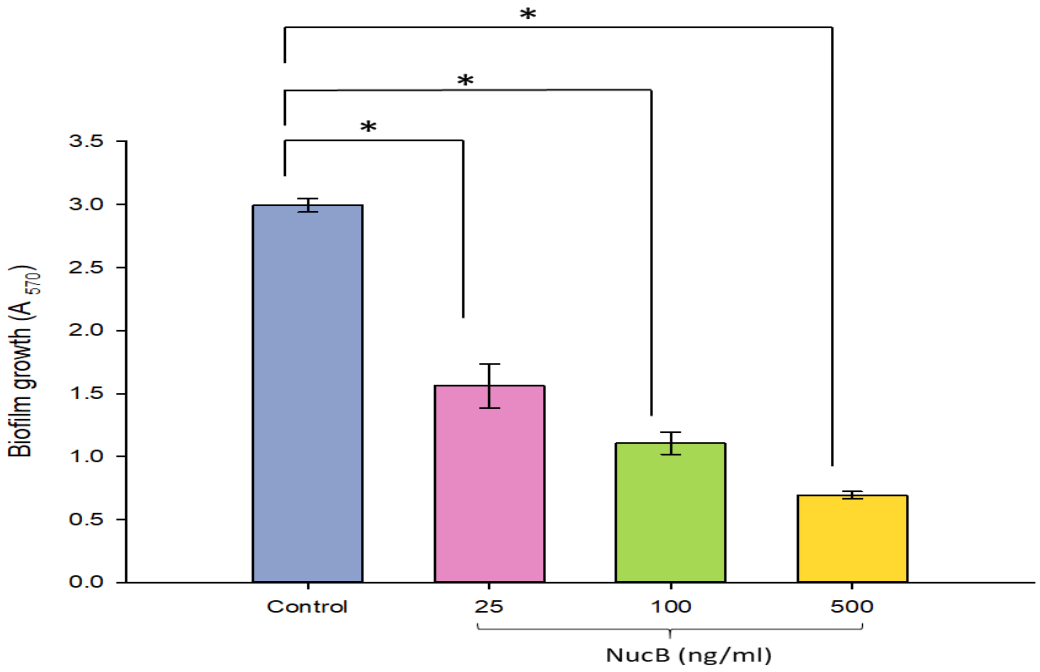

Figure 3.5 Concentration dependent dispersal of F. nucleatum biofilms by NucB. *A range of concentrations of NucB were incubated with preformed (4 days) F. nucleatum biofilms at 37°C for 1 h. Biofilms were stained with crystal violet, and the biofilm biomass was quantified by measuring A570. Bars represent mean values from three independent experiments and errors represent standard error. Statistical significance was calculated with One-way ANOVA with Tukey's Test (* p<0.05).*

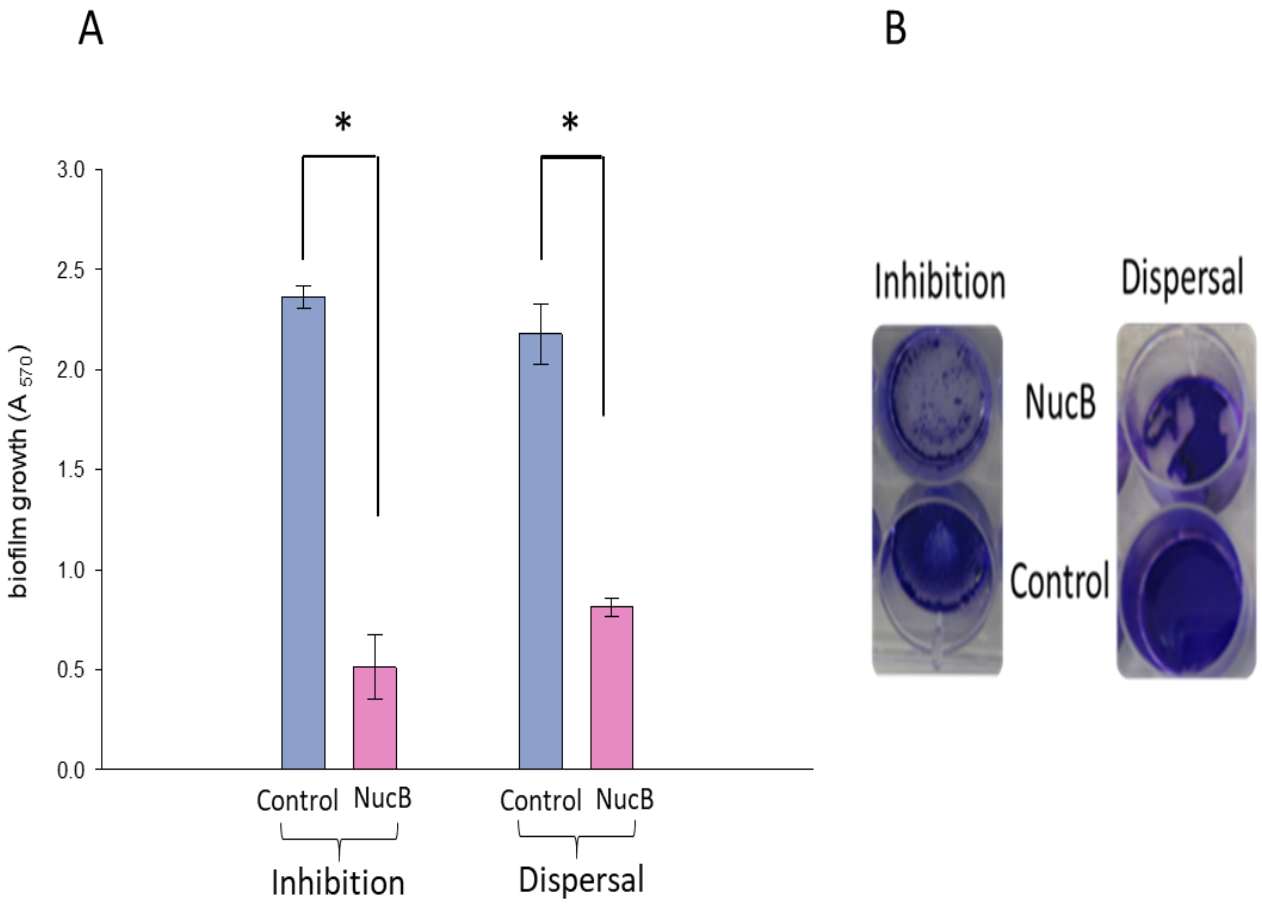

Figure 3.6 The inhibition and dispersal of F. nucleatum biofilms by NucB. *NucB 0.5 µg/ml was either included with the growth media and inoculum during biofilm formation (inhibition) or used to treat performed biofilms of F. nucleatum (dispersal). (A) Biofilms were stained with crystal violet, and the biofilm biomass was quantified by measuring A570. Points represent three independent repeats and error bars represent standard error. Statistical significance was calculated using the two-sample t-test. (B) F. nucleatum biofilms inhibition and dispersal were visualised by staining with crystal violet.*

3.6 Imaging analysis of NucB effect on F. nucleatum biofilms

In this section, confocal microscopy was used to visualize the effect of NucB on *F. nucleatum* biofilm architecture, and the images were quantified using a novel image analysis software program called BAIT (Biofilm Architecture Inference Tool) (Luo et al., 2019).

3.6.1 The inhibitory effect of NucB on *F. nucleatum* biofilms architecture

To evaluate the inhibitory effect of NucB on the architecture of *F. nucleatum* biofilms, biofilms were cultured on glass coverslips in the presence of 0.5 µg/ml NucB and compared to control biofilms which were grown without NucB. Biofilms grown in the presence of NucB looked sparse in comparison to control biofilms, but biofilm cells were distributed uniformly over the slide surface. Control biofilms were extensive, and clusters of loosely attached cells can be clearly seen at the top of these biofilms (Figure 3.7). BAIT was used to quantify the effects of NucB by measuring certain biofilm architectural parameters from biofilms cultured with NucB and comparing them to controls. These parameters were biovolume (number of voxels), surface area and total number of objects. All biofilm outcomes measured were significantly reduced by the presence of NucB during biofilm formation ($P < 0.05$). The control biofilm images measured an average of 3.27×10^7 voxels, while NucB biofilm images measured 1.97×10^7 voxels (40% reduction). The most notable difference measured was in the total objects detected, where there were about 70% fewer objects in NucB-treated than in control biofilms. Similarly, NucB caused a 50% reduction in the surface area which is defined as the sum of all biofilm voxel surfaces that are exposed to the liquid phase or substratum. It should be noted here that the outputs produced by BAIT are unitless as they refer to a number of either voxels, surfaces or objects.

Outcome	Control average (SE)	NucB average (SE)	P-value
Biovolume	3.27×10^7 (3.72×10^6)	1.97×10^7 (4.61×10^6)	0.01*
Number of objects	8.60×10^4 (4.42×10^3)	2.67×10^4 (7.30×10^3)	< 0.001*
Surface area	4.78×10^7 (1.35×10^6)	2.32×10^7 (4.33×10^6)	0.001*

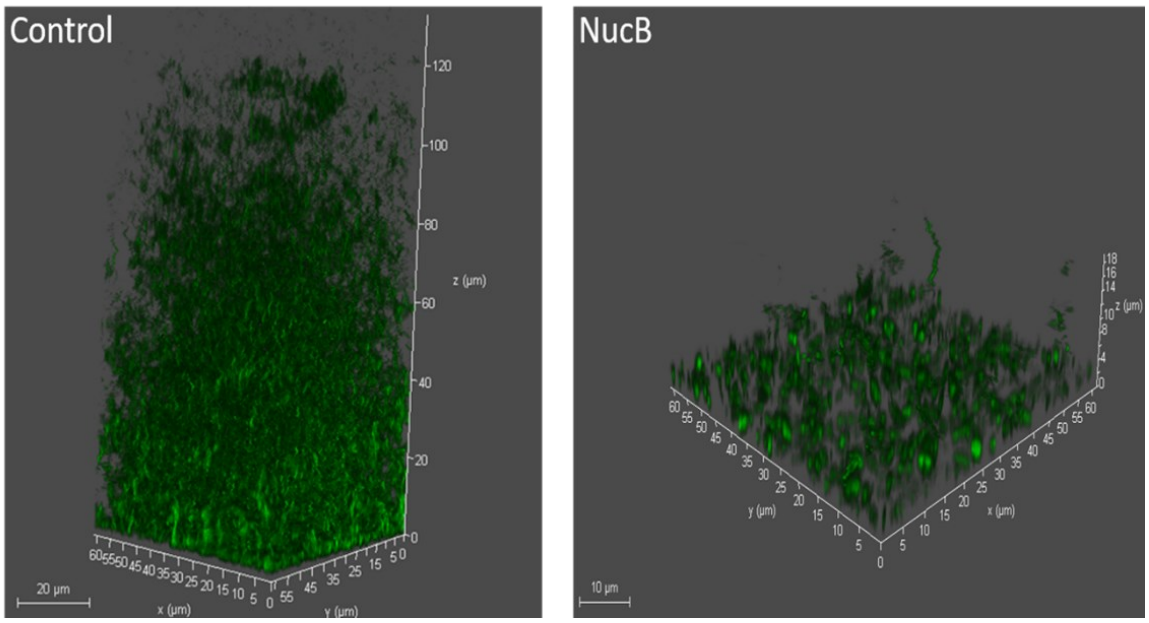

Figure 3.7 Quantification of F. nucleatum biofilms grown in the presence of NucB. *F. nucleatum biofilms were cultured on glass coverslips for 4 days in the presence of NucB. The biofilms were then incubated with SYTO 9 stain for 15 minutes, before CLSM image acquisition. Images were obtained using 3D imaging software (Las X) and quantified using BAIT software. The table shows data (means) derived from imaging of four randomly selected areas on the coverslip from three independent experiments. Statistical significance was calculated with the two-samples t-test with $p < 0.05$ considered statistically significant. SE= standard error.*

3.6.2 The effect of NucB on the architecture of preformed biofilms

To quantify the effect of NucB on the architecture of preformed biofilms, 0.5 µg/ml of NucB was incubated with 4 days old biofilms for 1 hour. Biofilms treated with NucB were much thinner than untreated controls and consisted of sparse single or double layer of cells (Figure 3.8). Control biofilms were extensive and dense covering the whole surface of the slide, and cells reached up to 100 µm in comparison to a maximum height of only 8 µm in case of NucB treated biofilms. BAIT analysis showed that treatment with NucB reduced all the measured biofilm architecture outcomes. In contrast to inhibition experiments where the maximum effect of NucB was on the number of objects detected, there was no significant difference in the total number of objects between control and NucB treated biofilms ($P = 0.05$). Although the difference was not statistically significant, the average number of objects detected in images of treatment biofilms was about 5-fold less than those of control biofilms. Biofilms treated with NucB showed a significant reduction in both biovolume and surface area ($P < 0.05$). The average number of voxels measured for NucB biofilms images were 80% less than those measured for control biofilms matching what was observed visually in the images. A comparable reduction was seen in the surface area of NucB treated biofilms which had about 85% less surface area than untreated controls.

Outcome	Control average (SE)	NucB average (SE)	P-value
Biovolume	4.34×10^7 (7.62×10^6)	7.55×10^6 (4.24×10^6)	0.015*
Number of objects	3.34×10^4 (8.27×10^3)	6.66×10^3 (5.25×10^3)	0.05
Surface area	3.47×10^7 (7.55×10^6)	5.12×10^6 (1.24×10^6)	0.01*

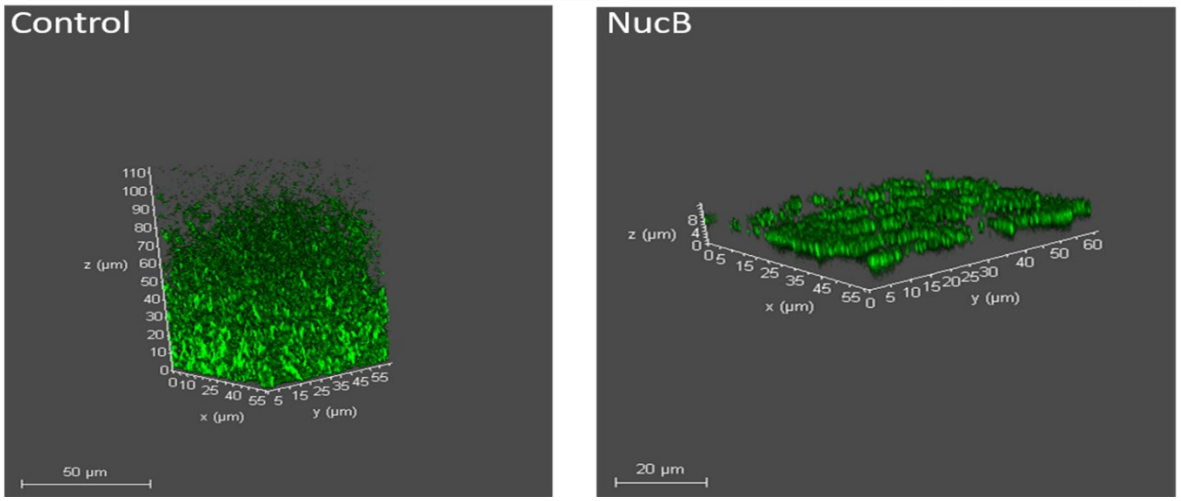

Figure 3.8 NucB effect on the architecture of preformed F. nucleatum biofilms*. F. nucleatum biofilms were cultured on glass coverslips for 4 days and then treated with NucB for 1 h. Biofilms were incubated with SYTO 9 stain for 15 minutes before CLSM image acquisition. Images were obtained using 3D imaging software (Las X) and quantified using BAIT software. The table shows average data derived from imaging of four randomly selected areas on the coverslip from three independent experiments. Statistical significance was calculated with the two-samples t-test with $p < 0.05$ considered statistically significant. SE= standard error.*

3.7 F. nucleatum biofilm formation in a microfluidic biofilm system

The previous sections showed the effect of NucB on static *F. nucleatum* biofilms. To assess the effect of the enzyme on biofilms developed under more physiologically relevant conditions, attempts were made to grow the biofilms in a microfluidic biofilm platform. *F. nucleatum* cells were seeded into the channels of a Bioflux 48-well plate and brightfield images were taken over 24 h of incubation to monitor the biofilm growth. Although cells were capable of adhesion and attachment, biofilm formation was not observed in any channel despite several attempts of optimising the bacterial inoculum concentration and media flow rate. It is worth mentioning that other oral bacteria such as *S. gordonii* were able to form biofilms inside the same Bioflux, indicating that the observed result was specific to *F. nucleatum*. This could be due to the inability to keep the growth conditions anaerobic as required for this species. Although the system was supplied by an anaerobic gas mixture, the experiment involved frequent removal of the plate from the Bioflux chamber, which could cause oxygen leakage into the system.

3.8 Establishing a dual species biofilm model to assess the effect of NucB on P. gingivalis entry into pre-existing biofilms.

Previous results by our group showed that *P. gingivalis* appear to be sensitive to NucB enzyme during biofilm formation. In this section, a mixed-species biofilm model made up of *S. gordonii* and *P. gingivalis* was employed to test this hypothesis.

3.8.1 Cultivation of mono-species biofilms of P. gingivalis

In order to decide which *P. gingivalis* strain to use, the biofilm formation ability of several strains was assesses using crystal violet assay. None of the *P. gingivalis* strains produced robust monospecies biofilms. Table 3.2 shows the absorbance values readings (A570):

Table 3.2 The absorbance values (A570) for monospecies biofilms of P. gingivalis strains after staining with crystal violet.

P. gingivalis strain	W50	381	ATCC 33227	100	control
A570	0.112	0.163	0.132	0.163	.064

3.8.2 Developing a qPCR method to enumerate *S. gordonii* and *P. gingivalis* in a dual species biofilm.

In order to quantify individual species in a mixed species biofilm, a qPCR assay was developed using pure DNA template containing the target sequence for the PCR primers and probe sets. The target DNA for *S. gordonii* and *P. gingivalis* had already been cloned in the pCR2.1 vector, labelled as plasmids pTOPO-*Sg* and pTOPO-*Pg*, respectively. Therefore, the first step was to extract these plasmids from *E. coli*. This DNA could then be used to prepare dilutions and to generate a standard curve for qPCR quantification of bacterial species within the mixed species biofilm. The plasmids were extracted from *E. coli* using a QiaPrep kit and eluted in 50 µl Buffer EB (10 mM Tris CI, pH 8.5). Plasmid samples were visualised by 1% agarose gel electrophoresis (Figure 3.9).

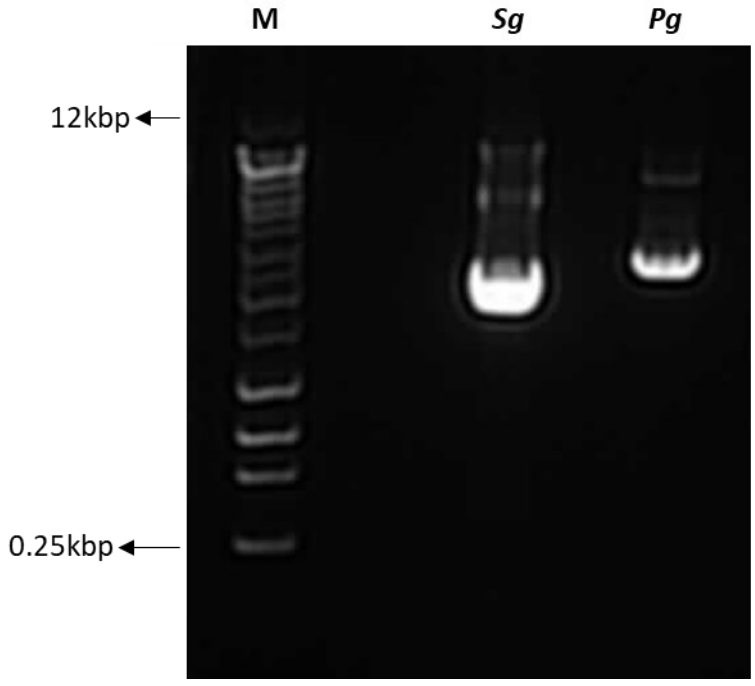

Figure 3.9 Agarose gel electrophoresis of P. gingivalis (Pg) and S. gordonii (Sg) plasmids*. Several bands were seen as it is expected with undigested plasmids. HyperLadder 1kb plus was used as a molecular weight marker (M).*

3.8.3 PicoGreen assay to measure plasmid DNA concentration.

In order to accurately measure the DNA concentration of the extracted plasmids, the concentrations were determined using the PicoGreen double stranded DNA assay kit (Life Technology) following the manufacturer's instructions. A PicoGreen standard curve was generated using Lambda DNA (Figure 3.10). The molecular weight of each plasmid was known, and it was therefore possible to calculate the concentrations of plasmid DNA in terms of the number of molecules present in a unit volume. The plasmid concentrations were: 9.62×10^{14} molecules/µl for *S. gordonii* and 1.23×10^{14} molecules/µl for *P. gingivalis*.

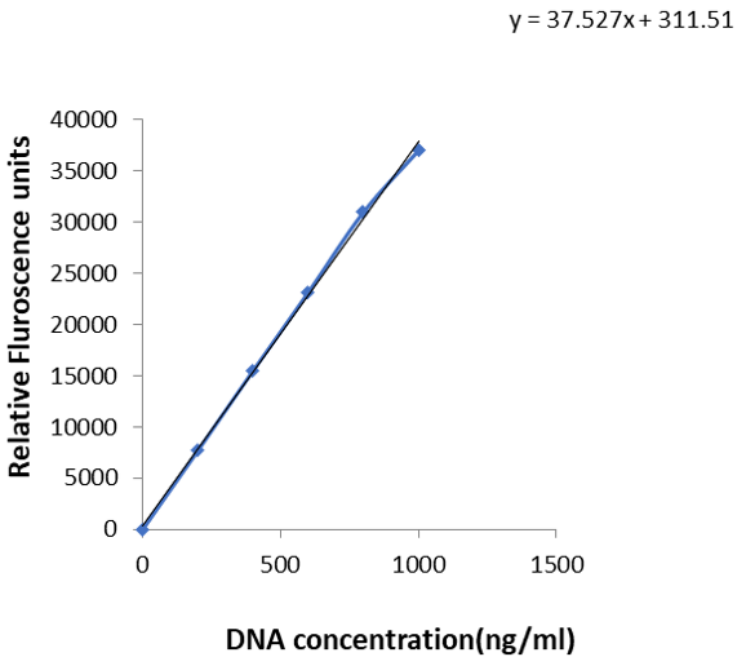

Figure 3.10 PicoGreen standard curve to measure the concentration of P. gingivalis and S. gordonii plasmids DNA*. The equation of the curve (shown on the graph) was used to calculate the unknown concentrations of plasmid DNA.*

3.8.4 Extraction of chromosomal DNA of *P. gingivalis* and *S. gordonii*

DNA was extracted from planktonic cultures of *P. gingivalis* and *S. gordonii* using the same method (see Materials and Methods) to ensure that one method can be used later to extract DNA of both species from a dual-species biofilm. Samples were visualised by 1% agarose gel electrophoresis. DNA extracted from both species appeared as destinctive and sharp band of high molecular weight (Figure 3.11).

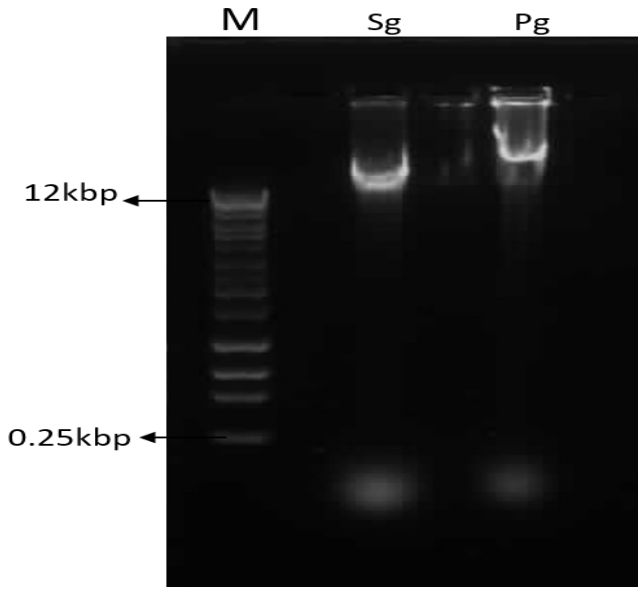

Figure 3.11 Gel electrophoresis of DNA extracted from P. gingivalis (Pg) and S. gordonii (Sg) *planktonic cultures. HyperLadder 1kbp plus was used as a molecular weight marker (M).*

3.8.5 qPCR optimisation

Standard curves were developed using ten-fold serial dilutions of plasmid DNA (Figure 3.12 A, Figure 3.13 A). Figures 3.12 B and 3.13 B, show the real-time reaction amplification plots which correlate the fluorescence signal with the PCR cycle number. The amplification plot consists of a baseline, exponential, linear and plateau phase. A threshold value is set amid the exponential phase and at a level sufficient to distinguish relevant amplification signals from the background noise. The cycle number at which the fluorescence signal of the reaction crosses the threshold value is known as the quantification cycle or threshold cycle (C_t). The concentration of the DNA standards was plotted against the corresponding threshold cycle (Figure 3.12 A, Figure 3.13 A) and it was therefore possible to

calculate the unknown concentrations of *P. gingivalis* and *S. gordonii* DNA extracted from the biofilm. For *S. gordonii*, the primer target was a restriction endonuclease fragment (standard gene), present at a single copy in the chromosome. However, in the case of *P. gingivalis*, the 16S rRNA gene was used as a target. There are four copies of the 16S rRNA gene per genome of *P. gingivalis,* and therefore, corrections were made for copy number.

The qPCR reaction efficiencies were 94.7% for *S. gordonii* and 96.44% for *P. gingivalis* indicating that the reaction was working well. The amplified plasmid fragments were run on 1% agarose gels to check the size of each amplicon. The amplified fragments were of the expected sizes: 96 bp for *S. gordonii,* and 83 bp for *P.* gingivalis (Figure 3.14).

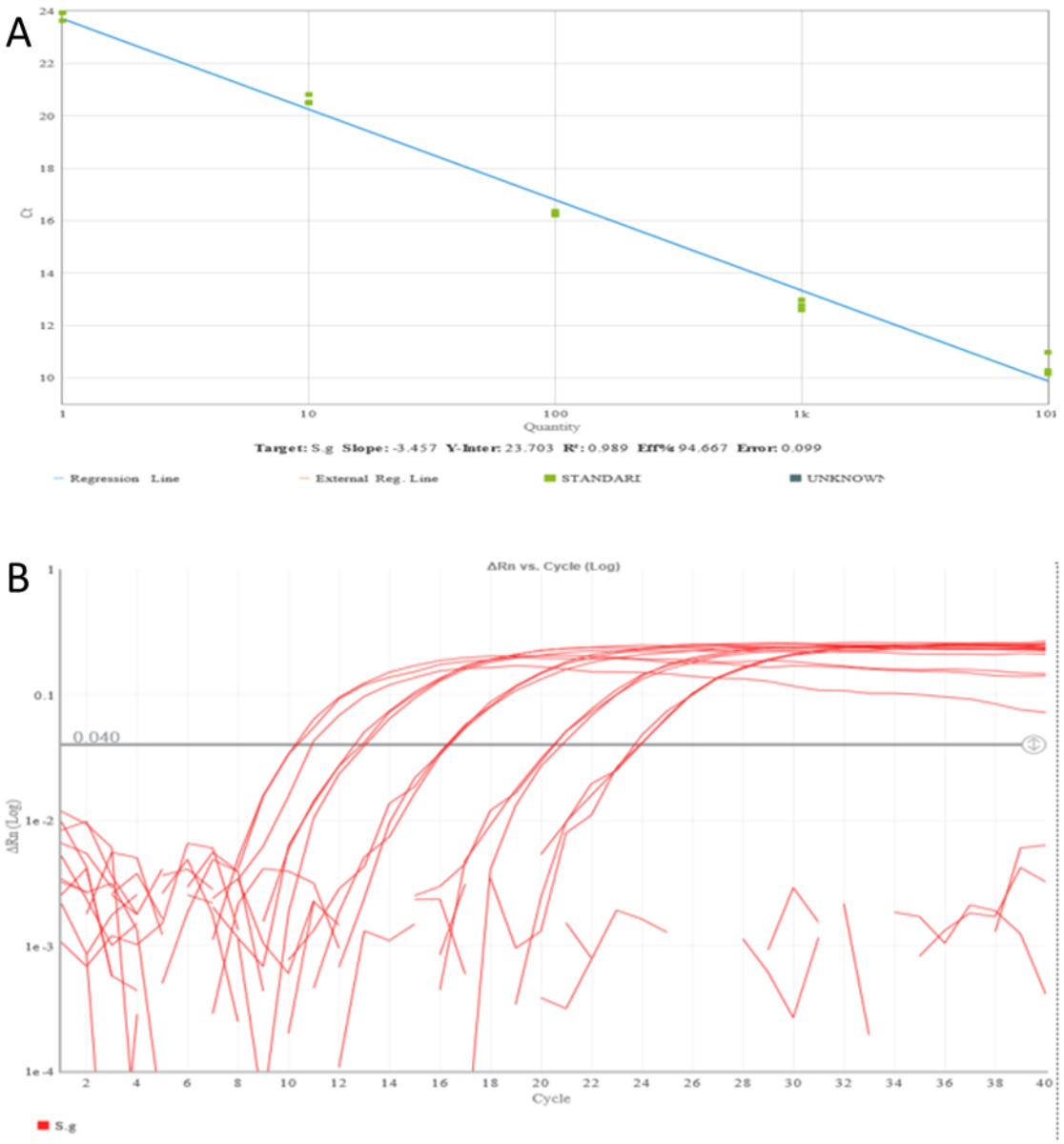

Figure 3.12 qPCR optimisation for S. gordonii *(A) a standard curve of ten-fold serial dilutions of pTOPO-Sg plasmid standards.* DNA concentration is plotted against threshold cycle [CT]. *(B) qPCR amplification plot for pTOPO-Sg plasmid standards. Fluorescence signal is plotted against PCR cycle number. A* threshold is set in the exponential phase of the amplification plot to distinguish relevant amplification signals from the background noise (horizontal grey line).

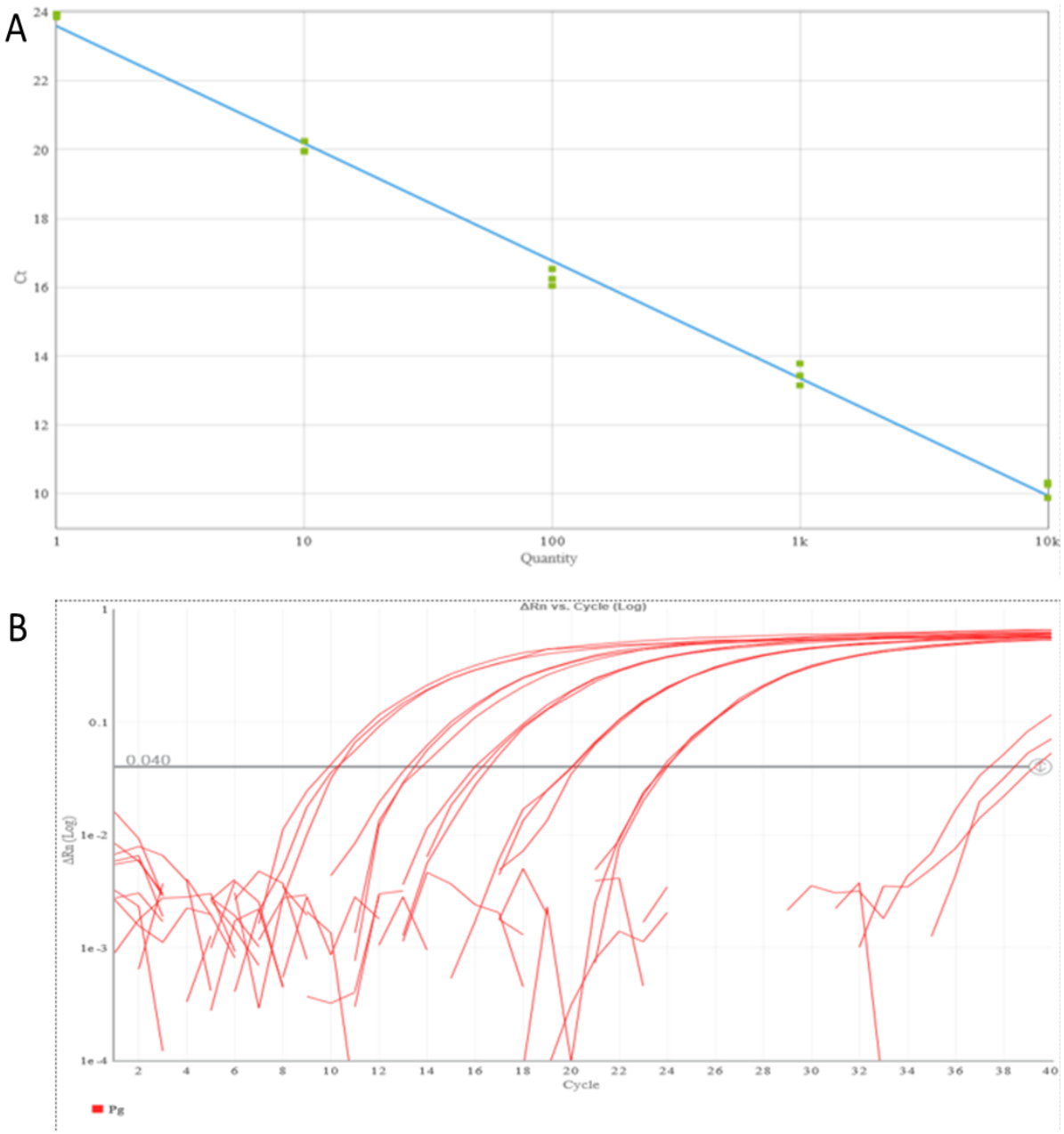

Figure 3.13 qPCR optimisation for P. gingivalis *(A) standard curve of ten-fold serial dilutions of pTOPO-Pg plasmid standards. (B) qPCR amplification* plot for *pTOPO-Pg plasmid standards. Fluorescence signal is plotted against PCR cycle number.*

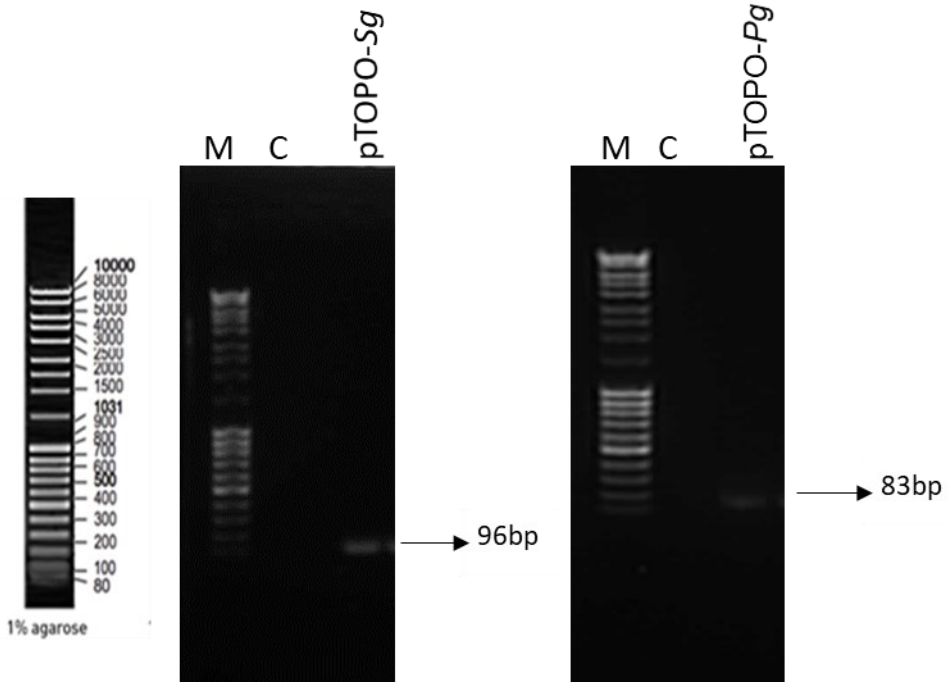

Figure 3.14. Agarose gel electrophoresis of S. gordonii (pTOPO-Sg) and P. gingivalis (pTOPO-Pg) amplified plasmids fragments. *The size of qPCR amplified targets was as expected: pTOPO-Sg 96bp, pTOPO-Pg 83 bp. No amplification was seen in the negative controls (C). MassRuler Ladder was used as a molecular weight marker (M).*

3.8.6 Sensitivity of *P. gingivalis* to NucB in a dual species biofilm model

Having optimised qPCR methods to quantify *P. gingivalis and S. gordonii* within the dual species biofilm model, it was possible to determine whether NucB can prevent *P. gingivalis* from entering preformed biofilms of *S. gordonii*. *P. gingivalis* and NucB were added to 24-h old biofilms of *S. gordonii* and the biofilms were grown for additional 20 h. DNA was extracted from the biofilms and both species were quantified as mentioned above. As can be seen in Figure 3.15, there was no significant difference ($P > 0.05$) in *P. gingivalis* numbers between NucB treated and untreated biofilms. Similar results were observed for *S. gordonii* numbers within the treatment and control biofilms. Additionally, there were much more *S. gordonii* than *P. gingivalis* cells (approximately 4000:1) in all biofilms.

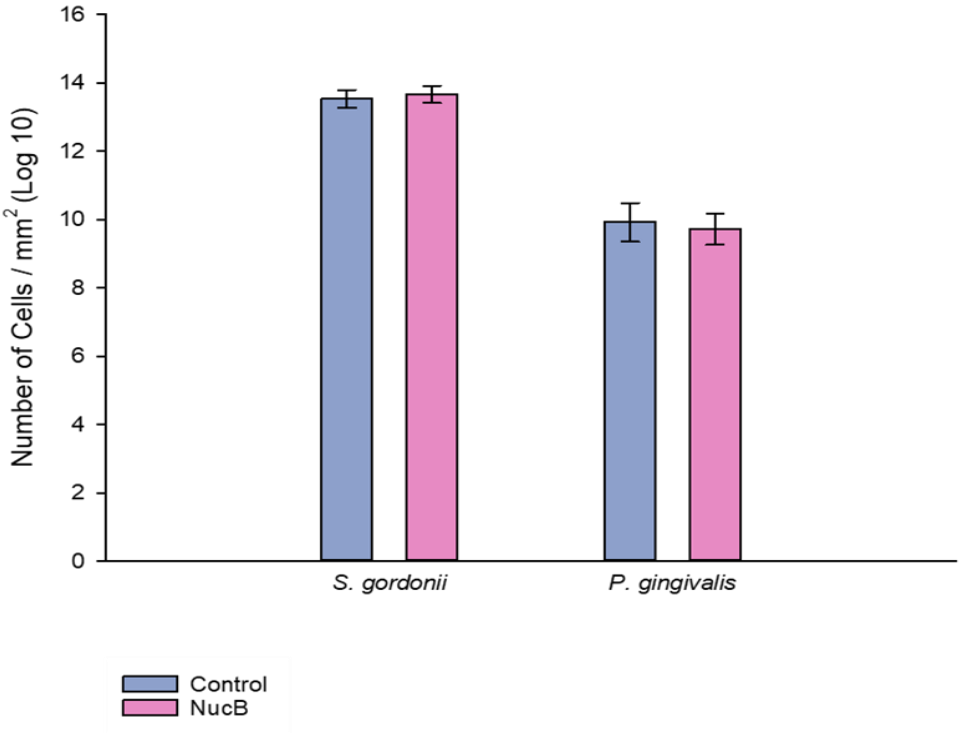

Figure 3.15 Effects of NucB on numbers of S. gordonii and P. gingivalis cells in a dual species biofilm. *S. gordonii biofilms were grown for 24 h before adding NucB and P. gingivalis. Dual-species biofilms were grown for 20 h and bacterial cells/mm^2 were quantified by qPCR. Bars represent means (± SD) from four independent experiments. The graph shows no significant differences in the number of P. gingivalis or S. gordonii between NucB and control biofilms.*

3.9 Discussion

Significant amounts of eDNA were obtained from *F. nucleatum* biofilms suggesting that eDNA is a major component in the biofilm matrices of these species. However, the Nanodrop Spectrophotometer does not specifically measure double stranded DNA as other molecules that absorb or scatter UV light at 260 nm wavelength such as RNA are also measured. Hence, the eDNA concentration measured might be partially due to RNA and other contaminants present in the sample. Using a PicoGreen assay to quantify the purified eDNA could give a more accurate measurement of the level of eDNA in the biofilms, since PicoGreen is specific for double stranded DNA (Singer et al., 1997, Dragan et al., 2010). The eDNA purified from *F. nucleatum* biofilms was of high molecular weight as shown with agarose gel electrophoresis. The results of this study are different from the only other study that measured eDNA in *F. nucleatum biofilms* where much lower concentrations and smaller size of eDNA in biofilms of the same strain of *F. nucleatum* were reported (Ali Mohammed et al., 2013). The biofilms here yielded up to1300 ng/µl of high molecular weight eDNA compared to the 25 ng/µl and 100 bp reported by Ali Mohammed et al. These contrasting results could be due to different culture conditions or different methods of eDNA extraction. First, the growth medium in the reported study was peptone based medium supplemented with hemin, while the biofilms in this project were cultured in brain heart infusion broth supplemented with sodium glutamate. Second, phenol-chloroform extraction was employed to obtain eDNA in this project, while Ali Mohammed et al used Fast DNA spin kit which involve completely different reagents and protocol. eDNA extraction requires biofilms to be harvested by scraping which could cause cell lysis and consequently leakage of intracellular DNA into the sample. Previous work in our group has measured the release of the intracellular enzyme lactate dehydrogenase in scraped biofilm samples and showed that scraping does not result in substantial cell lysis (Jamal Akhil's MClinDent thesis). Yuan et al. (2019) has recently used magnetic beads to extract eDNA from wastewater treatment plant and found this method superior to other methods at separating eDNA from intracellular DNA. In the future, this method could be employed to obtain a cleaner eDNA from microbial biofilms.

The units of activity generated by 1 mg of the NucB preparation used in this study were about 500 times of those produced by 1 mg of DNase I. Previously, the antibiofilm activity of NucB was compared to that of DNase I and it was found that the full dispersal of *B. licheniformis* biofilms requires 5 times higher DNase I than NucB w/v concentrations (Nijland et al., 2010). These results do not seem to be consistent with the mean specific activity measured for both enzymes in this thesis as NucB exhibited 500x more DNase activity, but only 5x more biofilm dispersal activity than DNase I. The discrepancy observed could be attributed to a structural difference between eDNA present in the bacterial biofilms and the DNA substrate digested by the enzyme in the activity assay. Measuring the specific activity of NucB using a robust assay can ensure that the same amount of activity is added in each experiment. However, this assay should be repeated regularly (ideally before starting a new group of experiments) to detect and make corrections for any drop in the activity during storage of the enzyme.

Previous investigations into the sensitivity of biofilms to DNase enzymes found that mature biofilms are somewhat resistant to DNase treatment (Whitchurch et al., 2002, Qin et al., 2007, Hall-Stoodley et al., 2008, Seper et al., 2011, Barnes et al., 2012, Peng et al., 2020b). In this work, however, 0.5 µg/ml NucB was sufficient to inhibit biofilm development as well as disperse 4 days old biofilms of *F. nucleatum*. Ali Mohammed et al. (2013) tested the sensitivity of *F. nucleatum* biofilms to DNase I and found that the biofilms were not inhibited or dispersed even when 1 mg/ml of DNase I was used. Together, these results support the notion that NucB has a superior antibiofilm activity over DNase I. Nevertheless, it might be difficult to compare different enzymes without using equal units of activity with exactly the same substrate. The low quantity and small size of eDNA produced within the biofilm matrices of Mohammed et al (2013) could also be behind the low sensitivity of their biofilms to DNase I.

Crystal violet staining has lots of limitations as a method of biofilm quantification, and therefore, its results must be supported by more reliable methods such as microscopy. Confocal laser scanning microscopy confirmed the significant inhibitory and dispersal effects of NucB observed with crystal violet staining. BAIT has recently been introduced by Luo et al. (2019) as a software programme that can provide rapid quantification of biofilms imaged with CLSM.

The parameters measured by BAIT represent the architectural aspects of the biofilms, and thus, can be utilized to assess how a treatment affect biofilms. In this study, NucB clearly altered the architecture of *F. nucleatum* biofilms when it was either present during the biofilm formation or added to pre-established biofilms. NucB significantly reduced the biovolume of developing or preformed *F. nucleatum* biofilms. Biovolume is defined as the number of voxels and it represents the bioburden of a biofilm, hence, the biovolume decreases as the biofilm growth is reduced. The measured biovolume reduction due to the presence of NucB during biofilm formation appear to be far less than the decrease in the biofilm growth observed in the images. The opposite was reported by Luo et al. (2019), where BAIT detected changes in the biofilm architecture that were not visible in the images. However, this observation was related to parameters other than the biovolume, hence, BAIT may have different sensitivity levels for different parameters of the biofilm architecture. An object is defined by BAIT as the sum of voxels that are connected to each other, and hence the total number of objects is an estimate of biofilm fragmentation. The lower the number of objects detected, the more increased the fragmentation. Although the difference in the total objects detected between biofilms developed with and without NucB was not statistically significant, the visual comparison of the images indicates that control biofilms were much more compact than NucB treated biofilms. Hence, the presence of NucB during or after *F. nucleatum* biofilm formation seems to increase biofilm fragmentation. Increasing biofilm fragmentation is suggested to reduce the average diffusion distance, and consequently, facilitate the transfer of solutes within the biofilm (Yang et al., 2000, Battin et al., 2003). This effect could be of importance in improving the diffusion of oral healthcare agents and penetration of biofilms by antimicrobials. NucB also caused *F. nucleatum* biofilms to have less surface area when compared to control biofilms. The surface area of a biofilm reflects the number of biofilm cells exposed to the bulk-liquid phase. Changes in surface area of the biofilm are likely to have implications on the physiology of the biofilm as exposed biofilm cell layers usually show greater metabolic activity than cells in the biofilm centre (Okabe et al., 1996, Neu and Lawrence, 1997, Werner et al., 2004, Rani et al., 2007, Bester et al., 2011). Also, the decrease in the surface area of the biofilm

might lessen the uptake of nutrients from the bulk-liquid (Battin et al., 2003). Overall, NucB could reduce not only the bioburden of the biofilms, but also change the biofilm architecture in a way that might render the biofilm physiologically less active and more susceptible to both antimicrobials as well as oral healthcare products.

P. gingivalis is strongly implicated in the initiation and progression of periodontitis, and therefore, the ability of excluding or removing them from the subgingival biofilm should allow for the change of the composition of the biofilm towards a healthy periodontal state. Identifying ways to inhibit the entry and or persistence of *P. gingivalis* in the dental biofilm could present a potential intervention that can prevent or reduce the severity of periodontitis. In this study, the presence of NucB when adding *P. gingivalis* to preformed biofilms of *S. gordonii* did not reduce the numbers of *P. gingivalis* within the resultant two species biofilm. These results suggest that eDNA does not play a role in the attachment of *P. gingivalis* to pre-existing biofilms of *S. gordonii*. Also, it appears that eDNA is not important for the persistence of *P. gingivalis* in the biofilm as 20 hour incubation with NucB was not sufficient to release *P. gingivalis* from the biofilm. In contrast to the results here, a previous study has shown that *in vitro* dental plaque models cultured in the presence of NucB comprise much less *P. gingivalis* than controls (Rostami et al., 2017). However, it might not be possible to compare the results of the two studies since the experimental approach was totally different. The experiment here was performed on a simple two species biofilm composed of *S. gordonii* and *P. gingivalis*, whereas the reported study employed a complex multispecies biofilm model which would involve unlimited interbacterial interactions. In this current study, the eDNA targeted by NucB is mainly produced by *S. gordonii* considering that *S. gordonii* have already formed a biofilm before NucB and *P. gingivalis* were added, and the eventual two species biofilm also consists predominantly of *S. gordonii*. *S. gordonii* produces an extracellular DNase that might digest some or all the eDNA released during the biofilm formation. Thus, it is possible that there were not substantial amounts of eDNA present in the biofilm when *P. gingivalis* were introduced, and therefore, the attachment of *P. gingivalis* to *S. gordonii* biofilms was not eDNA dependent. Instead, other well-known species-specific interactions might have taken place

between *P. gingivalis* and *S. gordonii* (Daep et al., 2008, Roky et al., 2020). Furthermore, eDNA could be shielded from NucB by binding to DNABII proteins which were shown to stabilize the complex structure of eDNA in many biofilms (Goodman et al., 2011, Gustave et al., 2013, Novotny et al., 2013, Devaraj et al., 2015, Rocco et al., 2017).This hypothesis is further supported by the recent observation that antibodies derived against DNABII proteins of *P. gingivalis* inhibited the attachment of these bacteria to preformed *S. gordonii* biofilms and disrupted the biofilm structure (Rocco et al., 2018). It is also noted here that NucB did not release *S. gordonii* from the biofilm. This result again is evidence that eDNA is not a major component in *S. gordonii* biofilms matrices or that it is bound to DNABII proteins. To provide a clearer insight into the dependence of *P. gingivalis* on eDNA for attachment and persistence, further investigations using models that involve early colonizing bacteria other than *S. gordonii* are required.

Chapter 4: Characterizing a Streptococcus gordonii deoxyribonuclease (SsnA)

4.1 Outline

As it has been shown by previous studies, and in this thesis, biofilms formed by oral bacteria are sensitive to treatment by exogenous DNases such as NucB. Several oral bacteria including *S. gordonii* were found to produce extracellular DNases (Palmer et al., 2012), however, the role of these DNases within the oral biofilm is not yet clear. In particular, it is unknown whether DNases naturally present in dental plaque have biofilm reducing effects on oral species. Understanding the natural role of theses DNases could lead to the development of strategies to modulate them for biofilm control.

The extracellular DNase of *S. gordonii* DL1 was identified as a LPxTG cell wall surface protein and was subsequently termed SsnA (streptococcal secreted nuclease) (Robert Shields, PhD thesis). The production of extracellular DNases appears to be common among pathogenic streptococci such as *Streptococcus suis* (Fontaine et al., 2004), *Streptococcus pyogenes* (Hasegawa et al., 2010), *Streptococcus pneumoniae* (Beiter et al., 2006) and *Streptococcus agalactiae* (Derré-Bobillot et al., 2013). Pathogenic bacteria mainly use their DNases to degrade the DNA backbone of NETs and facilitate the escape from killing or entrapment by neutrophils (Derré-Bobillot et al., 2013, de Buhr et al., 2014). However, *S. gordonii* exists as a part of the commensal flora of the oral cavity and it is involved in early colonization therefore, it is unlikely that the escape from NETs is the primary function of SsnA.

S. gordonii plays a major role in the formation of the early plaque as well as the subsequent mature polymicrobial biofilm. For instance, *S. gordonii* enhances the growth of some other commensals in the dental biofilm such as *Veillonella* spp. (Egland et al., 2004), while they compete with the cariogenic bacteria *S. mutans* (Wang and Kuramitsu, 2005, Kreth et al., 2008a, Zhuang et al., 2018, Chen et al., 2019b). There is also increasing evidence that *S. gordonii* could contribute to pathogenicity by exerting synergistic interactions with some pathogenic species such as *Aggregatibacter actinomycetemcomitans* and *P. gingivalis*

thereby directing the development of pathogenic oral biofilm community (Hajishengallis and Lamont, 2016, Bowen et al., 2018). Considering the role *S. gordonii* plays in shaping the oral biofilm composition, SsnA might be utilized to enhance this role by dispersing biofilms of eDNA dependent oral species.

In the previous chapter, *F. nucleatum* cells were sensitive to NucB indicating their dependence upon eDNA for biofilm formation and stability. *F. nucleatum* acts as a bridging organism between early and late colonizers of the dental biofilm, and hence, typically interacts with *S. gordonii*. Therefore, *S. gordonii* SsnA might be involved in the disruption of biofilms of *F. nucleatum*. This chapter aims to investigate the effect of SsnA on biofilms of *F. nucleatum* and characterize its DNase activity.

4.2 Testing the sensitivity of F. nucleatum biofilms to SsnA

To evaluate the inhibitory effect of SsnA on *F. nucleatum* biofilms, SsnA (5 µg/ml) was included with the growth media during the biofilm formation. To test dispersal, 5 µg/ml SsnA was added to 4 days old biofilms of *F. nucleatum*. The biomass of biofilms grown in the presence of SsnA was about 50% less than that of control biofilms. While a statistically significant reduction in the biofilm biomass was observed in case of inhibition ($P < 0.05$), SsnA had no effect on the dispersal of preformed biofilm (Figure 4.1).

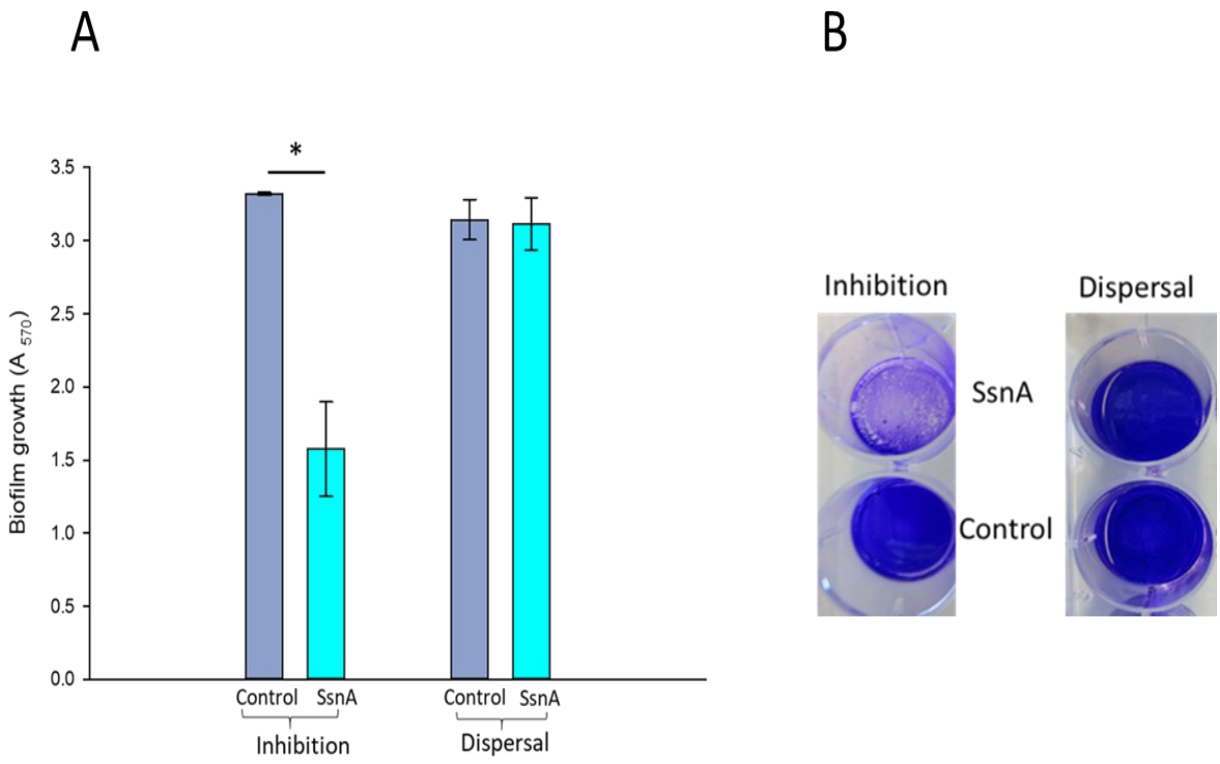

Figure 4.1 The antibiofilm activity of SsnA against F. nucleatum biofilms. *SsnA (5 µg/ml) was either included with the growth media and inoculum during biofilm formation (4 days) or used to treat 4 days old performed biofilms of F. nucleatum. (A) Biofilms were stained with crystal violet and the biofilm biomass was quantified by measuring A_{570}. Bars represent three independent repeats and error bars represent standard error. Statistical significance was calculated using the two-sample t-test. (B) F. nucleatum biofilm inhibition and dispersal were visualised by staining with crystal violet.*

4.3 Imaging analysis of SsnA effect on F. nucleatum biofilms

In this section, confocal laser scanning microscopy was used to visualise the effect of SsnA on *F. nucleatum* biofilm architecture, and the images were quantified using BAIT (Biofilm Architecture Inference Tool) (Luo et al., 2019).

4.3.1 **The inhibitory effect of SsnA on the architecture of F. nucleatum biofilms**

To determine whether the presence of SsnA during biofilm formation affects the architecture *of F. nucleatum biofilms,* biofilms were cultured on glass coverslips in the presence of 5 µg/ml SsnA, and the biofilm architecture was compared to that of biofilms grown without SsnA. Biofilms cultured in the presence of SsnA were much thinner than control biofilms, and the biofilm showed irregular thickness across the slide surface (Figure 4.2). In the absence of SsnA, biofilms grew extensively with cells projecting up to 120 µm, whereas the maximum height for SsnA-treated biofilms was about 45 µm. Clusters of loosely attached cells can be clearly observed at the top of both SsnA-treated and control biofilms. BAIT was used to quantify the effect of SsnA on the biofilms' architecture by measuring biovolume (number of voxels), surface area and total number of objects from biofilms cultured with and without SsnA. Whilst the visual inspection of the images shows SsnA biofilms to be about 3 times thinner than control biofilms, the biovolume measured was not significantly reduced by the presence of SsnA during biofilm formation. The total number of objects and surface area of the biofilms were both significantly reduced by SsnA, where the presence of SsnA during biofilm formation caused 70% and 50 % reduction in the total objects detected and surface area, respectively. This indicates that SsnA increased biofilm fragmentation and reduced its surface area.

Outcome	Control average (SE)	SsnA average (SE)	P-value
Biovolume	3.27×10^7 (3.72×10^6)	1.97×10^7 (4.61×10^6)	0.09
Number of objects	8.60×10^4 (4.42×10^3)	2.67×10^4 (7.30×10^3)	< 0.001*
Surface area	4.78×10^7 (1.35×10^6)	2.32×10^7 (4.33×10^6)	0.001*

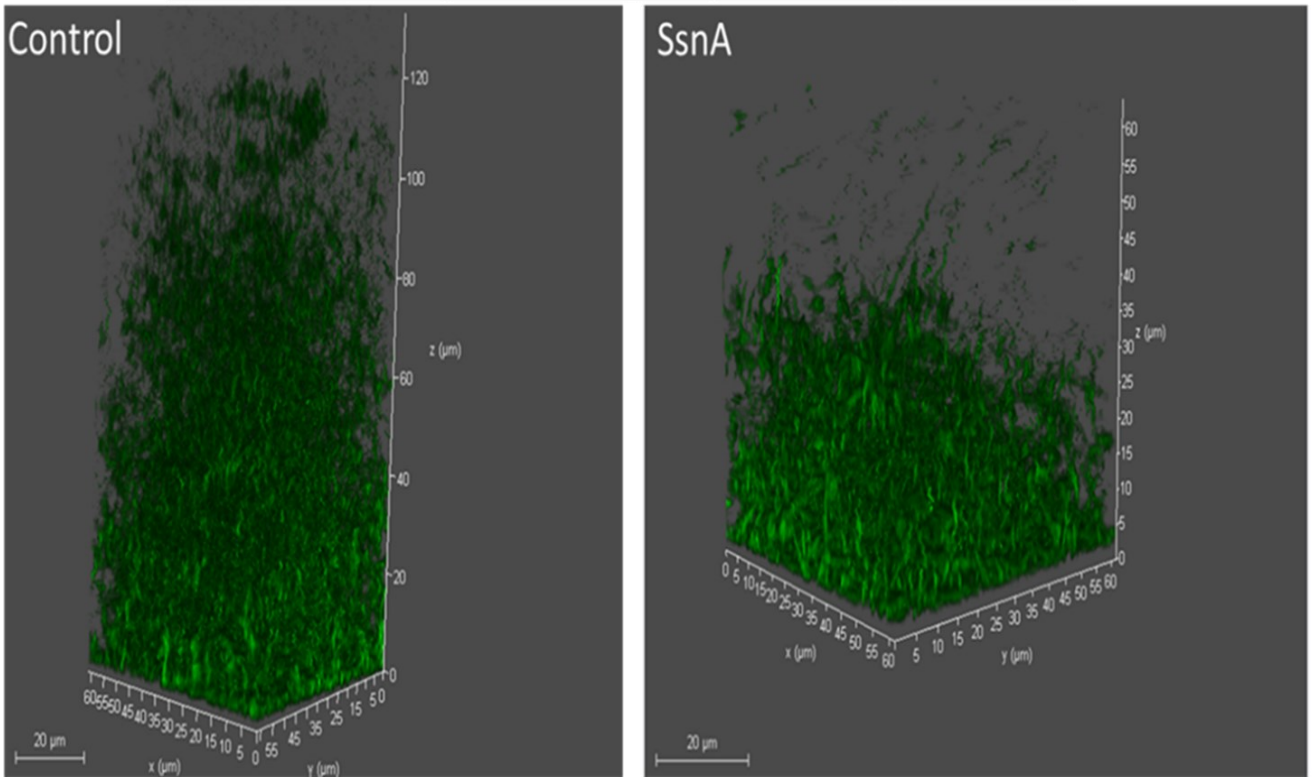

Figure 4.2. Quantification of the inhibitory effect of SsnA on the architecture of F. nucleatum biofilms by Confocal laser scanning microscopy. *F. nucleatum biofilms were cultured on glass coverslips for 4 days in the presence of 5 µg/ml SsnA. The biofilms were then incubated with SYTO 9 stain for 15 minutes, before CLSM image acquisition. Images were obtained using 3D imaging software (Las X) and quantified using BAIT software. The table shows data (means) derived from imaging of four randomly selected areas on the coverslip from three independent experiments. Statistical significance was calculated with the two-samples t-test with $p < 0.05$ considered statistically significant. SE = Standard Error*

4.3.2 The effect of SsnA on the architecture of preformed biofilms

To quantify the effect of SsnA on the architecture of preformed biofilms, 5 µg/ml of SsnA was incubated with 4 days old biofilms of *F. nucleatum* for 1 hour. In contrast to inhibition experiments, both SsnA treated and control biofilms were extensive and dense, covering the whole surface of the slide. Very little difference was observed in the thickness of biofilms (*Figure 4.3*). BAIT analysis also showed no significant difference in any of the measured architecture parameters between control and SsnA treated biofilms suggesting that incubation of preformed biofilms with SsnA did not change the architecture of the biofilms.

Outcome	Control average (SE)	SsnA average (SE)	P-value
Biovolume	$1.17x\ 10^8$ ($4.41\ x10^7$)	$1\ x\ 10^8$ ($3.14\ x\ 10^7$)	0.7
Number of objects	$7.76x\ 10^4$ ($4.88\ x\ 10^4$)	$1.33\ x\ 10^5$ ($6.1\ x10^4$)	0.5
Surface area	$9.22\ x\ 10^7$ ($3.59\ x\ 10^7$)	$8.78x\ 10^7$ ($4.04\ x\ 10^7$)	0.9

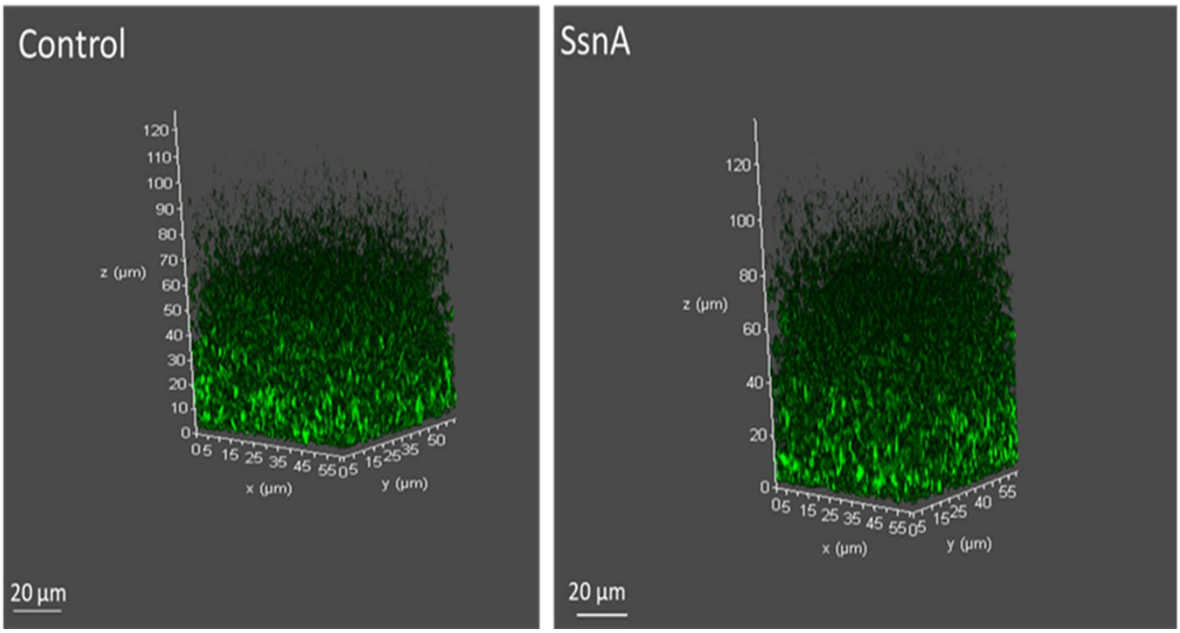

Figure 4.3 The effect of SsnA on the architecture of preformed biofilms of F. nucleatum. *F. nucleatum biofilms were cultured on glass coverslips for 4 days and treated with 5 µg/ml SsnA for 1 hour. The biofilms were then incubated with SYTO 9 stain for 15 minutes, before CLSM image acquisition. Images were obtained using 3D imaging software (Las X) and quantified using BAIT software. The table shows data*

(means) derived from imaging of four randomly selected areas on the coverslip from three independent experiments. Statistical significance was calculated with the two-samples t-test with p < 0.05 considered statistically significant. SE = Standard Error.

4.4 The activity of SsnA against calf thymus DNA

The previous section showed that SsnA could inhibit biofilm formation but not disperse preformed biofilms. This contrasts with the antibiofilm effect observed for NucB in the previous chapter where developing and preformed biofilms were both disrupted by NucB. To investigate the enzymatic activity of SsnA in comparison to NucB, the DNase activity of both enzymes was assessed *in vitro* against a commercially available high molecular weight DNA substrate, calf thymus DNA.

4.4.1 Time course degradation of calf thymus DNA by SsnA

To compare the DNA degrading activity of SsnA and NucB, 125 µg calf thymus DNA was incubated with 10 ng/ml SsnA or NucB at 37˚C over a 10-60 min time frame. Tris was used as a buffer in SsnA experiments, whereas for NucB the reaction buffer was HEPES. Ideally, the two enzymes should have been compared using the same buffer, however, Tris was not compatible with the high concentrations of manganese required for NucB`s optimal activity. Therefore, HEPES was used as an alternative. The use of Tris as a buffer for SsnA was continued to keep conditions consistent with previous experiments. When the digestion of Calf thymus DNA was monitored by agarose gel electrophoresis (Figure 4.4), the bands corresponding to the high molecular weight substrate were not reduced by SsnA even after 60 min of incubation, while NucB showed partial digestion of the calf thymus DNA at 10 min, and was capable of digesting the calf thymus DNA almost completely by 60 min. The activity of SsnA and NucB was also assessed by spectrophotometric measurement of the calf thymus DNA digestion products that are quantitatively solubilised by perchloric acid (Figure 4.5). SsnA demonstrated no detectable degradation of high molecular weight calf thymus DNA into low molecular weight DNA fragments at any timepoint. In contrast, more than 25 % of the 125 µg high molecular weight DNA was digested by NucB after 60 min. The

amount of calf thymus DNA degraded by NucB at 60 min of incubation was significantly greater than that measured for control ($P<0.05$).

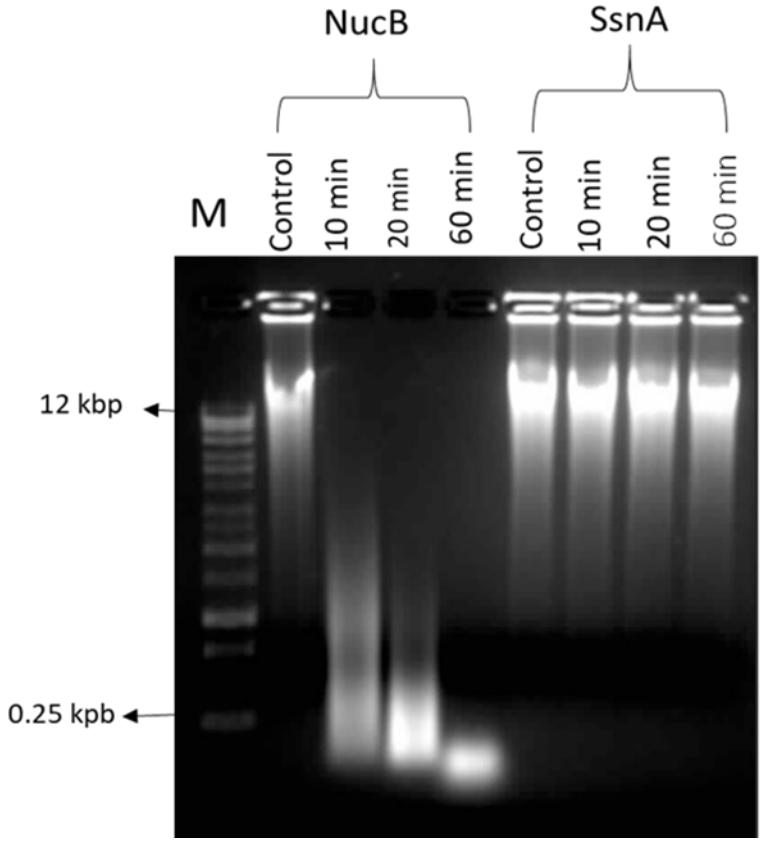

***Figure 4.4** Gel electrophoresis of the digestion products of CT DNA by SsnA. 10 ng/ml SsnA or NucB was incubated with 125 µg calf thymus DNA at 37˚C for (10, 20 and 60 min). The reaction buffer for NucB consisted of 25 mM HEPES (pH 8.0) and 5 mM $MnSO_4$. For SsnA, the reaction buffer contained 25 mM Tris (pH 8.0), 40 µM EDTA and 80 µM $MgCl_2$.Samples of the digestion products generated by the enzymes were separated by agarose (1% w/v) gel electrophoresis. Hyperladder 1kb plus was included as a molecular weight marker (M).*

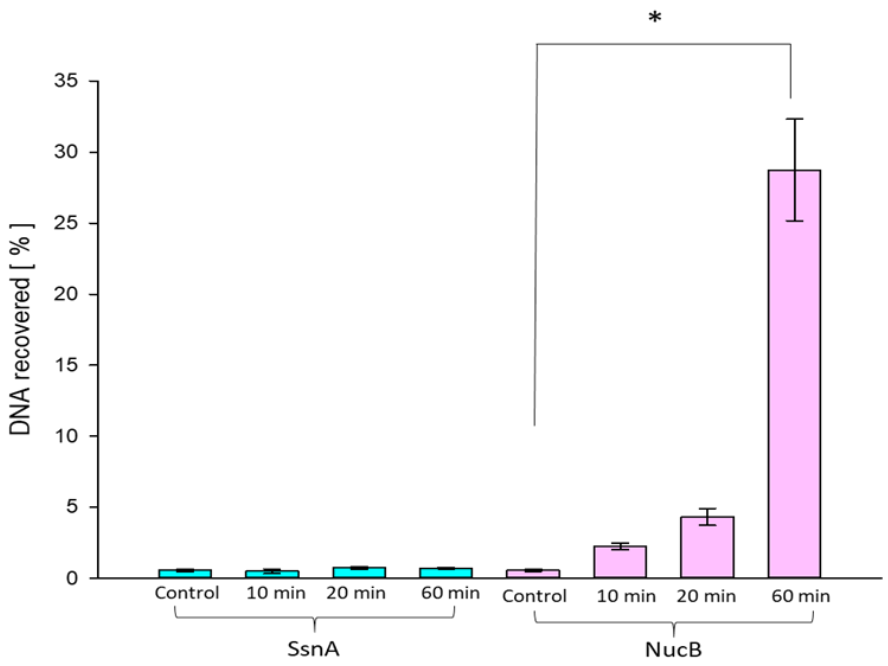

Figure 4.5 ***The activity of SsnA and NucB against Calf thymus DNA over time.*** *125 µg calf thymus DNA was incubated with a 10 ng/ml of SsnA or NucB at 37˚C, and the amount of low molecular weight DNA generated was measured in a spectrophotometer at 260 nm after 10, 20 and 60 min of incubation. The reaction buffer for NucB consisted of 25 mM HEPES (pH 8.0) and 5 mM $MnSO_4$. For SsnA, the reaction buffer contained 25 mM Tris (pH 8.0), 40 µM EDTA and 80 µM $MgCl_2$. Bars represent mean values from three independent repeats and errors represent standard error.*

4.4.2 Effects of SsnA concentration on the degradation of calf thymus DNA

To determine whether higher concentrations of SsnA could digest calf thymus DNA, varying concentrations of SsnA were incubated with 125 µg calf thymus DNA for 60 min. Agarose gel electrophoresis of the digestion products showed distinctive and bright bands of high molecular weight DNA with all SsnA concentrations. Conversely, all concentrations of NucB achieved total digestion of calf thymus DNA as highlighted by the absence of any visible DNA fragments (Figure 4.6). No influence on DNA degradation activity of SsnA was observed in the semi-quantitative analysis, even when concentrations increased up to 5 µg/ml (Figure 4.7). In contrast, about 70% of the calf thymus DNA was digested with concentrations as low as 0.1 µg/ml of NucB.

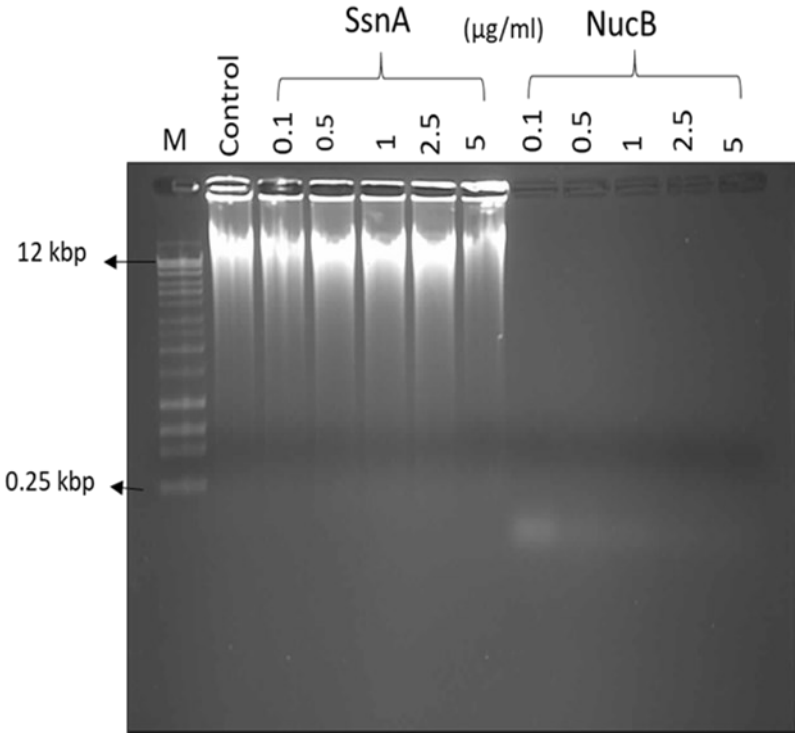

Figure 4.6 The effect of SsnA concentration on degradation of calf thymus DNA. *125 µg calf thymus DNA was incubated with a range of concentrations of SsnA or NucB at 37C° for 1 hour. The reaction buffer for NucB consisted of 25 mM HEPES (pH 8.0) and 5 mM $MnSO_4$. For SsnA, the reaction buffer contained 25 mM Tris (pH 8.0), 40 µM EDTA and 80 µM $MgCl_2$.Samples of the digestion products generated by the enzymes were separated by agarose (1% w/v) gel electrophoresis. Hyperladder 1kb plus was included as a molecular weight marker (M).*

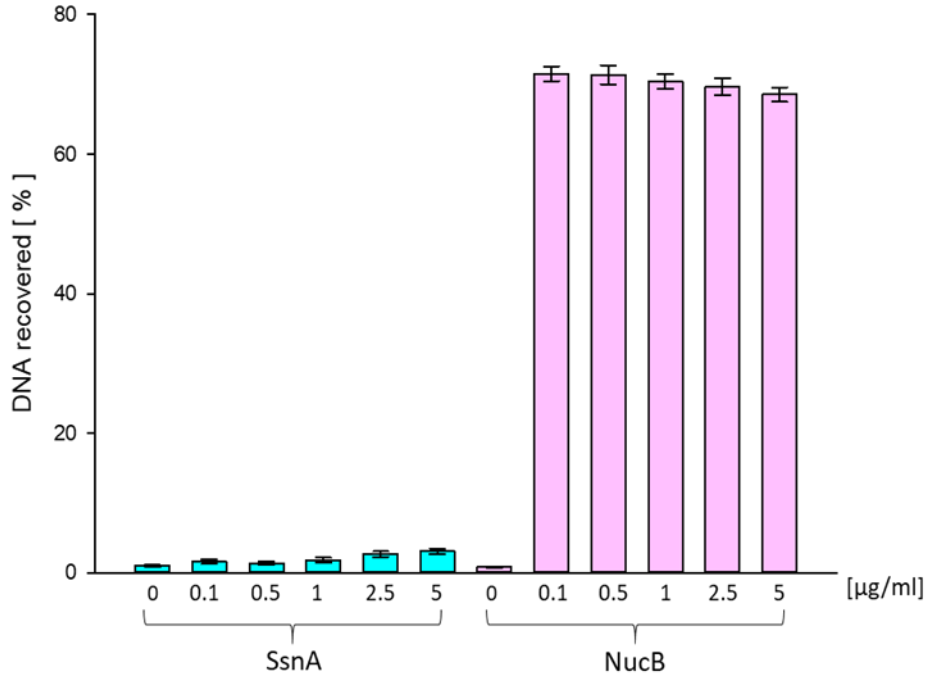

Figure 4.7 Concentration dependent activity of SsnA and NucB against calf thymus DNA*. 125 µg calf thymus DNA was incubated with a range of concentrations of SsnA or NucB at 37˚C for 1 h, and the amount of low molecular weight DNA generated was measured in spectrophotometer at 260 nm. Bars represent mean values from three independent repeats and errors represent standard error.*

4.5 The activity of SsnA against an Oligonucleotide probe

SsnA showed no degradation activity against calf thymus DNA which is primarily double stranded. To investigate the DNA degradation activity of SsnA against single stranded DNA, an oligonucleotide probe was used as a substrate and Förster resonance energy transfer (FRET) assay was employed (see Materials and Methods). NucB was also included to assess its activity against this single stranded substrate in comparison to SsnA. The degradation of the oligo probe was detected by an increase in fluorescence signal and fluorescence versus enzyme concentration curves were produced. The change in fluorescence was initially measured over 30 min (Figure 4.8). This revealed that most of the reaction has occurred by 0 time point in nearly all cases, and that there was little decrease in fluorescence over the 30 minutes. Also, the fluorescence produced by the

lowest NucB concentrations continued to increase up to 30 minutes. Therefore, a single 30-minute time point was used to measure the activity (Figure 4.9).

NucB demonstrated superior DNase activity over SsnA until a concentration of 0.5 µg/ml where NucB capability to degrade the probe started to drop, and SsnA activity continued to rise. Surprisingly, NucB activity was dramatically inhibited by concentrations higher than 1 µg/ml. Whilst SsnA achieved maximum degradation of the probe at ≥ 2.5 µg/ml, the observed DNase activity for NucB was maximum at much lower concentration (0.5 µg/ml). However, when comparing the activity of both enzymes at the points of maximum DNA degradation, SsnA showed significantly higher activity than NucB ($P < 0.05$).

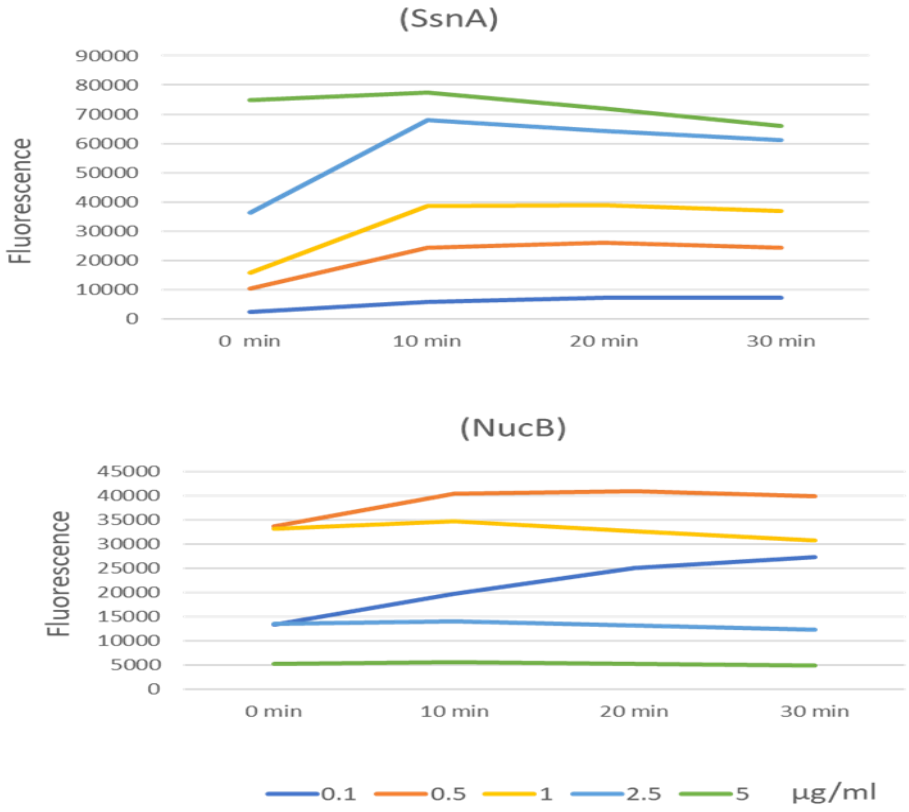

Figure 4.8 Förster resonance energy transfer assay of SsnA and NucB DNase activity against an oligo probe over time. *SsnA or NucB were incubated with an oligonucleotide probe at 37°C and the change of fluorescence was measured over 30 minutes using a microplate reader.*

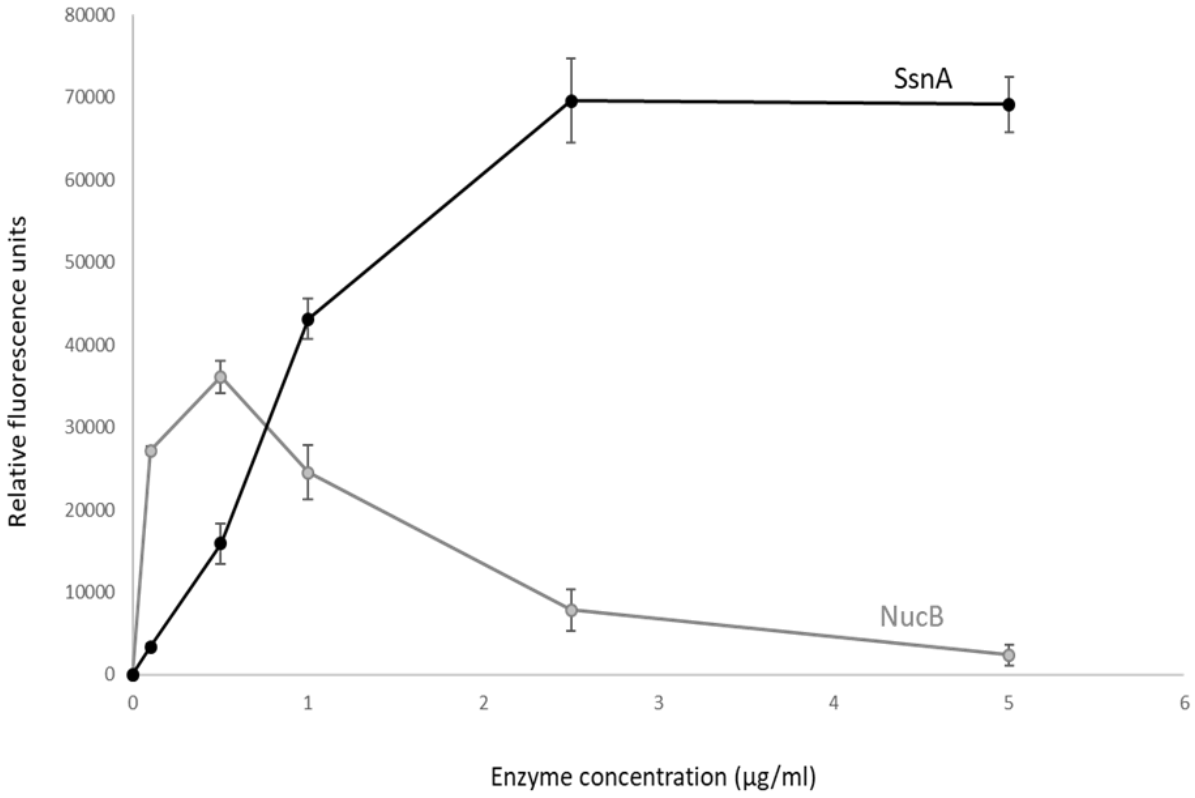

Figure 4.9. Förster resonance energy transfer assay of SsnA and NucB activity against an oligo probe at one time point. *SsnA or NucB were incubated with an oligonucleotide probe at 37°C and the amount of fluorescence produced was measured at 30 min using microplate reader. The reaction buffer for NucB consisted of 25 mM HEPES (pH 8.0) and 5 mM $MnSO_4$. For SsnA, the reaction buffer contained 25 mM Tris (pH 8.0), 40 µM EDTA and 80 µM $MgCl_2$. Error bars represent standard error from the mean (3 replicates).*

4.6 The activity of SsnA against Salmon sperm DNA

In the previous sections, SsnA could efficiently digest an oligo nucleotide probe which is a single stranded substrate, but not calf thymus DNA that is predominantly double stranded DNA. However, calf thymus DNA is also a different substrate to the oligo probe with regards to size. To further explore the substrate specificity of SsnA, agarose gel DNase activity assay was performed using 5 µg/ml of purified SsnA and 125 µg of double stranded Salmon sperm DNA

as a substrate. SsnA or NucB was incubated with either low or high molecular weight salmon sperm DNA at 37 C° for 1 hour. Gel electrophoresis of the digestion products shows that low molecular weight salmon sperm DNA was only partially digested by SsnA, while no degradation activity was observed against the high molecular weight DNA. In contrast, NucB fully digested both low and high molecular weight DNA leaving no detectable fragments (Figure 4.10 A). Increasing the incubation time to 24 hours caused SsnA to achieve complete degradation of low molecular weight salmon sperm DNA but had no obvious impact on the enzyme activity against the high molecular weight DNA (Figure 4.10 B). However, it should be noted here that the loss of LMW DNA is much easier to detect considering its appearance as a sharp band rather than a smear as it is the case with the HMW substrate. In summary, our results suggest that SsnA can fully digest double stranded DNA, but only if it is of low molecular weight.

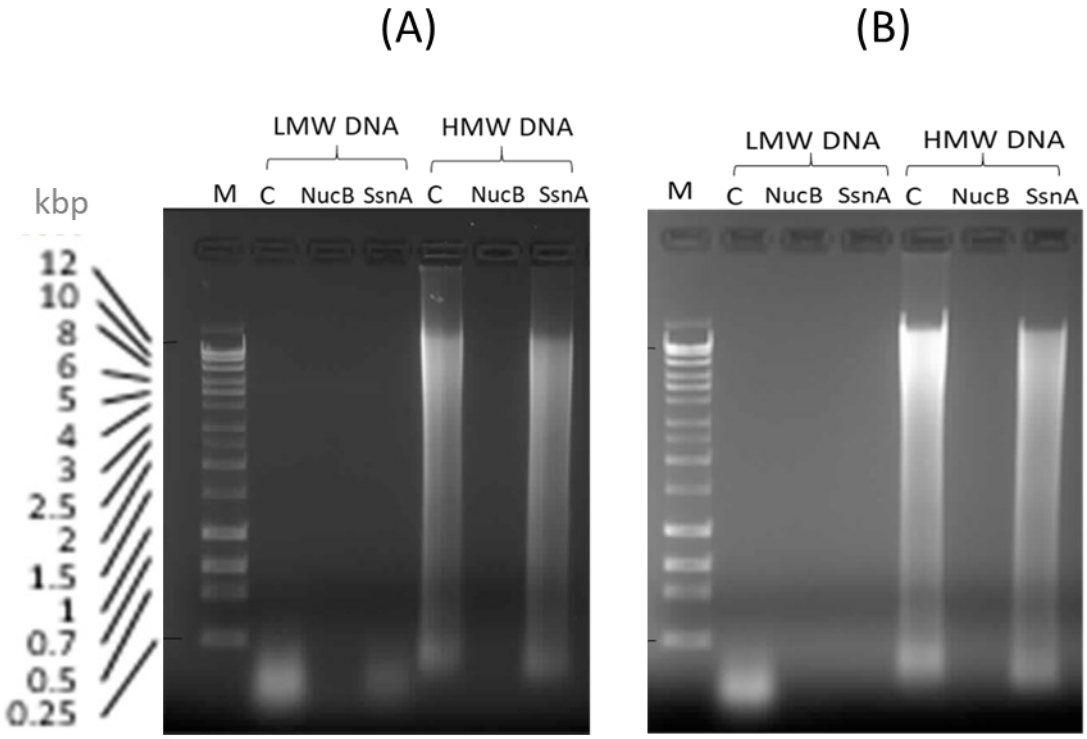

Figure 4.10. The DNase activity of SsnA against Salmon sperm DNA. *Low molecular weight (LMW) or High molecular weight (HMW) Salmon sperm DNA (125 µg) was incubated with SsnA (5 µg/ml) at 37 C° for (A) 1 hour and (B) 24 hours. The reaction buffer for NucB consisted of 25 mM HEPES (pH 8.0) and 5 mM $MnSO_4$. For SsnA, the reaction buffer contained 25 mM Tris (pH 8.0), 40 µM EDTA and 80 µM $MgCl_2$.Samples of the digestion products were separated by agarose gel electrophoresis and 1kb plus DNA ladder is included as a marker.*

4.7 The activity of SsnA against plasmid DNA

To give more insight into the preference and specificity of the DNA substrate for SsnA, a supercoiled plasmid was used as a substrate for the activity assay in this section. Varying concentrations of SsnA and NucB were incubated with 1 µg/ml plasmid DNA at 37 °C for 1 hour, and digestion products were visualised by agarose gel electrophoresis. For SsnA, the band corresponding to the supercoiled plasmid disappeared by concentrations ≥ 1 µg/ml, but two new bands possibly indicating open circle (nicked) and linear forms of DNA appeared (Figure 4.11 A). As expected, all concentrations of NucB could digest the supercoiled plasmid substrate completely leaving no detectable fragments or smear. When SsnA was incubated with the plasmid DNA over a time period of 24 hours, the band of the supercoiled substrate disappeared by 30 min, and only a nicked form of DNA was detectable until 4 hours of incubation. By 24 h, another band indicating linearized plasmid appeared just below the nicked open circular (Figure 4.11 B). It should be noted that in the latter case, some nicked DNA pre-exists in the plasmid sample explaining the OC signal observed in the control. Overall, the supercoiled plasmid substrate was converted to either linear or nicked form, however, it was never fully degraded by SsnA at any of the time points.

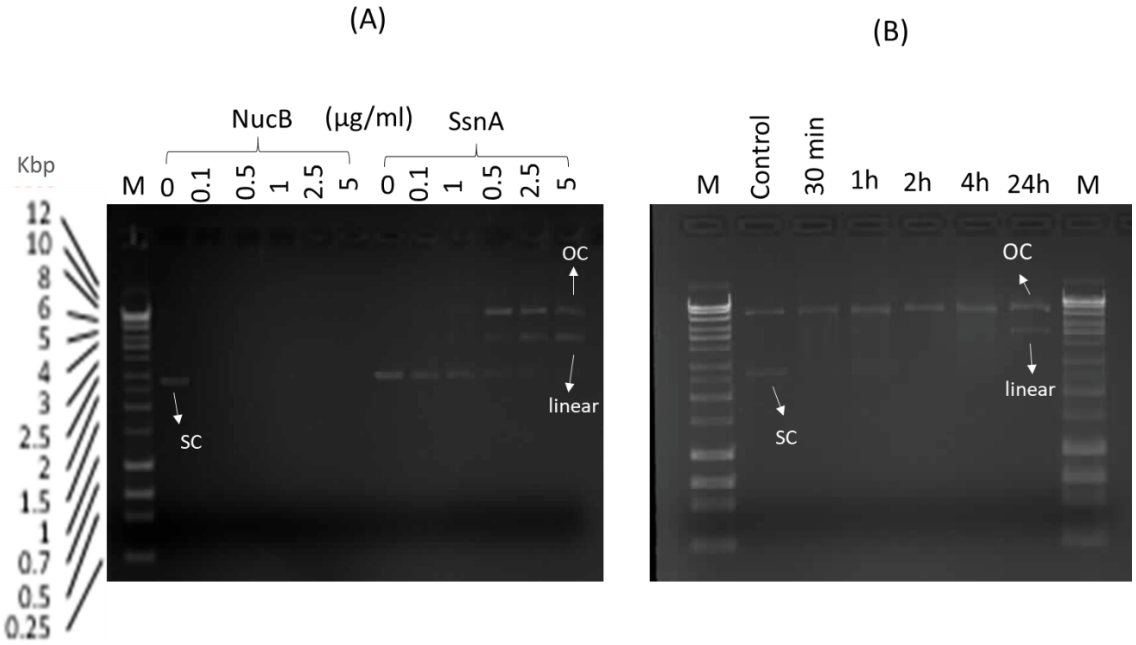

Figure 4.11 The DNase activity of SsnA against plasmid DNA*. (A) varying concentrations of SsnA or NucB were incubated with (1 µg) supercoiled plasmid at 37C° for 1 hour. (B) SsnA (5 µg/ml) was incubated with the plasmid over 24 hours. The reaction buffer for NucB consisted of 25 mM HEPES (pH 8.0) and 5 mM $MnSO_4$. For SsnA, the reaction buffer contained 25 mM Tris (pH 8.0), 40 µM EDTA and 80 µM $MgCl_2$.Samples of the digestion products were separated by agarose gel electrophoresis. 1kb plus DNA ladder is used as a marker. The supercoiled plasmid (SC) was converted by SsnA to open circle (OC) fragments and linear DNA.*

4.8 Thermal stability of SsnA versus NucB

Our previous results showed that SsnA exhibits different antibiofilm and DNase activity to NucB. In this section, the thermostability of the two enzymes was investigated. A sample of SsnA was heat-treated at 80°C for 25 min, whereas NucB was heat-treated for 5 to 60 min at 100°C (heating NucB at 80°C for 25 min had no effect on the enzyme activity). To assess the activity of the enzymes after heating, SsnA and NucB samples were tested for the ability to degrade an oligo nucleotide probe and calf thymus DNA, respectively. Heating at 80°C for 25 min was sufficient to completely inactivate SsnA (data not shown), whereas all heat-treated NucB samples were able to degrade the DNA substrate (Figure 4.12). These observations highlight another property where SsnA is different to NucB.

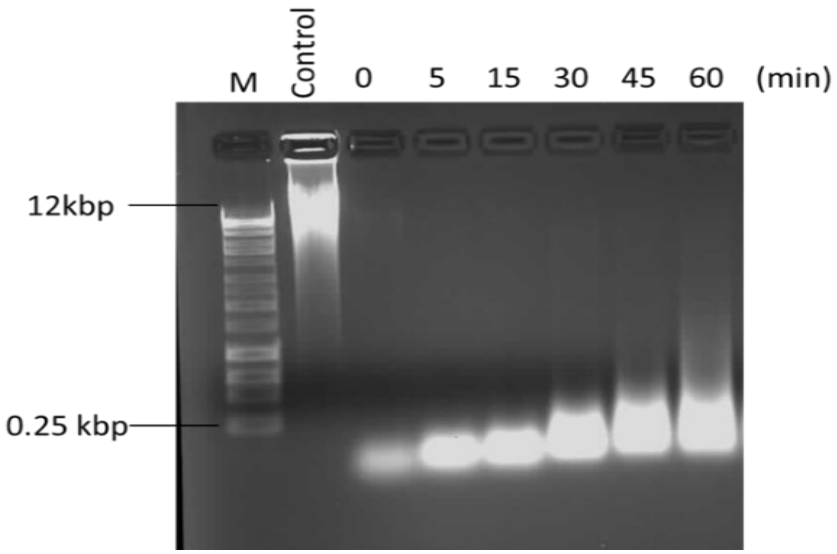

Figure 4.12. The thermal stability of NucB. *NucB was heated at 100˚C for (5, 15, 30,45 and 60) min. Samples from each time point were incubated with calf thymus DNA for at 37˚C for 1h and digestion products were visualised with 1% agarose gel electrophoresis.*

4.9 *Discussion*

Extracellular DNases are secreted by many oral bacterial species; however, their exact biological role remains elusive. One potential function of these DNases is that they could be involved in the prevention of new biofilm formation or targeting pre-established biofilms of eDNA dependent species in the oral biofilm. This hypothesis is based on previous observations that biofilms formed by oral bacteria are sensitive to exogenous DNases such as DNase I and NucB. This chapter investigated the ability of SsnA, a deoxyribonuclease secreted by *S. gordonii,* to disrupt the formation and the stability of *F. nucleatum* biofilms.

SsnA inhibited the formation of *F. nucleatum* biofilms and influenced the resultant biofilm architecture. Whilst the bioburden of the biofilm was not significantly reduced by SsnA, the presence of SsnA produced significantly more fragmented biofilms with reduced surface area. However, SsnA, unlike NucB, could not change the architecture of or disperse pre-established biofilms. The resistance of mature biofilms to DNase enzymes have been previously observed in many biofilms (Mann et al., 2009, Whitchurch et al., 2002, Qin et al., 2007) (Seper et al., 2011, Barnes et al., 2012, Binnenkade et al., 2018). This phenomenon could be explained by the substitution or protection of eDNA by other components of the biofilm matrix such as proteins (Devaraj et al., 2015, Rocco et al., 2017, Devaraj et al., 2019, Devaraj et al., 2018), polysaccharides (Peng et al., 2020b, Wang et al., 2015) and lipoteichoic acids (Castillo Pedraza et al., 2017) which are present in increased amounts in a mature biofilm matrix. However, our model of *F. nucleatum* biofilms was readily dispersed by NucB in the previous chapter (see section 3.5,3.6). With these observations, it was clear that there is a marked difference in the ability of SsnA and NucB to degrade biofilms. Therefore, the rest of the work in the chapter aimed to understand the substrate specificity and enzymatic activity of these two polypeptides.

To better understand the enzymatic properties of SsnA that might affect its antibiofilm activity, the degradation of different DNA substrates by SsnA was analysed in comparison to NucB. The results here indicate that SsnA is not a potent DNase as it could only digest single stranded DNA and low molecular weight double stranded DNA, when much lower concentrations of NucB showed

very efficient degradation of all DNA substrates including high molecular weight DNA and a supercoiled plasmid. Nevertheless, the DNase activity of SsnA against single stranded DNA was considerably higher compared to NucB. This is consistent with a previous report demonstrating that double stranded DNA is a better substrate for NucB though it can still digest single stranded DNA (Baslé et al., 2017). (Zweig et al., 2014) showed that single stranded DNA plays a vital role in the initial phases of biofilm formation, but the authors found that as the biofilm matures, single stranded DNA is not retained in the biofilm and double stranded DNA becomes more abundant and more important for the biofilm integrity. Since single stranded DNA is the substrate that was most efficiently degraded by SsnA, it is possible that SsnA could not disperse mature *F. nucleatum* biofilms due to the predominance of double stranded DNA once the biofilm had developed. Nonetheless, while interpreting the results, the limitations of the FRET activity assay used here should also be taken in consideration. The degradation of the single stranded substrate was measured by monitoring the increase in fluorescence which should result from the fluorophore and quencher at each end of the oligo probe being separated. Therefore, the observed increase in fluorescence could not only result from cleaving the probe, but also from cutting the fluorophore or the quencher.

The lack of any biofilm dispersal effect is also likely to be due to the failure of SsnA to efficiently degrade high molecular weight DNA. This hypothesis is supported by the evidence that most eDNA of bacterial biofilms is probably chromosomal DNA released during cell lysis, and it is therefore, of high molecular weight (Allesen-Holm et al., 2006). Furthermore, biofilms of *S. aureus* were demonstrated to require high molecular weight DNA (>11 kb) to act as structural support and maintain the biofilm integrity (Izano et al., 2008a). This concept might also apply to *F. nucleatum* biofilms considering that eDNA purified from this biofilm model was high molecular weight DNA (see Section 3.3). Additionally, plasmid DNA is suggested to contribute to eDNA in biofilms as the presence of a plasmid has been reported to cause an increase in the biofilm production (Seper et al., 2011). Here, it was demonstrated that SsnA is unable to achieve total degradation of a circular plasmid. Taken together, these findings further emphasize the correlation between the observed antibiofilm behaviour of SsnA

and its biochemical properties. Yet, it is also possible that the inability of SsnA to disrupt preformed biofilms or digest high molecular DNA is simply caused by the concentration of SsnA being low. Therefore, it would be interesting to see whether using much higher concentrations could make any difference.

Our observations also raise questions about the role of SsnA in *S. gordonii* particularly the ability of SsnA to convert a supercoiled plasmid to nicked and linearized DNA. Other membrane localised bacterial DNases including EndA of *Streptococcus pneumoniae* and NucA of *Bacillus subtilis*, appear to behave similarly to SsnA when degrade supercoiled plasmids. These enzymes are well-known for their role in DNA uptake and natural competence (Lacks et al., 1974) (Lacks et al., 1975) (Puyet et al., 1990) (Provvedi et al., 2001). DNA uptake in gram positive bacteria is thought to consist of two steps which are binding and entry. During the binding step, exogenous DNA binds on the outside of the competent cell and undergoes double stranded breaks (Provvedi et al., 2001) or single stranded nicks followed by double stranded cleavages (Lacks and Greenberg, 1976) (Morrison and Guild, 1973). A single strand of that cleaved DNA is pulled into the cell in the entry step, while the complementary strand is degraded extracellularly (Chen and Dubnau, 2004). EndA has proven important for transporting the donor DNA to the interior of the cell (Lacks and Neuberger, 1975) (Lacks et al., 1975), whereas NucA was suggested to be responsible for making double-stranded breaks in the cell bound DNA to produce linear DNA thereby increasing the rate of DNA uptake (Provvedi et al., 2001). Similar process of DNA uptake could occur in *S. gordonii* with SsnA being responsible for the entry of the transforming DNA or generating linear DNA during the binding step. Competence in *S. gordonii* was found to be regulated by CcpA which plays a well-recognized role in the regulation of sugar metabolism and carbon catabolite repression in Gram-positive bacteria (Zheng et al., 2012). Interestingly, CcpA has also been shown to be also responsible for regulating SsnA expression (Robert shields, PhD thesis). Previous work in our group has investigated the importance of SsnA for genetic transformation of *S. gordonii* and found that the deletion of SsnA gene did not reduce the transformation efficiency (David Taylor, unpublished work). However, our collaborator Angela Nobbs is currently shedding

new light on this aspect of SsnA and seems to come across contrasting results (Angela Nobbs, personal communication).

Bacteria also take up DNA to use it as a source of carbon, nitrogen and phosphorous (Ibáñez de Aldecoa et al., 2017). Since SsnA degrades single stranded DNA very efficiently, this membrane-localized DNase could help *S. gordonii* obtain nutrients by degrading the non-transforming DNA strand remaining outside the cell. This hypothesis is further supported by the previous observation that SsnA is downregulated when an optimal source of sugar is available for *S. gordonii* (Robert shields, PhD thesis). Although the findings in this chapter do not prove that SsnA plays either of the above-mentioned roles, they will undoubtedly contribute to investigate this potential with more in-depth studies.

Overall, this study revealed distinct differences between NucB and SsnA regarding their biochemical and antibiofilm properties. Further characterization of the differences between NucB and other native nucleases of the dental biofilm will be critical for understanding whether the modulation of DNases produced by oral bacteria could help control dental plaque. At present, the exact contribution of SsnA to *S. gordonii* and to the whole dental biofilm remains unclear and will require further investigations.

Chapter 5: Characterizing eDNA in the matrix of model plaque biofilms

5.1 Outline

The role of eDNA in oral biofilms has been investigated almost solely by studying single- species biofilm models. However, dental plaque is formed by multiple bacterial species, and the biofilm matrix production by oral bacteria is unlikely to be the same when they live within a complex community. A better understanding of the structure and function of eDNA in mixed species oral biofilm models could help elucidate the potential of eDNA being used as a target for dental plaque control.

Exopolysaccharides, mainly *S. mutans* derived glucans, have been long recognized as the major matrix components in cariogenic plaque biofilms. *S. mutans* and a few other bacteria use sucrose as a substrate to produce glucans and build the biofilm matrix (Koo et al., 2013, Cugini et al., 2019). eDNA and extracellular matrix proteins including amyloids and host proteins and glycoproteins are also present in cariogenic biofilms (Liao et al., 2014, Castillo Pedraza et al., 2017, Besingi et al., 2017, Kim et al., 2018), however, their biological roles within the biofilm are poorly understood. Information about the matrix composition of non-cariogenic biofilms such as subgingival dental plaque is scarce, but since very low levels of sugars are available for subgingival bacteria, the matrix of subgingival biofilms might not contain substantial amounts of polysaccharides. Nonetheless, some bacteria in the subgingival biofilm produce non-glucan exopolysaccharides such as PNAG (ß- 1,6- N- acetyl- D- glucosamine) which are not dependent on dietary sugars for synthesis (Izano et al., 2008b).

eDNA results from death and lysis of microbial cells or is actively secreted into the biofilm matrix by viable cells (Allesen-Holm et al., 2006, Perry et al., 2009a, Liao et al., 2014). Previous research has shown that extracellular DNA production in multispecies biofilms is species dependent and that eDNA abundance in these

biofilms is not always related to the amount present in single-strain biofilm models (Steinberger and Holden, 2005). Some bacteria might contribute more than others to eDNA production in multispecies biofilms such as dental plaque, therefore, eDNA might be enriched in specific sequences. Investigating the sequence of eDNA in dental plaque will enhance our understanding of its functions and importance as a target for antibiofilm strategies.

DNA present inside the periodontal bacterial cell is known to contribute to the pathogenesis of periodontitis by stimulating the release of proinflammatory mediators such as tumour necrosis factor alpha (TNF-α), interleukin -6 and interleukin-8 from human immune cells (Nonnenmacher et al., 2003a, Sahingur et al., 2010, Sahingur et al., 2012, Kim et al., 2012b, Soto-Barreras et al., 2017). It is possible that eDNA also reach and interact with the host cells involved in the inflammatory process of periodontitis, therefore, the host immune reaction towards eDNA in dental plaque needs to be investigated. Macrophages are vastly involved in the immune response-mediated periodontal bone damage in periodontitis primarily because of their ability to produce several destructive cytokines including TNF-α, IL-6, and IL-1β (Zhuang et al., 2018). *F. nucleatum* have long been implicated in the pathogenesis of periodontal disease, and this thesis has showed that *F. nucleatum* biofilms contain substantial amounts of eDNA. This eDNA might interact with immune cells such as macrophages and provoke the release of inflammatory cytokines, thereby, contributes to the periodontal tissues destruction.

This chapter aims to characterise eDNA as a component of supra and subgingival dental plaque by addressing the following objectives:

1. Assess the effect of DNase enzymes, NucB and SsnA, on aerobic and anaerobic *in vitro* dental plaque models.
2. Investigate whether eDNA is enriched in sequences from particular species in the dental plaque.
3. Investigate ability of eDNA from periodontal bacteria to stimulate host cell responses.

5.2 Visualisation of in vitro dental plaque models

An *in vitro* dental plaque model was developed to help investigate the abundance and function of eDNA within dental plaque biofilms. Mixed-species biofilms were cultured on inert (glass) surfaces, using human saliva as an inoculum. Biofilms were grown for 24 h either aerobically in the presence of 2% (w/v) sucrose as a cariogenic supragingival dental plaque model, or anaerobically without sucrose to model subgingival dental plaque. These model plaque biofilms were visualised under scanning electron microscopy (SEM). The aerobic biofilms consisted predominantly of long chains of coccoid cells possibly streptococci (*Figure 5.1*).

Short rods were also observed sometimes. One prominent feature is large yeast cells that were found consistently throughout biofilm samples and likely to be *Candida*. Occasionally, extracellular material was seen at high magnification either encompassing some cells or as thin strands (Figure 5.1). The anaerobic biofilm samples showed more diverse cell morphology than aerobic biofilms, but *Candida* were not observed in any of the samples (Figure 5.2). Strands of extracellular materials were more frequent and more obvious throughout the anaerobic biofilms. It should be noted that the biofilms here (Figure 5.1, Figure 5.2) are visualized in their collapsed state as they were dehydrated for SEM.

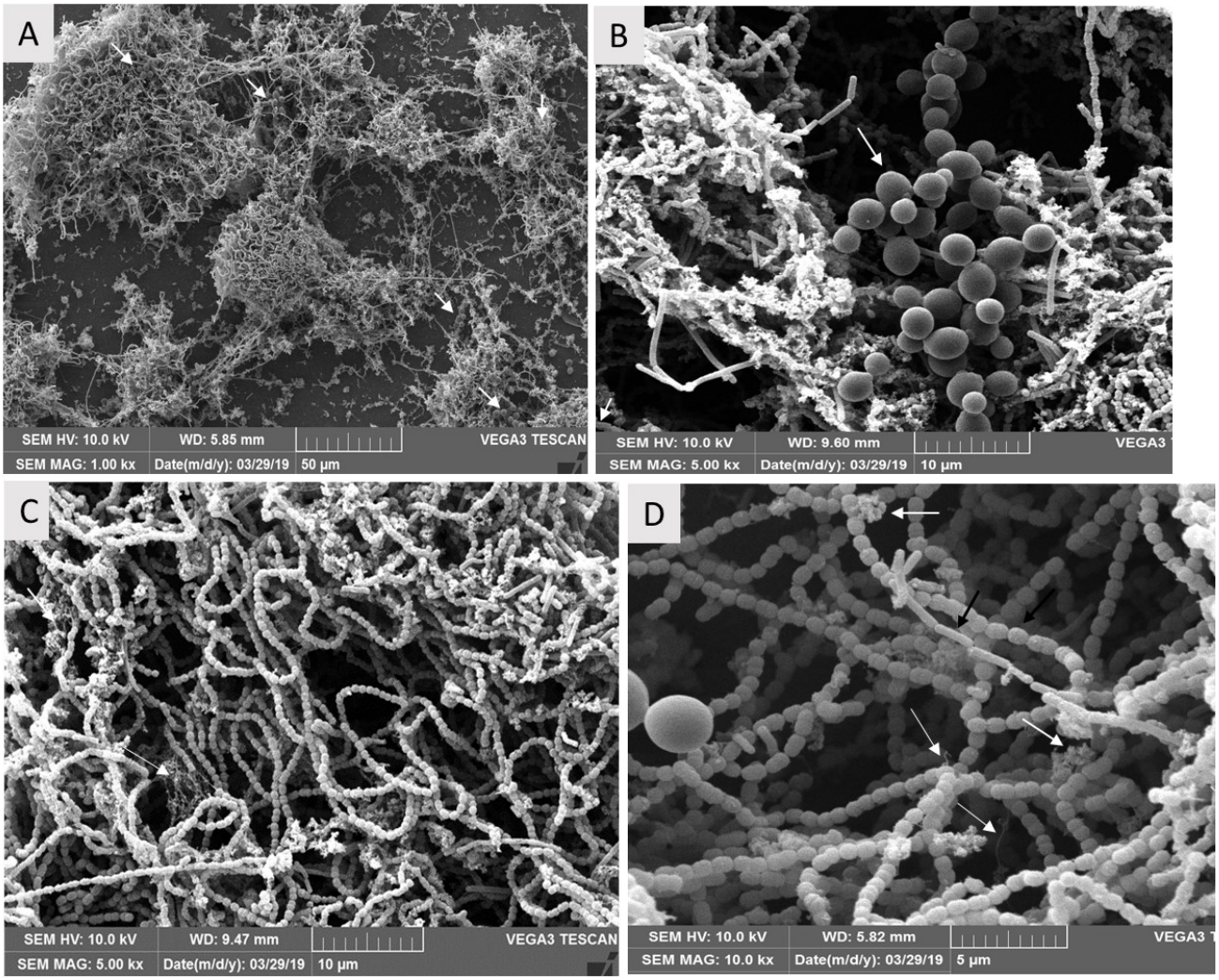

Figure 5.1 Scanning electron micrographs of model supragingival dental plaque. *Mixed species saliva biofilms were grown aerobically in the presence of 2% sucrose to model supragingival dental plaque. [A,B] Yeast and hyphae cells were seen throughout the biofilm (indicated by arrows) [C,D] Chains of Streptococci and rod shaped cells (black arrows), Extracellular material was seen at high magnification as thin strands or covering some cells(white arrows).*

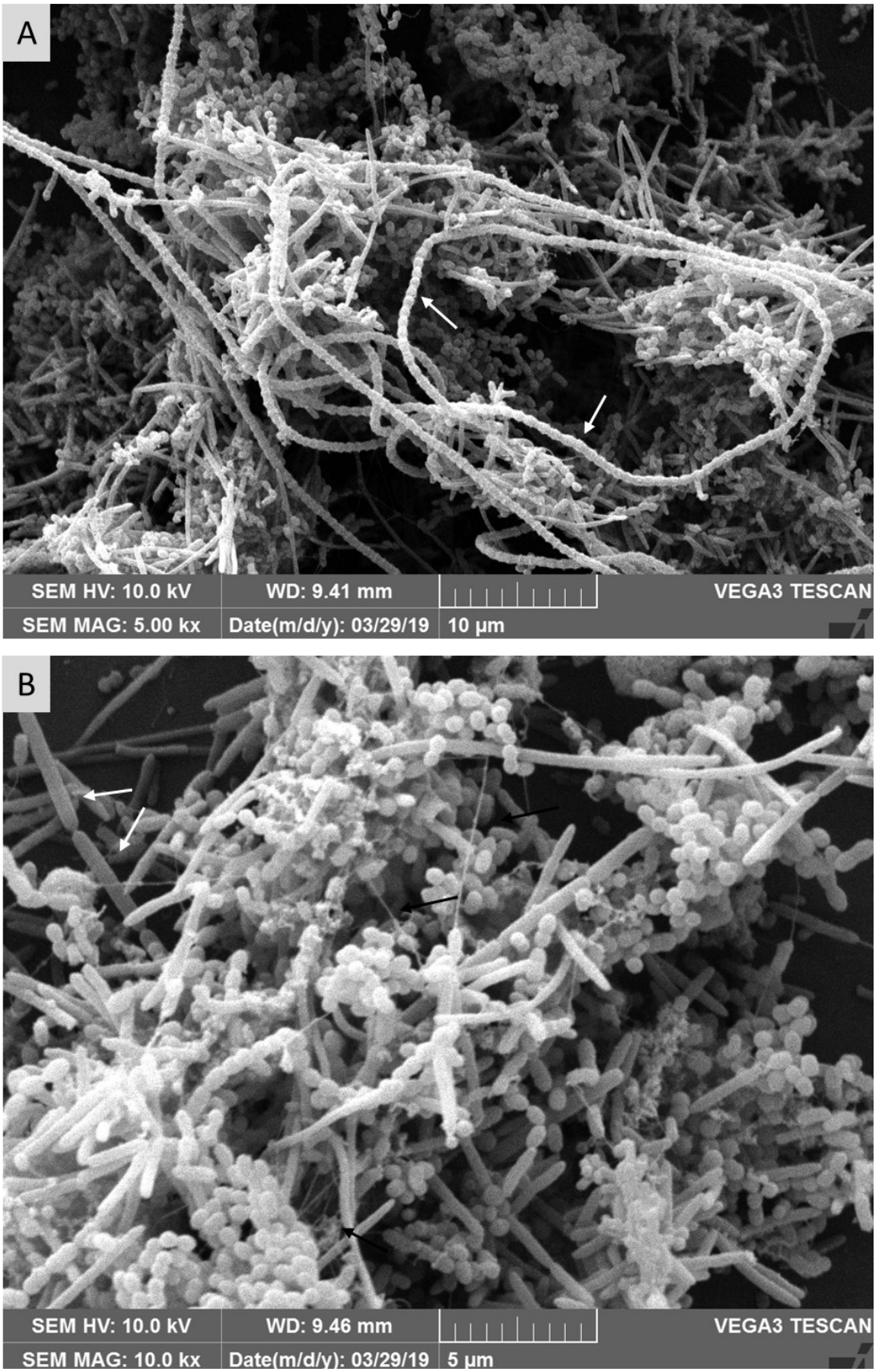

Figure 5.2 Scanning electron micrographs of model subgingival biofilms. *Mixed species saliva biofilms were grown on glass surfaces for 24 h in anaerobic conditions to*

model subgingival dental plaque and were visualised with SEM. [A] long chains of cocci (white arrows) [B] long rods can be seen throughout the biofilm (white arrows) and Strands of extracellular material was also observed at high magnification (Black arrows)

5.3 Testing the sensitivity of in vitro dental plaque models to NucB and SsnA

To investigate whether eDNA has a role in the development of dental plaque biofilms, model biofilms were grown in the presence of NucB or SsnA DNase enzymes. These two enzymes were found in the previous chapter to have different DNA substrate preference and distinct antibiofilm activity against *F. nucleatum* biofilms. Therefore, they were both used here to compare their effect on plaque biofilms. To grow these biofilms, human saliva samples from three different volunteers were diluted 1:100 in artificial saliva and cultured for 24 h. Biofilms were cultured aerobically in the presence of 2% sucrose for supragingival biofilm modelling. To model subgingival dental plaque, biofilms were grown in anaerobic conditions and the biofilm growth was assessed by crystal violet staining. As can be seen in (Figure 5.3), only NucB reduced the formation of the anaerobic biofilms (70% reduction), whereas no effect was observed for either enzyme in case of aerobic biofilms. The role of eDNA in the stabilization of these saliva biofilms was assessed by treating preformed biofilms (24 h old) with NucB or SsnA for one hour. Here, neither aerobic nor anaerobic biofilms were dispersed by NucB or SsnA treatment (Figure 5.4).

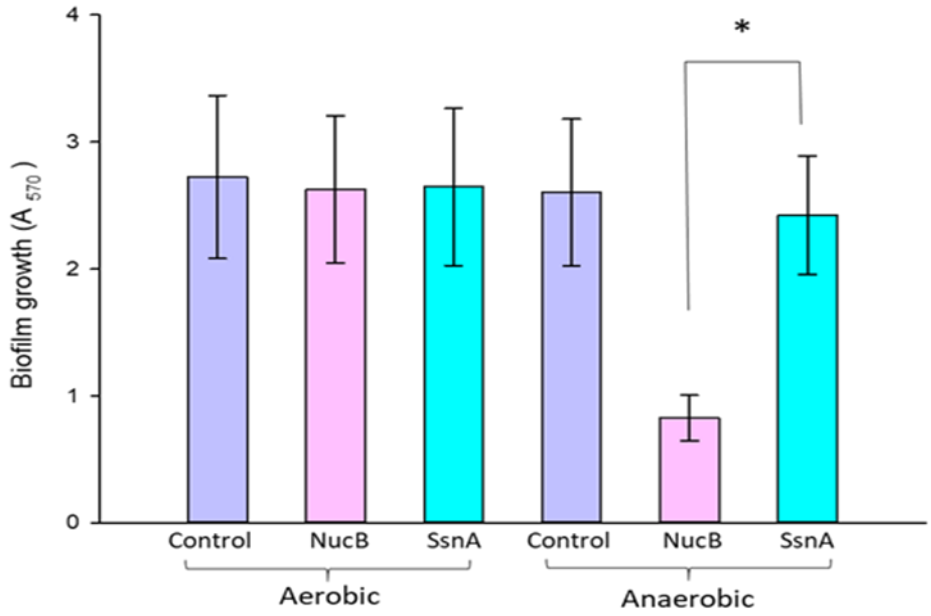

Figure 5.3 Inhibition of model dental plaque biofilms with NucB or SsnA*. Artificial saliva media were inoculated from 3 different natural human saliva samples and grown either aerobically in the presence of 2% sucrose to model supragingival dental plaque or anaerobically without sucrose modelling subgingival dental plaque. 0.5 µg/ml NucB or 5 µg/ml SsnA were included during biofilm formation (24 h) and biofilms were quantified by staining with crystal violet. Bars represent mean values from three independent experiments and standard errors are indicated. Statistical significance was calculated with the one-way ANOVA and the post-hoc Tukey`s test.*

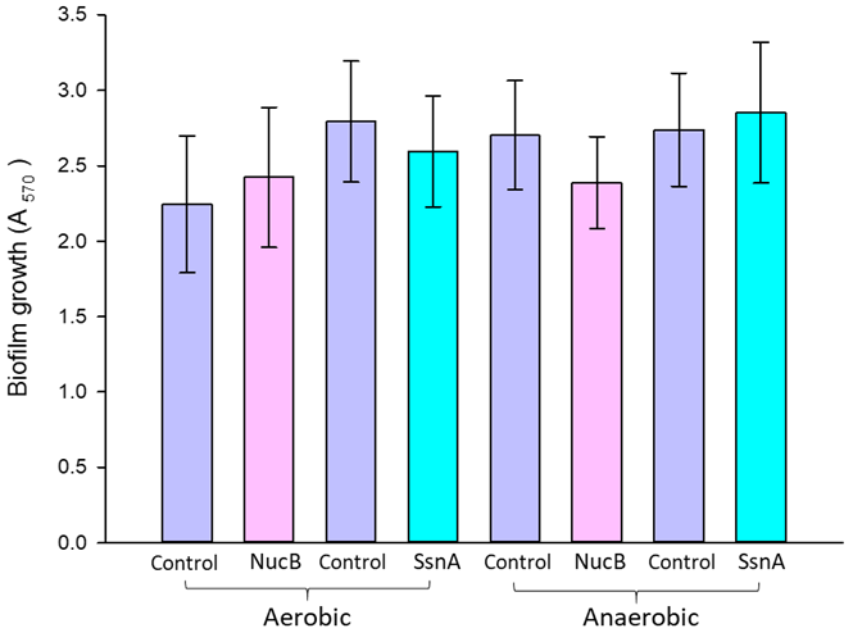

Figure 5.4 Dispersal of model dental plaque biofilms with NucB and SsnA*. Artificial saliva media were inoculated from 3 different natural saliva samples and grown for 24 h either aerobically in the presence of 2% sucrose to model supragingival dental plaque or anaerobically without sucrose modelling subgingival dental plaque. Preformed biofilms were treated by NucB and SsnA for 1 hour at 37˚C and quantified by staining with crystal violet. Bars represent mean values from three independent experiments and standard errors are shown. Statistical significance was calculated with the one-way ANOVA and the post-hoc Tukey`s test.*

.

5.4 Imaging of NucB and SsnA effect on in vitro dental plaque biofilms

To visualise the effects of NucB and SsnA on the development and the structural integrity of model dental plaque biofilms, the biofilms were cultured on glass coverslips and analysed by SEM. NucB and SsnA were either present during the development of the biofilm to view the inhibitory effect of the enzymes or used to treat preformed biofilms for 1 h to assess dispersal. Aerobic biofilms grown in the presence of NucB or SsnA consisted of large cells aggregates that covered most of the slide surface, similar to what was observed in control biofilms (Figure 5.5). At high magnification, the biofilms showed a dense network of microbial cells which were closely aggregated. Extracellular material was evident in both control and treatment images. For the anaerobic biofilms, the presence of NucB during biofilm formation resulted in the biofilms containing few isolated cells or small clusters of cells scattered over the surface of the slide (Figure 5.5). In contrast, SsnA did not reduce biofilms developed under anaerobic conditions, although extracellular material observed in control biofilms was not obvious in SsnA biofilms. When testing dispersal, neither NucB nor SsnA could disperse any of the biofilms (Figure 5.6).

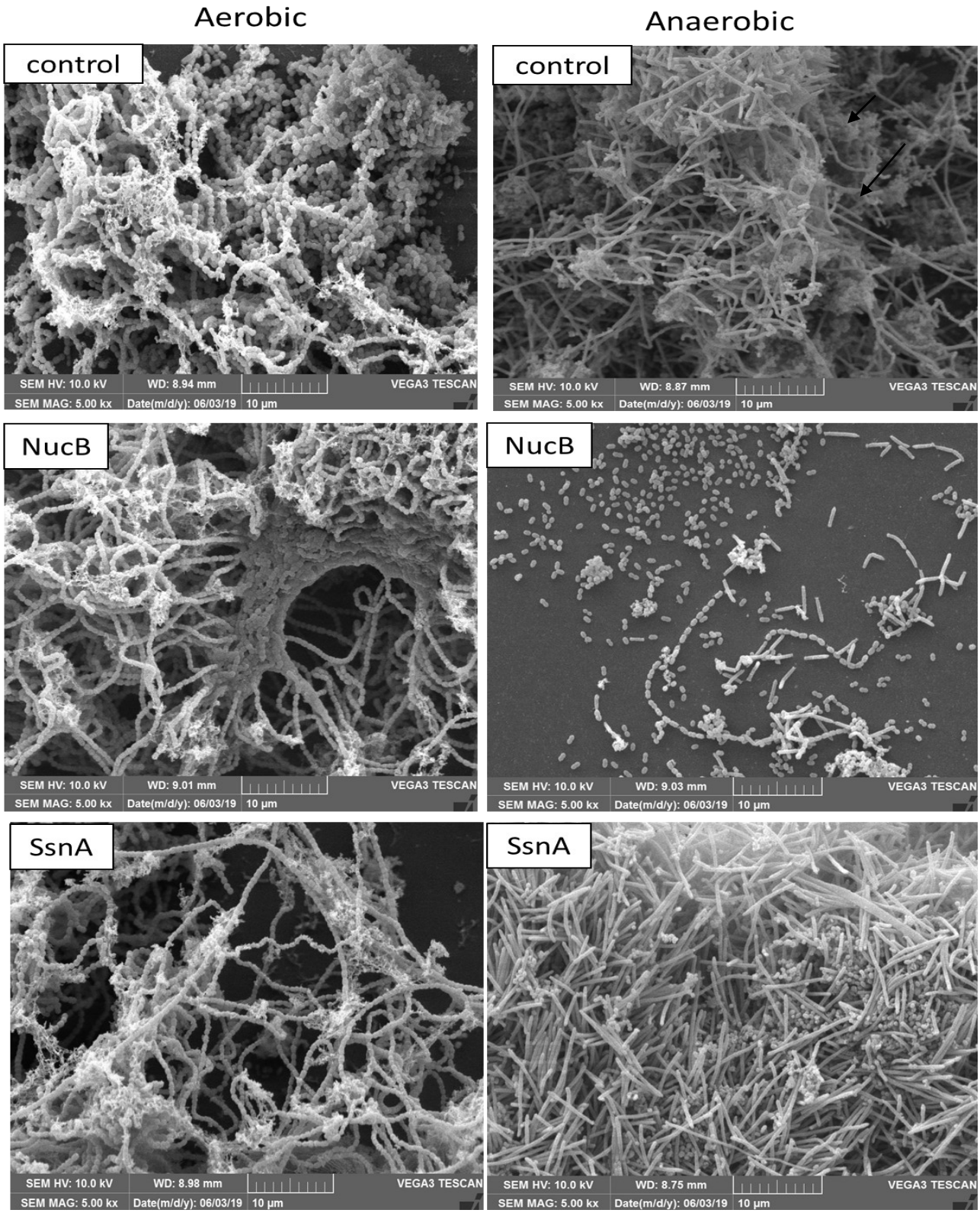

Figure 5.5 Scanning electron microscopy of mixed species saliva biofilms grown in the presence NucB or SsnA. *Mixed species saliva biofilms were grown in artificial saliva for 24 h and viewed with SEM. The biofilms were either cultured aerobically in the presence of 2% sucrose to model supragingival dental plaque or anaerobically as a subgingival biofilm model. The aerobic biofilms were not reduced by NucB or SsnA. The anaerobic biofilms formation was only inhibited by NucB. Although the anaerobic*

biofilm formation was not reduced by SsnA, the extracellular material seen surrounding the cells in control biofilms (black arrows) was not obvious in SsnA-treated biofilms.

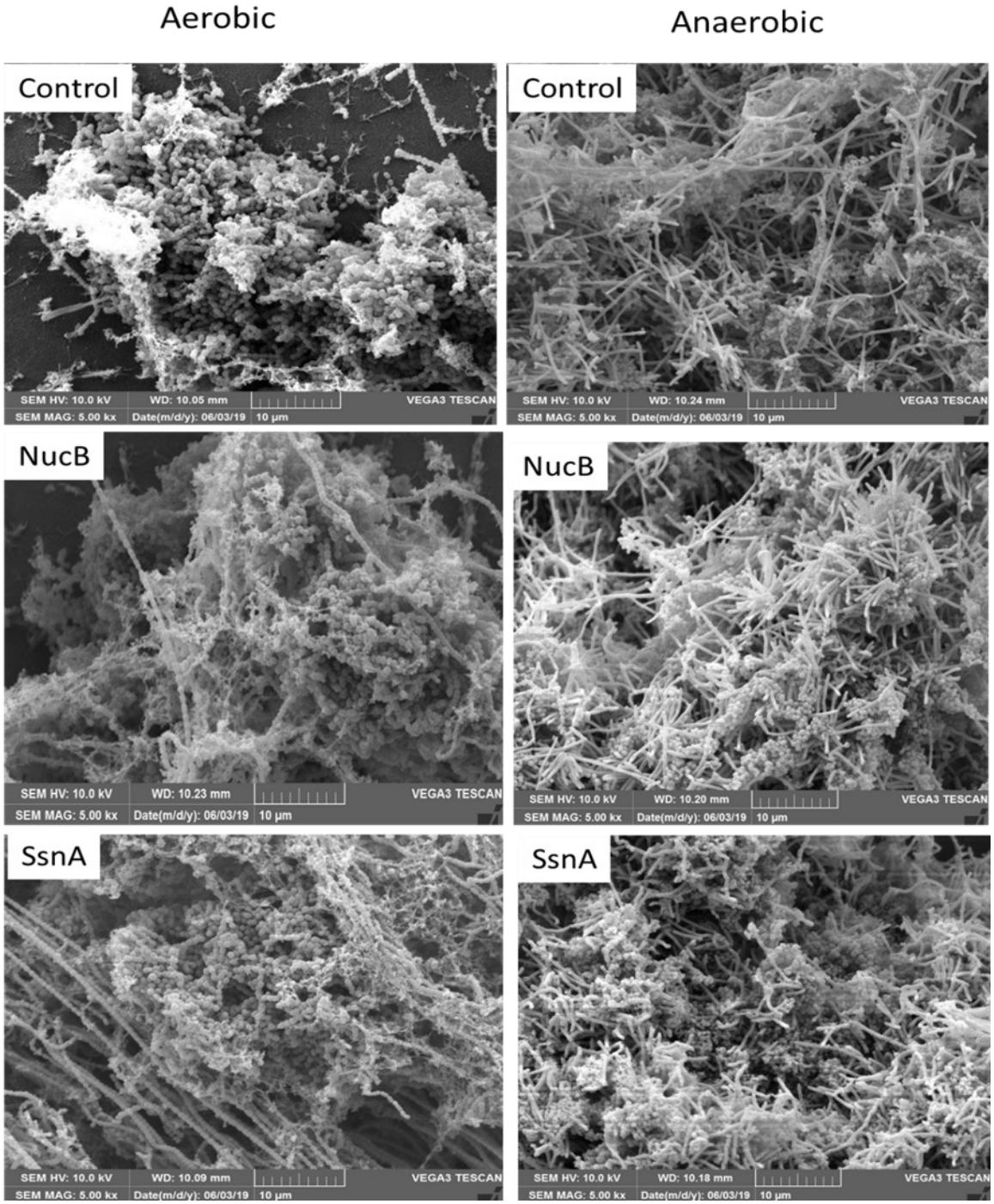

Figure 5.6 Scanning electron microscopy of preformed in vitro plaque biofilms treated with NucB or SsnA. *Mixed species saliva biofilms were grown in artificial saliva for 24 h, treated with NucB or SsnA for one hour and viewed with SEM. The biofilms were either cultured aerobically in the presence of 2% sucrose to model supragingival dental plaque or anaerobically as a subgingival biofilm model. Biofilms were not dispersed by NucB or SsnA.*

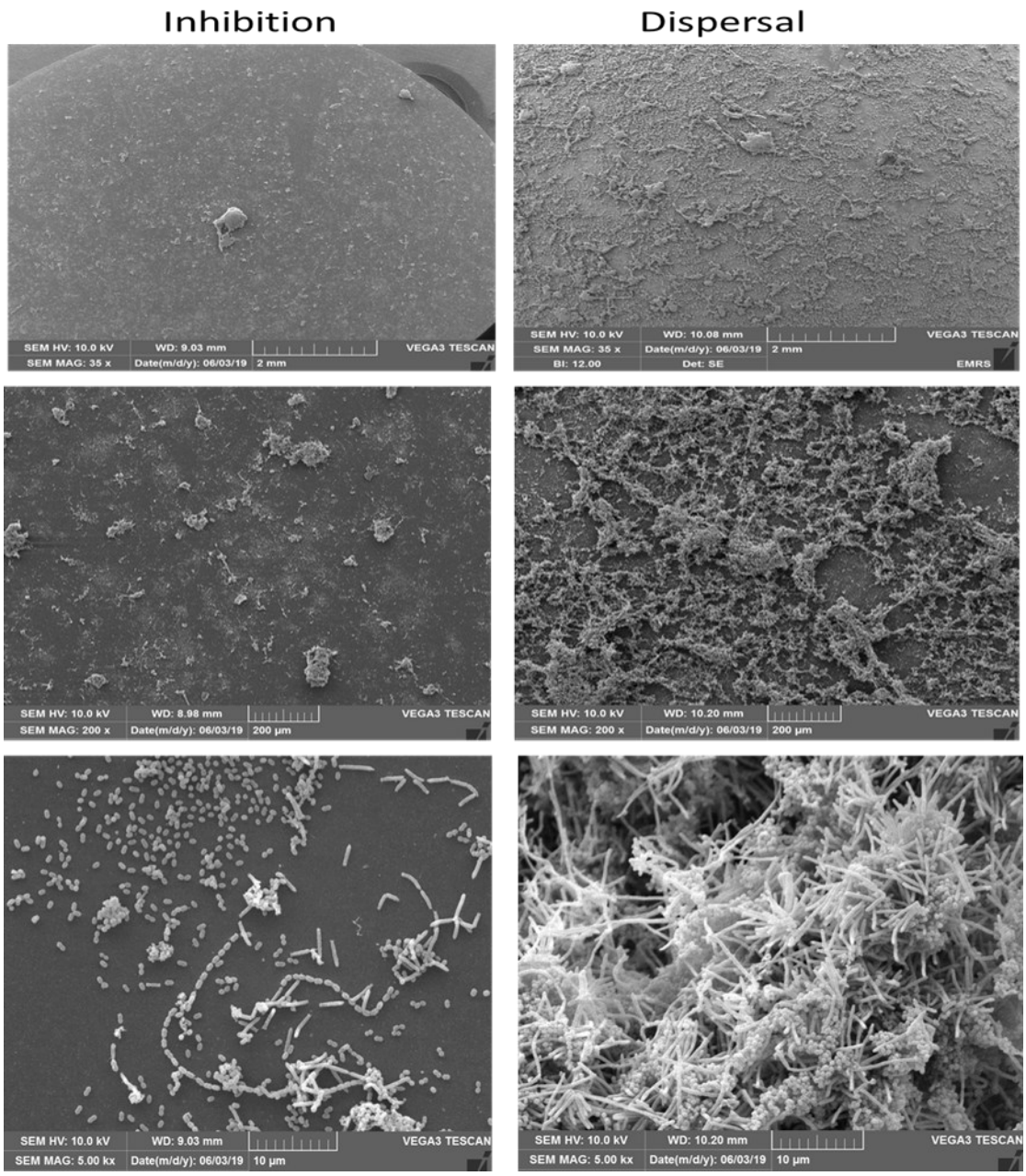

Figure 5.7 ***The inhibitory versus the dispersal effect of NucB on anaerobic model dental plaque biofilms at different magnification****. Biofilm formation was inhibited by NucB, but preformed biofilms were not removed by the enzyme.*

5.5 Purification of eDNA from in vitro dental plaque models

Our previous results suggest that subgingival biofilms are more dependent on eDNA than supragingival biofilms. To further investigate this hypothesis, eDNA was extracted from 3 independent supragingival (aerobic) and subgingival (anaerobic) model dental plaque biofilms. To extract eDNA, biofilms were harvested and collected in 1 ml PBS. This mixture was centrifuged to separate cells and eDNA was purified from the supernatants. The eDNA extracted was analysed by agarose gel electrophoresis (Figure 5.8). *eDNA purified from the anaerobic biofilms appeared as a sharp band of high molecular* weight, while no distinctive bands were observed for eDNA of aerobic biofilms. Instead, small fragments were seen at the bottom of the gel possibly indicating low molecular weight DNA or RNA. Interestingly, when eDNA extracts were measured by a Nanodrop 1000 spectrophotometer, eDNA from aerobic biofilms showed higher concentrations in comparison to eDNA purified from the anaerobic biofilms.

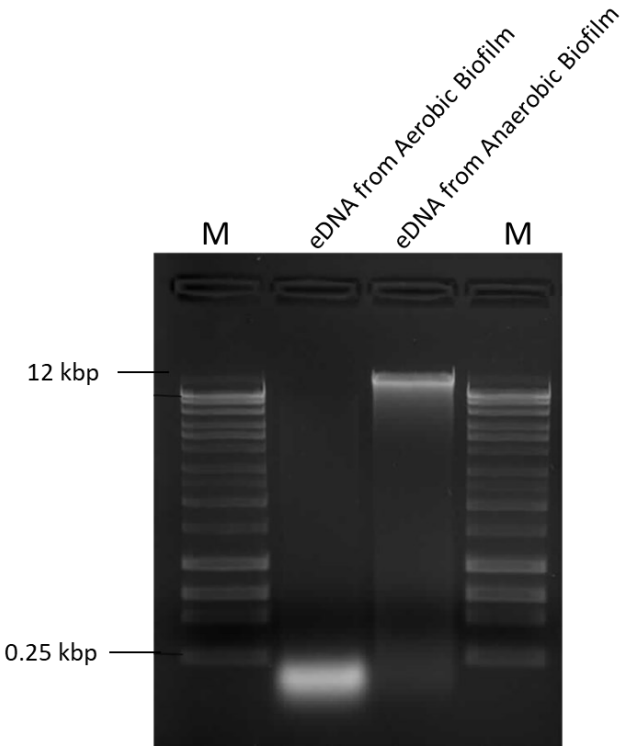

***Figure 5.8 Extracellular DNA extraction from model supra and subgingival biofilms**. eDNA was extracted from in vitro supragingival (aerobic) and subgingival (anaerobic) model plaque biofilms and visualized with 1% agarose gel electrophoresis. Hyperladder 1kb plus was included as a molecular weight marker (M). The image is a representative of eDNA purification from 3 biofilms derived from saliva of three different volunteers.*

5.6 Measuring proteins in the matrix of in vitro dental plaque models

In the previous section, it was possible to extract eDNA from the anaerobic biofilms, but not from the aerobic biofilms suggesting that eDNA is more abundant in subgingival biofilms. To further explore the differences in the matrix composition of supra and subgingival biofilms, the extracellular protein content in aerobic and anaerobic model plaque biofilms was measured using the Bradford Coomassie brilliant blue assay. Samples were derived from 3 different saliva volunteers where each saliva inoculum was used to grow one aerobic and one anaerobic biofilm. Cells were separated from the biofilms as mentioned in Section 5.6. A standard curve was generated using bovine serum albumin, and protein concentrations in biofilms were calculated from the standard curve generated (Figure 5.9 A, B). The protein concentrations in aerobic biofilms samples were about 6 times the protein concentrations for the anaerobic biofilms (Figure 5.9 C). Protein gel electrophoresis was also used to analyse the proteins present in the biofilm samples (Figure 5.10). No bands were observed for the anaerobic biofilms, while the aerobic biofilms showed unclear bands pattern probably due to the proteins being degraded. However, the overall staining intensity was much higher in the aerobic biofilms. In general, these results indicate that the supragingival biofilms are rich in extracellular proteins when compared to the subgingival ones.

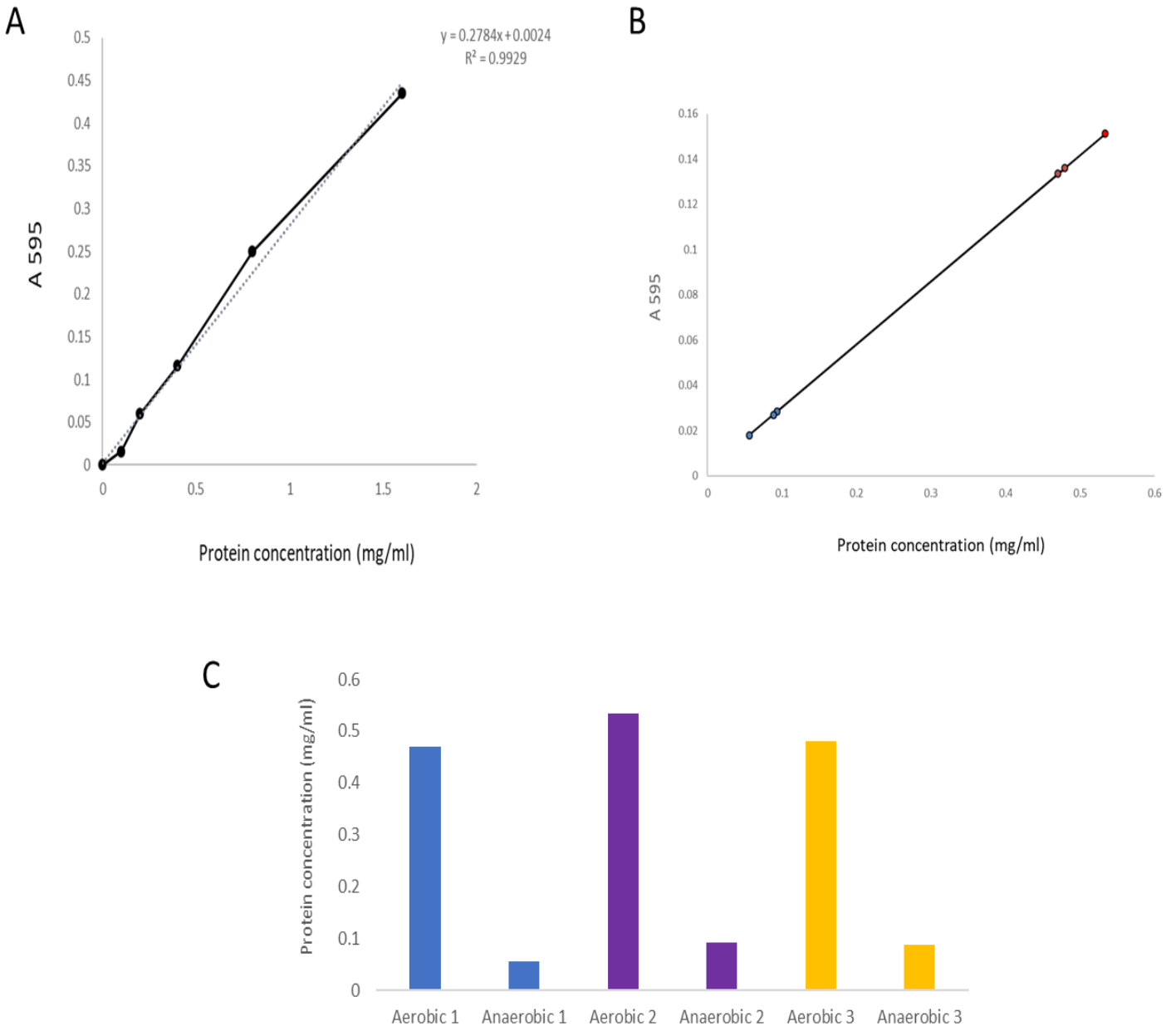

Figure 5.9 Measuring extracellular proteins in in vitro dental plaque biofilms. *(A) a standard curve was generated using concentrations of bovine serum albumin ranging from 0.1 to 1.6 mg/ml. (B) Protein concentrations in three independents microcosm biofilms were calculated from the equation of standard curve. (C) All 3 aerobic biofilms were richer in proteins than their corresponding anaerobic biofilms.*

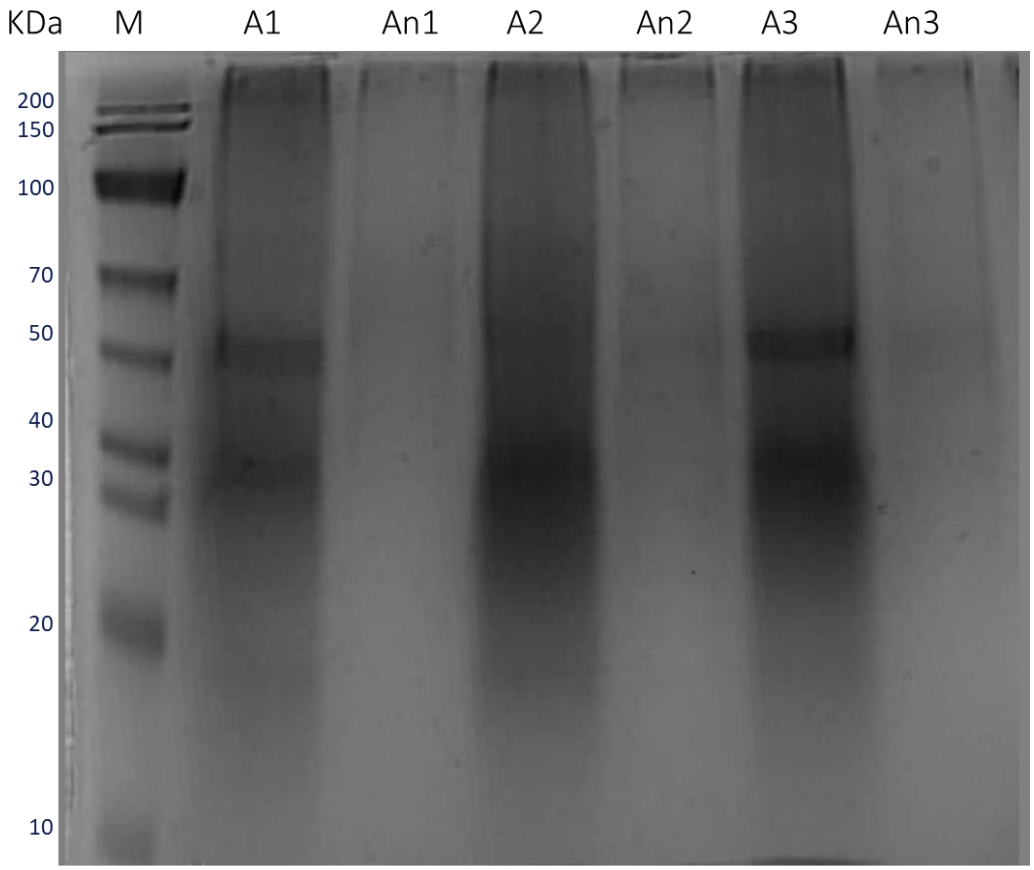

Figure 5.10 SDS PAGE gel comparing protein content of in vitro supragingival vs subgingival biofilms*. Saliva from three different volunteers was used to grow supra and subgingival model biofilms. Supragingival (aerobic) biofilms (A1,2,3) showed much higher staining intensity than subgingival (anaerobic) biofilms (An1,2,3). Ladder is PageRuler unstained Broad Range protein ladder (10-200 kDa). Gel was stained using Coomassie InstantBlue.*

5.7 Sequencing of intracellular DNA (iDNA) and eDNA from in vitro dental plaque models

To further characterize eDNA in dental plaque, this section investigated the microbial composition of eDNA purified from model dental plaque biofilms. eDNA and iDNA were purified from saliva derived subgingival biofilms (12 different saliva volunteers). The 24 samples were analysed for their microbial composition by 16S rRNA gene sequencing (see Materials and Methods). The bacterial composition of the DNA samples was analysed at genus level (Figure 5.11). The relative abundance of genera in the samples showed a noticeable difference between eDNA and iDNA bacterial composition. eDNA samples were highly dominated by genus *Veillonella* which surprisingly made up more than 90% of the total composition in all samples. In contrast, the relative abundance of *Veillonella* in iDNA samples was only 4 to 6%. Genus *Fusobacterium* was also present in eDNA, but at very low abundance and in only 6 of the 12 samples. Other genera detected in eDNA composition were *Streptococcus* in 3 samples, *Prevotella 6* and *Gemella* in one sample each. For iDNA samples, Genera *Haemophilus*, *Fusobacterium*, *Veillonella and Streptococcus w*ere present in all samples with Streptococcus being the most abundant Genus. *Porphyromonas* was detected in all but 2 samples. The controls which consist of the buffer alone (PBS) were dominated by bacterial DNA. Although these samples are likely to contain environmental DNA, the bacterial DNA observed here is primarily from oral bacteria and the pattern is close to that of iDNA samples indicating that there might have been some cross-sample contamination. In addition, a large proportion of the bacterial genera in iDNA samples were unassigned.

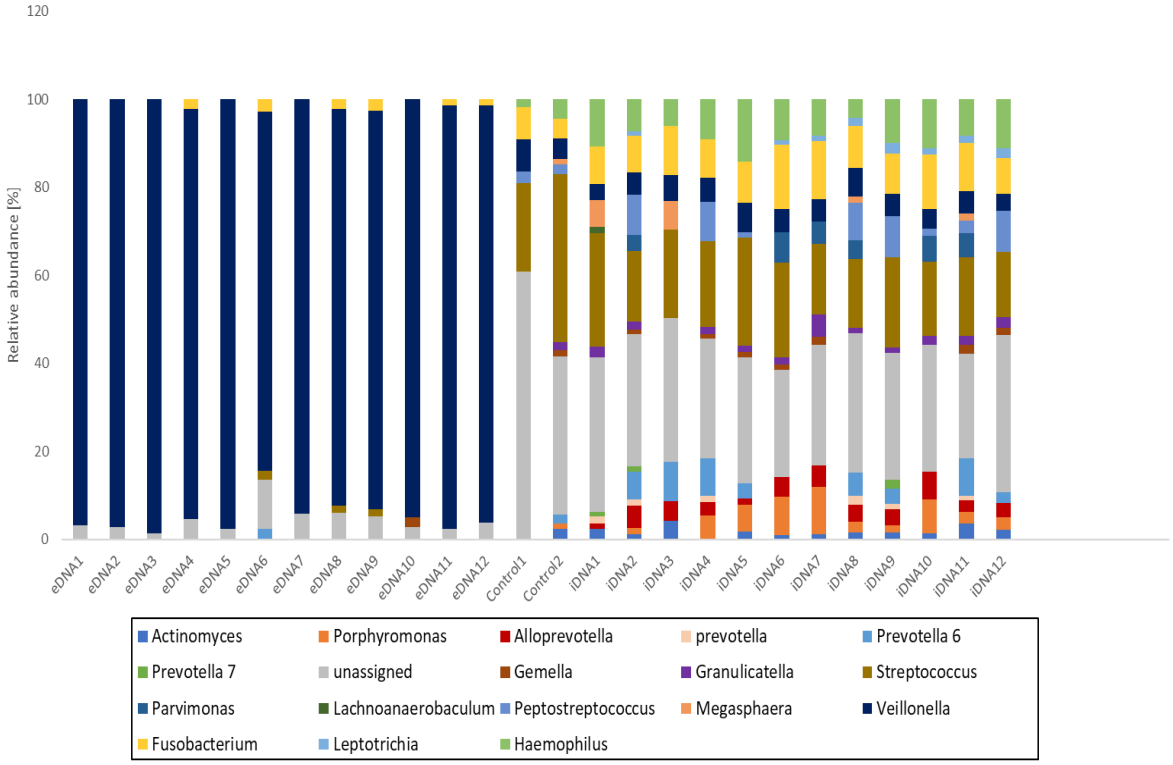

Figure 5.11 The bacterial composition of eDNA and iDNA samples purified from model subgingival biofilms*. The percentages represent the relative abundance of major genera in the samples. Control 1 and control 2 are buffer only samples.*

5.8 The immunostimulatory effect of eDNA

This thesis has shown that eDNA is important for the development and stability of oral biofilms. In this section, it was investigated whether eDNA is also involved in stimulating the host immune response towards these biofilms. To explore this possibility, the immunogenic potential of eDNA extracted from *F. nucleatum* biofilms was assessed on macrophages. Macrophages were exposed to three independent eDNA preparations, and levels of the proinflammatory cytokine tumour necrosis factor-α (TNF-α) were measured using enzyme linked immunosorbent assay (ELISA). LPS from *E. coli* was included as a positive control. As can be seen in Figure 5.12 A, macrophages exposed to two of the three eDNA samples released higher levels of TNF-α than the unstimulated controls. However, eDNA showed less inflammatory potential compared to LPS. These

experiments were repeated later using different eDNA preparations and including eDNA digested with the DNase enzyme NucB as a control to confirm that the stimulating effect observed was caused by eDNA and not by any contaminants in the eDNA samples (e.g LPS). NucB was also included to exclude that any observed effect on the cells being caused by the enzyme. Surprisingly, this time none of the eDNA samples had a stimulating effect on the release of TNF-α although the cells were still appropriately stimulated by LPS (Figure 5.12 B).

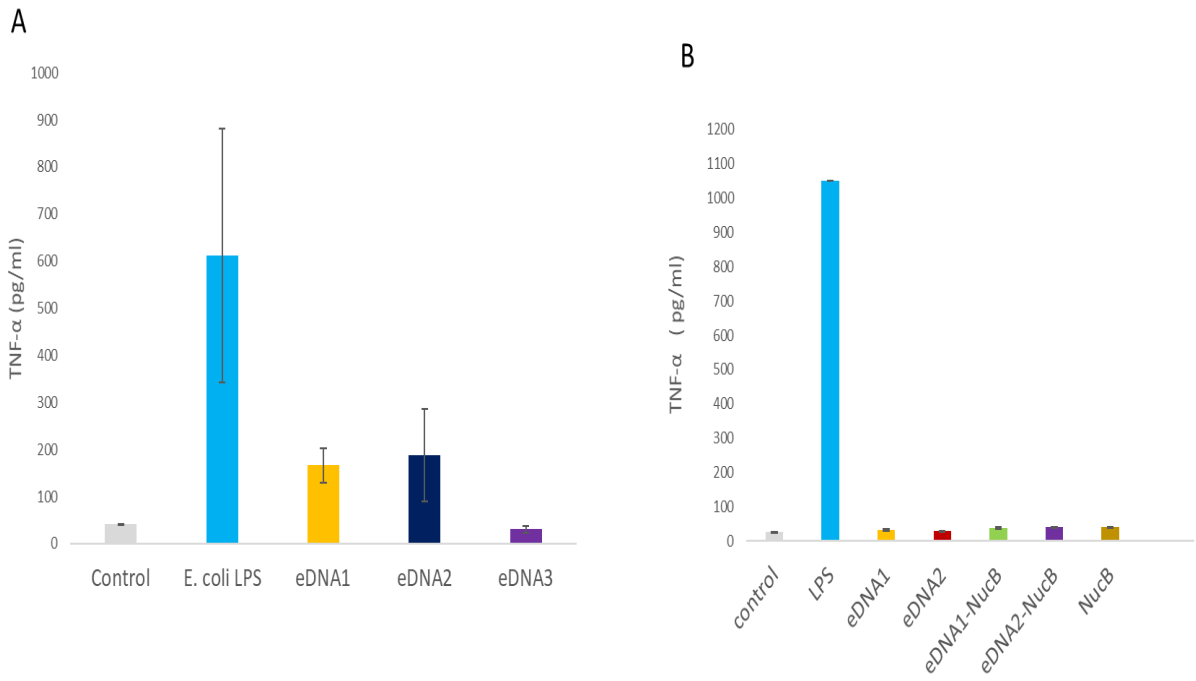

Figure 5.12 The inflammatory response of macrophages to eDNA. *Macrophages were exposed to eDNA, and TNF-a production was measured by ELISA. E. coli LPS is a positive control. (a) TNF-a release was moderately stimulated by two out of the three eDNA samples tested. Bars represent averages from two repeats and standards errors are indicated (B) Experiments were repeated using different eDNA preparations, and digested eDNA samples were included as controls. no stimulation effect was observed for eDNA.*

5.9 Discussion

The role of eDNA in oral biofilms has been widely studied in laboratory monospecies biofilms, however, there is a lack of studies on eDNA in multispecies oral biofilms which are more representative of natural dental plaque. This chapter aimed to characterize eDNA as a component of saliva derived model dental plaque biofilms. Studying eDNA in such polymicrobial community will enhance our understanding of the functions and structure of eDNA in dental plaque and could help evaluate the potential for eDNA to be used as a target to prevent or reduce dental plaque.

The general structure of the supragingival plaque model used here is consistent with what was previously reported in other electron microscopy studies of natural dental plaque (Listgarten et al., 1975, Lie, 1977, Zee et al., 1997) .These studies found that early dental plaque consisted predominantly of cocci with few rod-shaped cells and filaments. Subgingival natural plaque was found to contain diverse morphotypes including cocci, rods, fusiform and spirochetes (Listgarten, 1976, Wecke et al., 2000) which is close but much more complex than the structure observed in the SEM images in this chapter. Extracellular material was also detected covering the bacterial cells in previous SEM studies of natural dental plaque. Candida do not appear to be a common observation in SEM images of natural dental plaque. When fluorescence in situ hybridization (FISH) was employed, Candida was evident in supragingival and not in subgingival natural dental plaque (Zijnge et al., 2010). However, Candida was detected by cultivation of subgingival samples (Canabarro et al., 2013, Dahlén and Wikström, 1995) and metagenomic sequencing (Dabdoub et al., 2016).

When the impact of the DNase enzymes NucB and SsnA on the growth of simulated subgingival biofilms was evaluated, only NucB inhibited the formation of the biofilms. Interestingly, SsnA has proven effective at inhibiting monospecies biofilms of *F. nucleatum* in chapter 4, yet it could not interfere with the development of saliva derived biofilms in this chapter. This might reveal that eDNA of mixed species biofilms has different structure to eDNA released by bacteria in their monospecies biofilms. SsnA has been found in the previous chapter to only digest low molecular weight or single stranded DNA, therefore,

eDNA in mixed species communities is likely to be predominantly double stranded high molecular DNA even in the early stages of biofilm formation. Another likelihood is that double stranded high molecular weight DNA is the key structural form of eDNA in these biofilms. The observed difference in the effect of SsnA and NucB could also be due to the pH or metals present during the biofilm growth being supportive for the activity of NucB rather than SsnA, or the release of SsnA inhibitors by the numerous species present in the biofilm. This observation emphasizes that single species biofilm models might not well represent polymicrobial communities.

While subgingival biofilms were reduced by NucB, supragingival biofilms were not inhibited by either of the enzymes. These observations are consistent with a previous report where NucB was found to strongly inhibit the formation of anaerobic oral biofilms, but had little effect on biofilms grown aerobically in the presence of sucrose (Rostami et al., 2017). One possible explanation for these findings is that eDNA is less abundant or less important in supragingival cariogenic biofilms when compared to subgingival biofilms. Our knowledge of the biofilm matrix composition in supragingival cariogenic biofilms has been based mainly on studies of biofilms of the cariogenic bacteria, *S. mutans*. Polysaccharides, particularly glucans synthesized by *S. mutans* were found to be the major matrix components in these biofilms (Bowen et al., 2018, Cugini et al., 2019). eDNA is also found in cariogenic biofilms, however, how important it is for the development and stabilization of the biofilm is not well understood. Previous studies showed that eDNA is responsible for bacterial cell adhesion and enhancing cariogenic biofilm formation via interactions with glucans (Das et al., 2011, Liao et al., 2014). Another report suggested that eDNA can enhance the formation of glucans independent biofilms of *S.mutans* in low pH conditions (Kawarai et al., 2016). Castillo Pedraza et al. (2017) demonstrated that eDNA acts cooperatively with exopolysaccharide in *S. mutans* biofilms in the early stages of the biofilm development, but as the biofilm matures, eDNA`s role is taken over by lipoteichoic acid (LTA). Recently, targeting LTA during biofilm formation lessened the accumulation of *S. mutans* biofilms and this effect was more pronounced when the LTA treatment was combined with an exopolysaccharide synthesis inhibitor (Castillo Pedraza et al., 2020b). The previous findings are

mostly related to single-species *S. mutans* biofilms, and therefore, not necessarily valid in the mixed- species biofilm model used here. Furthermore, eDNA content was negatively correlated to the biofilm biomass in both single and multispecies cariogenic biofilms suggesting that eDNA is not critical for the biofilm accumulation (Castillo Pedraza et al., 2017). It is possible that eDNA has only a secondary role in building the matrix of cariogenic biofilms with exopolysaccharides being the key component, and therefore, digesting eDNA was not sufficient to prevent the formation of our model biofilms.

NucB could effectively prevent the accumulation of the anaerobic model biofilms in this study suggesting that eDNA is important structural component of subgingival biofilms. Information about the matrix composition of subgingival plaque is scarce, but it is unlikely that polysaccharides make a major component of the subgingival biofilm matrix. This is because bacteria in subgingival biofilm are only exposed to small amounts of sugars from diet to use it as a substrate and produce polysaccharides. Also, subgingival biofilms are rich in asaccharolytic bacteria which cannot metabolise carbohydrates. Still, some biofilm polysaccharides are not synthesised from dietary sugars. An example of these is PNAG which is a GlcNAc polymer produced by the subgingival bacteria *A. actinomycetemcomitans* (Izano et al., 2008b). One recent study developed an *in vitro* mixed species biofilm model that is gradually converted from supragingival to subgingival by changing the growth environment from aerobic to anaerobic conditions. In this study, exopolysaccharides decreased as the biofilm grew from the aerobic to the microaerophilic stage and became undetectable in the anaerobic biofilms (Thurnheer et al., 2016). Here, preformed model plaque biofilms were not removed by NucB or SsnA regardless of whether they were cultured to mimic supra or subgingival dental plaque. This might have occurred because eDNA is less abundant or less accessible in mature biofilms perhaps due to its replacement by or forming complexes with other components of the matrix such as exopolysaccharides, LTA and proteins (Castillo Pedraza et al., 2017, Rocco et al., 2017, Peng et al., 2020a)

The dependence of supra and subgingival model biofilms on eDNA was further investigated by assessing the presence of eDNA in their matrices. eDNA purified from both biofilms was quantifiable by NanoDrop spectrophotometry, however,

only eDNA of anaerobic biofilms was visible on agarose gels. According to these observations, it is likely that subgingival biofilms contain higher levels of eDNA than supragingival biofilms, which could contribute to subgingival biofilms being more sensitive to the DNase enzyme NucB. Interestingly, eDNA from aerobic biofilms showed high concentrations in spectrophotometry which could imply that eDNA might be abundant in supragingival biofilms, but it is of low molecular weight. Biofilms were found to require high molecular weight DNA to provide structural support (Izano et al., 2008a). This might again clarify why the growth of the aerobic biofilms was not affected by the presence of NucB. It is unclear if the environmental conditions can affect the production of eDNA in biofilms. The levels of eDNA in *S. mutans* biofilms were dependent on the amount and type of sugars in the growth medium (Kim et al., 2018). Recently, Ramirez et al. (2019) have found that biofilms grown under aerobic conditions contain greater amounts of eDNA compared to anaerobic biofilms. However, those were monospecies biofilms and therefore the observed effects might be species-specific.

The results in this chapter demonstrated that proteins are abundant in aerobic biofilms when compared to anaerobic biofilms, which once more highlights a distinct compositional difference between the supra and subgingival model biofilms evaluated here. Proteins have long been recognised as important biofilm components along with exopolysaccharides and eDNA. Cariogenic biofilms were found to contain several amyloid forming proteins that have significant effects on the biofilm development and integrity (Besingi et al., 2017, Chen et al., 2019a). DNABII proteins have also been observed in association with eDNA within the biofilm matrix of several oral bacteria including *S. intermedius* (Nur et al., 2013) *S. gordonii*, and *P. gingivalis* (Rocco et al., 2017) (Rocco et al., 2018). These proteins are thought to stabilize and maintain the integrity of the biofilm matrix and could shield eDNA from the action of DNase enzymes. Karygianni et al. (2020) have shown that the density of 6 species cariogenic biofilm was substantially reduced by the combined presence of DNase I and proteinase K during biofilm formation when compared to biofilms grown with DNase I or proteinase K alone.

The work in this chapter also investigated whether specific bacterial genera in subgingival biofilms are more responsible for the release of eDNA than others.

Identifying these genera might enhance our knowledge of eDNA`s functions in dental plaque. The microorganism diversity of iDNA and eDNA extracted from model subgingival biofilms was explored via 16S rRNA gene sequencing. The sequencing data analysis revealed a substantially different constitution between eDNA and iDNA. eDNA was predominantly released by *Veillonella* which in contrast made a small proportion of iDNA composition. *Veillonella* species are early colonizers that were previously reported to have a vital role in multispecies biofilm formation (Periasamy and Kolenbrander, 2010). Although there is no information available in the literature regarding the production of eDNA in *Veillonella* biofilms, the export of vesicle-associated DNA is prevalent among gram negative bacteria such as Veillonella (Dorward and Garon, 1990b). The pioneer colonizers of dental plaque, streptococci, constituted large proportion of iDNA. However, very low quantities of eDNA, and just in few samples, were released by this genus indicating that streptococcal species might not be key producers of eDNA when colonising mixed species biofilms. In fact, streptococci are well known for possessing multiple adhesins that facilitate their attachment to tooth surface as well as their interactions with other species in the dental biofilm, and therefore, they may not require to release large quantities of eDNA. Yet, eDNA release was demonstrated in several streptococcal species when growing in monocultures (Kreth et al., 2009b, Itzek et al., 2011b, Xu and Kreth, 2013b). Interestingly, *F. nucleatum* which have shown to produce large amounts of eDNA in their biofilms in chapter 1, released very small proportions of eDNA in this polymicrobial environment. This variation of eDNA production between single ad multispecies bacterial biofilms was previously reported for other bacterial species (Steinberger and Holden, 2005). The contribution of different bacteria to eDNA in multispecies biofilms might be influenced by several factors such as variations in growth rate, interbacterial interactions and different rate of cell death and lysis. It is worth to note that the composition of eDNA and iDNA was not donor dependent as the microbial composition was consistent throughout the samples despite the biofilms being derived from various saliva inoculums.

To give more insight into the functions of eDNA in dental plaque, the work here examined the ability of eDNA from *F. nucleatum* biofilms to induce an inflammatory response from macrophages. Considering that DNA inside the

bacterial cells can stimulate host immune response, DNA present in the extracellular matrix of biofilms is expected to have similar immunogenic potential. Ideally, eDNA purified from the model plaque biofilms should have been used in these experiments, however, it was difficult to obtain sufficient concentrations . Ramirez et al. (2019) assessed the inflammatory potential of eDNA from *Enterococcus faecalis* monospecies biofilms on macrophages. The authors found that eDNA stimulated low-grade inflammatory response when compared to planktonic bacteria and LPS. Similarly, the results of our initial experiments showed that eDNA can elicit moderate response from macrophages as demonstrated by promoting the release of the proinflammatory cytokine TNF-α. However, in our second set of experiments, eDNA had no effect on the secretion of TNF-α. This might have occurred because the eDNA samples were not as pure as the samples used in the initial experiments or due to a technical issue. Therefore, further work is required to clarify the immunogenic potential of eDNA in dental plaque and its relationship with the pathogenesis of periodontitis.

Overall, this chapter explored the functions of eDNA in supra and subgingival biofilms in terms of its importance for the formation and stability of the biofilms, and its role in stimulating the release of destructive cytokines from immune cells. It also investigated whether particular species are more responsible for the release of eDNA in the biofilm. The results here demonstrated that eDNA is an important structural component of subgingival biofilms and highlighted that eDNA structure in the mixed species plaque biofilm is likely to be different to what is usually released in single species biofilm models. The contribution of different oral bacteria to eDNA production needs further studies, ideally on natural dental plaque.

Chapter 6. General Discussion and Future Work

6.1 General Discussion

This work characterised a novel oral microbial DNase and showed that its DNA digestion and anti-biofilm activity was distinct from another bacterial DNase that has been explored for biofilm control. In addition, the work started to characterise the role of microbial eDNA in more complex situations such as mixed-species biofilms and the presence of host cells.

F. nucleatum was selected to form a model biofilm to test the effect of DNase enzymes as it is known to act as a bridging biofilm component by binding early and late colonizers of dental plaque thereby promoting biofilm maturation (Kolenbrander et al., 2010, Guo et al., 2014, Brennan and Garrett, 2019a). Digesting eDNA could be a useful approach in reducing *F. nucleatum* colonization and consequently reduce dental plaque. *F. nucleatum* was found to consistently form thick biofilms that seemed to be abundant in eDNA, and therefore, was a suitable model to assess the efficacy of the DNase enzymes NucB and SsnA on oral biofilm formation and stability. Yet, *F. nucleatum* contributed to very low percentage of eDNA purified from model plaque biofilms in chapter 5. eDNA release and dependence might be strain-dependent since some strains may have a strong requirement for eDNA, whereas others may utilize different approaches for adhesion and biofilm formation. Therefore, future work would need to screen multiple strains of these species. The dependence of *F. nucleatum* on eDNA might also vary according to the growth conditions. A previous study that employed the same strain of *F. nucleatum,* but with different culture medium reported low quantities of eDNA in biofilms of *F. nucleatum* and no effect of DNase I on the biofilm structure (Ali Moh*a*mmed et al., 2013*)*. Therefore, it is not clear how important is eDNA for the colonization and biofilm formation of *F. nucleatum* in their natural growing environment in the oral cavity. Also, *F. nucleatum*, is typically present within a mixed biofilm population *in vivo*, thus, it is not known whether DNases would have the same effect on *F. nucleatum* when it is participating in so many bacterial interactions. Additionally, it is unknown whether disrupting these species alone would disrupt the whole biofilm.

However, using *F. nucleatum* biofilms as a model provided a great opportunity to test and compare NucB and SsnA effects on eDNA rich biofilms formed by oral bacteria.

As this thesis is focussed on the role of eDNA in the plaque biofilm and the subsequent importance of targeting eDNA, investigating the effect of NucB on periodontal bacteria was pursued further. Some periodontal bacteria were previously found to decrease when NucB was present during the growth of salivary microcosms (Rostami et al., 2017). Therefore, it was hypothesized that eDNA could have a role in the integration of these bacteria into the dental biofilm. Among these species, *P. gingivalis* was chosen for investigation due to its well-known role as a keystone pathogen, and therefore, its special importance in the pathogenesis of periodontitis. Attempts were first made to test the effect of NucB on monospecies biofilms of *P. gingivalis*, however, these were thin and therefore difficult to assess. *P. gingivalis* is a late colonizer that is usually incorporated into the dental biofilm by binding to early colonizers commonly streptococci (Demuth et al., 2001, Lamont et al., 2002). It was, therefore, decided to use a dual species biofilm model of *P. gingivalis* and *S. gordonii* to test the dependence of *P. gingivalis* on eDNA. NucB treatment did not reduce the colonization or the persistence of *P. gingivalis* into the dual species biofilm with *S. gordonii*. *S. gordonii* were also not released from the biofilm following NucB treatment. Possible explanations for these observations were discussed in chapter 3. Of note, *S. gordonii* produces extracellular nuclease which could digest and reduce the amount of eDNA prior to introducing *P. gingivalis* making it difficult to see any obvious effect for NucB. To investigate this prospect, *S. gordonii* in this biofilm model could be replaced in the future by an isogenic mutant lacking DNase or other early colonizers that do not produce DNases. Gödeke et al. (2011) found that the amount of eDNA in biofilms of a nuclease mutant of Shewanella was significantly higher than that of the wild type. Additionally, the observed differences in the effects of NucB between *F. nucleatum* on one side and *S. gordonii* and *P. gingivalis* on the other side might reflect differing approaches to the formation and maintenance of biofilms among different oral species.

Several oral bacteria produce DNase enzymes, but whether these DNases have biofilm degrading functions within the dental plaque is yet to be clear. If these native DNases have biofilm degrading properties, there might be an opportunity to develop a strategy to modulate them and control dental plaque. SsnA, a DNase produced by *S. gordonii* was investigated here to provide an initial insight into this potential. A monospecies biofilm model of *F. nucleatum* was employed to test the antibiofilm effect of SsnA, hence, a comparison with NucB was possible. SsnA was able to inhibit the formation of the biofilm, but in contrast to NucB, it showed no obvious effects on preformed biofilms. This observation has led to investigating the DNase activity of both enzymes against various DNA substrates. It is well known that different nucleases can have different substrate specificities and preference. For example, two extracellular nucleases form *V. cholerae* appear to have different specificity for DNA substrates as one is capable of degrading circular and linearized plasmid DNA, whereas the other can only digests linearized DNA (Seper et al., 2011). Also, SWAN from *S. sanguinis* shows superiority at digesting single-stranded DNA (Morita et al., 2014). To use an enzyme for biofilm control, it is important to identify the key enzyme characteristics that correspond to good biofilm control. Here, NucB was shown to have stronger activity against complex double-stranded DNA, whereas SsnA appeared to have better activity against the single stranded substrate. Bovine DNase I which has proven to be effective as an antibiofilm enzyme, just like NucB, prefers degrading double-stranded substrates (Suck, 1994). Taken together, these findings suggest that the antibiofilm activity of DNases is positively correlated to their ability to hydrolyse double stranded DNA. This hypothesis was further emphasized in chapter 5 where SsnA lacked any antibiofilm effect with model plaque biofilms, while NucB maintained its high capacity at preventing biofilm formation. Whether DNases produced by other bacteria in dental plaque possess the same preference as SsnA for single stranded DNA is currently unknown, but if demonstrated by future investigations, it could indicate specific functions for these enzymes in the dental plaque.

The superior efficiency of NucB against the model plaque biofilms used in this project might imply that eDNA in dental plaque is largely double stranded DNA or that this type of DNA is the one critical for biofilm structure/scaffold. The

form of eDNA released in the biofilm matrix is likely to be reliant on the mechanism of eDNA release. For instance, single stranded DNA was shown to be released by a type IV secretion system (Zweig et al., 2014). It is anticipated that most eDNA in early biofilm growth is actively secreted since cell lysis should not yet be significant. However, this concept might be more relevant to single species biofilms than multiple species communities as the vast interbacterial interactions taking place could affect the rate of growth and cell death and lysis. The presence of different forms of eDNA in the extracellular matrix of multispecies biofilms could also be influenced by the different constituted species. Mature biofilms of *Sulfolobus acidocaldarius* were found to contain a mixture of both single and double stranded DNA, whereas only double stranded DNA was detected in *N. gonorrhoeae* biofilms (Zweig et al., 2014). Information about mechanisms of eDNA release are currently based on studies of single species biofilm models, however, the process is likely to be more complex in multispecies biofilms such as dental plaque, and hence requires further investigations. In the future, the forms of eDNA present in model plaque biofilms could be identified using the same methodology as employed by Zweig et al. (2014). The authors used fluorescently labelled ssDNA and ss/dsDNA-binding proteins to visualize ssDNA and total DNA in biofilms. This information could be valuable when selecting which DNases are best to target eDNA in dental plaque.

While the differing substrate specificity is one property that could be contributing to the observed distinct antibiofilm activity of NucB and SsnA. Other properties that could affect the DNase activity such as the pH range and the thermal stability also appear to be variable between the two enzymes. NucB was found to be a thermally robust enzyme that regain its activity after a heat cool cycle (Baslé et al., 2017). In this project, NucB was still active after heating at 100˚C for up to 60 min, whereas heating SsnA at 80˚C for 25 min was sufficient to completely inactivate the enzyme. To the best of my knowledge, the pH range of NucB activity has not yet been determined. However, the homologous protein NucB from *Bacillus subtilis s*hows high activity over the range pH 6.5-8.0, with an optimal pH of 7.5 and rapid drop-off in activity above pH 8 (Akrigg, 1978). On the other hand, work by our lab group has shown that SsnA exhibits its activity

over the range pH 6.5-9.5, with a rapid inhibition of activity between pH 5.5 and 4.5.

This work has shed light on specific situations where the use of DNases for biofilm control is favoured. NucB appears to be specifically useful for reducing subgingival plaque as indicated by its exclusive effect on anaerobic biofilms in chapter 5. On the other hand, the aerobic biofilms which represent supragingival plaque were not affected. Additionally, early plaque biofilms are likely to be more susceptible to degradation by DNases than mature biofilms as NucB only reduced biofilm formation but could not disperse mature plaque biofilms. These observations suggest that eDNA is an important structural constituent of subgingival plaque, but it might be more important or more accessible to degradation during the early phases of biofilm formation. Consequently, DNases are likely to be useful as a preventive strategy to reduce the accumulation of subgingival dental plaque or as an adjunctive periodontal therapy after the mechanical removal of subgingival plaque. An effective delivery system for the enzyme to the subgingival area is, therefore, of great importance. Methods used for the local delivery of antimicrobial agents in periodontal therapy including fibres, films, microparticles or gels could be valuable. Furthermore, early data form this project indicates that there may be some sensing of eDNA by host cells. There is evidence that sensing of microbial nucleic acids by innate immune cells is a unique inflammatory pathway in periodontal disease pathogenesis (Crump and Sahingur, 2015). If eDNA is proven to possess immunostimulatory effects, it could represent a new therapeutic target in the treatment of periodontitis. DNase enzymes could then be utilized to modulate the host response and reduce inflammation which has recently been emphasized as a key factor in the early pathogenesis of periodontitis (Bartold and Van Dyke, 2019). However, the results here were inconsistent and therefore further work is required to clarify this aspect of eDNA.

Another area where DNases could be valuable is enhancing the penetration of the dental biofilm by antimicrobials and oral health care agents. This work has shown that NucB made *F. nucleatum* biofilms more fragmented, an effect that could be of importance in improving the diffusion of therapeutics to the tooth surface. It is well known that dental plaque is poorly penetrated by therapeutic

agents such as triclosan and fluoride (Robinson, 2011). This presents an obstacle to preventing oral infections as well as increasing tooth tissue resistance to dental caries. Future work could investigate the use of NucB as a mean of facilitating the mass transport of therapeutics through oral biofilms. Additionally, the surface area of the biofilms was reduced with NucB treatment. This could have implications on the physiology of the biofilm as the surface area of a biofilm reflects the number of biofilm cells exposed to the bulk-liquid phase. Exposed biofilm cell layers usually show greater metabolic activity than cells in the biofilm centre (Okabe et al., 1996, Neu and Lawrence, 1997, Werner et al., 2004, Rani et al., 2007, Bester et al., 2011). Also, the decrease in the surface area of the biofilm might lessen the uptake of nutrients from the bulk-liquid (Battin et al., 2003). Consequently, NucB might not only reduce the bioburden of biofilms but also render what remained of the biofilm physiologically less active. Currently, antibiotics are recommended as an adjunctive therapy in severe periodontal disease (Pretzl et al., 2019). However, the administration of systemic antibiotics is known to be associated with the risk of antibiotic resistance that affect the entire human microbiome. The effect of NucB on the biofilm physiology imply that the biofilm cells are likely to be more susceptible to antibiotic therapy if the treatment is combined with DNases, hence, a lower dose of antibiotics is required. The effect of DNases on increasing the susceptibility of biofilms to antimicrobials has been reported in several studies (Waryah et al., 2017, Kaplan et al., 2018, Yu et al., 2019).

Identifying the major genera responsible for eDNA release in the dental biofilm could reveal unknown functions of eDNA and provide information on which organisms are likely to be targeted by DNases. The microorganism diversity of eDNA extracted from saliva derived biofilms here was noticeably different from that of iDNA from the same biofilms. eDNA from different species could have different functions. For example, in some species including *S. gordonii*, the release of eDNA appears to be related to the development of competence, while a relationship between eDNA production and motility has been highlighted in others (Ibáñez de Aldecoa et al., 2017). Here, the genus *Veillonella* made the most contribution to eDNA in the biofilm matrix. There is no information with regards to eDNA production by *Veillonella* in the literature, but this species is

known to play a crucial role in multispecies community formation (Periasamy and Kolenbrander, 2010). The extensive release of eDNA by *Veillonella* could be related to their ability to perform this role. One important finding is that eDNA release appears to be influenced by the growth condition. For instance, streptococci which constituted most of the biofilm composition here are known to be substantial eDNA producers when grown in monoculture (Kreth et al., 2009b, Itzek et al., 2011b, Xu and Kreth, 2013b), however, they only released very low amounts in this multispecies environment. Therefore, work investigating the composition of eDNA extracted from natural dental plaque is needed. Natural dental plaque could be scraped off extracted teeth and the microbial composition of eDNA purified from this plaque can be analysed.

The results of the SsnA activity assays in chapter 4 provided interesting information regarding a potential role for SsnA in DNA uptake by *S. gordonii*. Instead of digesting a circular plasmid, SsnA converts it to open circle and linear DNA, in a similar way to EndA of *Streptococcus pneumoniae and* NucA of *Bacillus subtilis* which are well known for their role in genetic competence (Lacks et al., 1974, Lacks et al., 1975, Puyet et al., 1990, Provvedi et al., 2001). The potential roles for SsnA in DNA uptake were discussed in chapter 4. Of note, only a single strand of DNA (the transformation strand) is pulled into the cell during DNA uptake while the complementary strand is degraded extracellularly. SsnA might be responsible for digesting this remaining DNA strand considering that SsnA is most efficient at degrading single stranded DNA. In this way, SsnA could also help *S. gordonii* obtain the necessary nutrient supply of carbon, nitrogen and phosphorous which make up the backbone of DNA. SsnA was downregulated when an optimal source of sugar is available for *S. gordonii* (Robert shields, PhD thesis) emphasizing the hypothesis that it is involved in providing *S. gordonii* with nutrients. However, further studies are required to verify this potential.

In conclusion, the role of eDNA as a structural support for oral biofilms and the potential of controlling dental plaque using DNase enzymes were supported by this work. Whether DNases produced naturally by oral bacteria can be modulated to help control dental plaque remains to be elucidated. In this project, SsnA from *S. gordonii* had an inhibitory antibiofilm effect against single species biofilms, but it lacked this effect with mixed species oral biofilms. Likewise, SsnA showed

effective DNA degradation action only against single stranded and low molecular weight double stranded DNA providing an indication that high molecular weight DNA double stranded is the predominant or the key structural form of eDNA in multispecies oral biofilms. This information could help identify which DNases can reduce dental plaque, however, more work needs to be done to fully understand this prospective. In the future, the antibiofilm activity and DNA specificity of other native DNases in the dental biofilm could be investigated to provide clearer insight into whether modulating native DNases could be utilized to reduce or control dental plaque.

6.2 Future work

Future work should focus further on what can be achieved with bacterial DNases in terms of controlling dental plaque and combating periodontitis and dental caries. The effects of DNases were examined here primarily using monospecies biofilms of only one strain of *F. nucleatum,* and therefore, investigating the sensitivity of other strains and other oral species is important. Also, the mixed species biofilms used here were *in vitro* biofilms in which bacteria probably use different strategies to what they naturally use to produce and utilize eDNA. The role of eDNA in oral biofilm formation and stability is likely to be affected by host factors which were absent in the models used in this project. Therefore, the results obtained here should be verified in more natural settings. This would confirm that the observations seen in this work were not the consequences of the conditions under which the experiments were performed. A first step toward studying eDNA in more natural conditions would be evaluating the effect of NucB and SsnA on biofilms grown in situ or in animal models. Schlafer et al. (2017) tested the effect of DNase I on biofilms grown on custom-made glass slabs mounted on the buccal flanges of individually designed lower jaw splints. This in situ model could similarly be used in the future to test the effect of NucB and SsnA. The safety aspect of DNases is also an issue of paramount importance as host toxicity is among the obstacles facing the transfer of many antibiofilm therapies form *in vitro* assays into clinical use. Gingival epithelial or fibroblastic cell lines could be employed to test the effect of different concentrations of DNases on host cells viability.

Throughout this work, it was emphasized that extracellular proteins could be utilized by oral bacteria to support the structural integrity of eDNA and consequently biofilms. Model supragingival biofilms, which were demonstrated to be rich in proteins in comparison to the subgingival one, were resistant to the action of NucB. Also, *P. gingivalis* and *S. gordonii,* both are known to have DNA binding proteins within their biofilm matrices, were not released from the biofilm when treated with NucB. Therefore, targeting eDNA-protein interactions is likely to play a crucial role in enhancing the sensitivity of oral biofilms to DNases. Antibodies derived against the *P. gingivalis* DNABII protein, Huß and *S. gordonii* DNABII protein Hu reduced monospecies biofilms of these species (Rocco et al., 2017). Combining NucB with antibodies against DNA binding proteins could have augmenting effects on biofilm reduction. Future work could also investigate the effect of antibodies against DNA binding proteins on model supra- and subgingival biofilms using the same methodology of Rocco et al. (2017).

Expanding our knowledge of DNases originating from oral bacteria is important. SsnA demonstrated weak antibiofilm effect when compared to NucB suggesting that DNases present naturally in dental plaque might have less capacity against oral biofilms than DNases from non-oral bacteria. Future projects could investigate this hypothesis and compare the antibiofilm and DNase activity of NucB to that of DNases produced by other oral bacteria. SsnA Also exhibited distinct DNase activity and varying specificities for different forms of DNA, an observation that might be related to functions of this DNase in *S. gordonii* and in dental plaque.

Finally, this research showed that the activity of bacterial DNases may differ depending on the producing species. Future studies should therefore find out if there are any differences between the activity of DNases produced by bacteria classified as pathogens and those come from commensal oral bacteria. Such differences could reveal the contribution of native DNases to pathogenicity. Of interest is the effect of native DNases on NETs which are thought to play a crucial defence role against bacterial invasion and were abundant in disease periodontal sites (Vitkov et al., 2009). DNases from periodontal bacteria were reported to degrade NETs suggesting that these DNases might be associated with pathogenicity (Palmer et al., 2012). Inhibitors of bacterial DNases have been

recently developed to reduce the virulence of species such as *S. pyogenes* and *S. pneumoniae* (Sharma et al., 2019). The DNase activity of periodontal bacteria could be inhibited in a similar way to treat periodontitis. However, the impairment of NETs clearance in inflammatory diseases including periodontitis could also contribute to tissue damage by triggering an autoimmune response (White et al., 2015). The modulation of DNase activity in dental plaque is therefore an area of future investigations and could contribute to the development of alternative therapeutic approaches for periodontitis.

References:

ACKART, D. F., HASCALL-DOVE, L., CACERES, S. M., KIRK, N. M., PODELL, B. K., MELANDER, C., ORME, I. M., LEID, J. G., NICK, J. A. & BASARABA, R. J. 2014. Expression of antimicrobial drug tolerance by attached communities of Mycobacterium tuberculosis. *Pathogens and Disease,* 70**,** 359-369.

AKRIGG, A. 1978. Purification and properties of a manganese-stimulated deoxyribonuclease produced during sporulation of Bacillus subtilis. *Biochemical Journal,* 172**,** 69-76.

ALAKOMI, H. L., SKYTTÄ, E., SAARELA, M., MATTILA-SANDHOLM, T., LATVA-KALA, K. & HELANDER, I. M. 2000. Lactic Acid Permeabilizes Gram-Negative Bacteria by Disrupting the Outer Membrane. *Applied and Environmental Microbiology,* 66**,** 2001.

ALI MOHAMMED, M. M., NERLAND, A. H., AL-HARONI, M. & BAKKEN, V. 2013. Characterization of extracellular polymeric matrix, and treatment of Fusobacterium nucleatum and Porphyromonas gingivalis biofilms with DNase I and proteinase K. *Journal of Oral Microbiology,* 5**,** 20015.

ALLESEN-HOLM, M., BARKEN, K. B., YANG, L., KLAUSEN, M., WEBB, J. S., KJELLEBERG, S., MOLIN, S., GIVSKOV, M. & TOLKER-NIELSEN, T. 2006. A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms. *Molecular Microbiology,* 59**,** 1114-1128.

BAELO, A., LEVATO, R., JULIÁN, E., CRESPO, A., ASTOLA, J., GAVALDÀ, J., ENGEL, E., MATEOS-TIMONEDA, M. A. & TORRENTS, E. 2015. Disassembling bacterial extracellular matrix with DNase-coated nanoparticles to enhance antibiotic delivery in biofilm infections. *Journal of Controlled Release,* 209**,** 150-158.

BARNES, A. M. T., BALLERING, K. S., LEIBMAN, R. S., WELLS, C. L. & DUNNY, G. M. 2012. Enterococcus faecalis Produces Abundant Extracellular Structures Containing DNA in the Absence of Cell Lysis during Early Biofilm Formation. *mBio,* 3**,** e00193-12.

BARTOLD, P. M. & VAN DYKE, T. E. 2019. An appraisal of the role of specific bacteria in the initial pathogenesis of periodontitis. *Journal of Clinical Periodontology,* 46**,** 6-11.

BASLÉ, A., HEWITT, L., KOH, A., LAMB, H. K., THOMPSON, P., BURGESS, J. G., HALL, M. J., HAWKINS, A. R., MURRAY, H. & LEWIS, R. J. 2017. Crystal structure of NucB, a biofilm-degrading endonuclease. *Nucleic Acids Research,* 46**,** 473-484.

BATTIN, T. J., KAPLAN, L. A., NEWBOLD, J. D., CHENG, X. & HANSEN, C. 2003. Effects of current velocity on the nascent architecture of stream microbial biofilms. *Applied and environmental microbiology,* 69**,** 5443-5452.

BEENKEN, K. E., SPENCER, H., GRIFFIN, L. M. & SMELTZER, M. S. 2012. Impact of Extracellular Nuclease Production on the Biofilm Phenotype of

Staphylococcus aureus under In Vitro and In Vivo Conditions. *Infection and Immunity,* 80**,** 1634.

BEITER, K., WARTHA, F., ALBIGER, B., NORMARK, S., ZYCHLINSKY, A. & HENRIQUES-NORMARK, B. 2006. An Endonuclease Allows Streptococcus pneumoniae to Escape from Neutrophil Extracellular Traps. *Current Biology,* 16**,** 401-407.

BERNABE, E., MARCENES, W., HERNANDEZ, C. R., BAILEY, J., ABREU, L. G., ALIPOUR, V., AMINI, S., ARABLOO, J., AREFI, Z., ARORA, A., AYANORE, M. A., BÄRNIGHAUSEN, T. W., BIJANI, A., CHO, D. Y., CHU, D. T., CROWE, C. S., DEMOZ, G. T., DEMSIE, D. G., DIBAJI FOROOSHANI, Z. S., DU, M., EL TANTAWI, M., FISCHER, F., FOLAYAN, M. O., FUTRAN, N. D., GERAMO, Y. C. D., HAJ-MIRZAIAN, A., HARIYANI, N., HASANZADEH, A., HASSANIPOUR, S., HAY, S. I., HOLE, M. K., HOSTIUC, S., ILIC, M. D., JAMES, S. L., KALHOR, R., KEMMER, L., KERAMATI, M., KHADER, Y. S., KISA, S., KISA, A., KOYANAGI, A., LALLOO, R., LE NGUYEN, Q., LONDON, S. D., MANOHAR, N. D., MASSENBURG, B. B., MATHUR, M. R., MELES, H. G., MESTROVIC, T., MOHAMMADIAN-HAFSHEJANI, A., MOHAMMADPOURHODKI, R., MOKDAD, A. H., MORRISON, S. D., NAZARI, J., NGUYEN, T. H., NGUYEN, C. T., NIXON, M. R., OLAGUNJU, T. O., PAKSHIR, K., PATHAK, M., RABIEE, N., RAFIEI, A., RAMEZANZADEH, K., RIOS-BLANCAS, M. J., RORO, E. M., SABOUR, S., SAMY, A. M., SAWHNEY, M., SCHWENDICKE, F., SHAAHMADI, F., SHAIKH, M. A., STEIN, C., TOVANI-PALONE, M. R., TRAN, B. X., UNNIKRISHNAN, B., VU, G. T., VUKOVIC, A., WAROUW, T. S. S., ZAIDI, Z., ZHANG, Z. J. & KASSEBAUM, N. J. 2020. Global, Regional, and National Levels and Trends in Burden of Oral Conditions from 1990 to 2017: A Systematic Analysis for the Global Burden of Disease 2017 Study. *Journal of Dental Research,* 99**,** 362-373.

BESINGI, R. N., WENDERSKA, I. B., SENADHEERA, D. B., CVITKOVITCH, D. G., LONG, J. R., WEN, Z. T. & BRADY, L. J. 2017. Functional amyloids in Streptococcus mutans, their use as targets of biofilm inhibition and initial characterization of SMU_63c. *Microbiology,* 163**,** 488-501.

BESTER, E., KROUKAMP, O., HAUSNER, M., EDWARDS, E. A. & WOLFAARDT, G. M. 2011. Biofilm form and function: carbon availability affects biofilm architecture, metabolic activity and planktonic cell yield. *Journal of Applied Microbiology,* 110**,** 387-398.

BINNENKADE, L., KREIENBAUM, M. & THORMANN, K. M. 2018. Characterization of ExeM, an Extracellular Nuclease of Shewanella oneidensis MR-1. *Frontiers in Microbiology,* 9.

BOS, R., VAN DER MEI, H. C. & BUSSCHER, H. J. 1999. Physico-chemistry of initial microbial adhesive interactions – its mechanisms and methods for study. *FEMS Microbiology Reviews,* 23**,** 179-230.

BOWEN, W. H., BURNE, R. A., WU, H. & KOO, H. 2018. Oral Biofilms: Pathogens, Matrix, and Polymicrobial Interactions in Microenvironments. *Trends in Microbiology,* 26**,** 229-242.

BOWEN, W. H. & KOO, H. 2011. Biology of Streptococcus mutans Derived Glucosyltransferases: Role in Extracellular Matrix Formation of Cariogenic Biofilms. *Caries Research,* 45**,** 69-86.

BRADSHAW, D., MARSH, P., WATSON, G. & ALLISON, C. 1997. Oral anaerobes cannot survive oxygen stress without interacting with facultative/aerobic species as a microbial community. *Lett Appl Microbiol,* 25**,** 385-387.

BRADSHAW, D. J., HOMER, K. A., MARSH, P. D. & BEIGHTON, D. 1994. Metabolic cooperation in oral microbial communities during growth on mucin. *Microbiology,* 140**,** 3407-3412.

BRADSHAW, D. J., MARSH, P. D., WATSON, G. K. & ALLISON, C. 1998. Role of Fusobacterium nucleatum and Coaggregation in Anaerobe Survival in Planktonic and Biofilm.Oral Microbial Communities during Aeration. *Infection and Immunity,* 66**,** 4729.

BRECX, M., RÖNSTRÖM, A., THEILADE, J. & ATTSTRÖM, R. 1981. Early formation of dental plaque on plastic films. *Journal of Periodontal Research,* 16**,** 213-227.

BRENNAN, C. A. & GARRETT, W. S. 2019a. Fusobacterium nucleatum -- symbiont, opportunist and oncobacterium. *Nature Reviews Microbiology,* 17**,** 156.

BRENNAN, C. A. & GARRETT, W. S. 2019b. Fusobacterium nucleatum - symbiont, opportunist and oncobacterium. *Nature reviews. Microbiology,* 17**,** 156-166.

BROWN, J. L., YATES, E. A., BIELECKI, M., OLCZAK, T. & SMALLEY, J. W. 2018. Potential role for Streptococcus gordonii-derived hydrogen peroxide in heme acquisition by Porphyromonas gingivalis. *Molecular Oral Microbiology,* 33**,** 322-335.

BUI, F. Q., ALMEIDA-DA-SILVA, C. L. C., HUYNH, B., TRINH, A., LIU, J., WOODWARD, J., ASADI, H. & OJCIUS, D. M. 2019. Association between periodontal pathogens and systemic disease. *Biomedical Journal,* 42**,** 27-35.

BUSSCHER, H. J., NORDE, W. & VAN DER MEI, H. C. 2008. Specific Molecular Recognition and Nonspecific Contributions to Bacterial Interaction Forces. *Applied and Environmental Microbiology,* 74**,** 2559.

BUSSCHER, H. J. & VAN DER MEI, H. C. 1997. Physico-Chemical Interactions in Initial Microbial Adhesion and Relevance for Biofilm Formation. *Advances in Dental Research,* 11**,** 24-32.

BYRNE, S. J., DASHPER, S. G., DARBY, I. B., ADAMS, G. G., HOFFMANN, B. & REYNOLDS, E. C. 2009. Progression of chronic periodontitis can be predicted by the levels of Porphyromonas gingivalis and Treponema denticola in subgingival plaque. *Oral Microbiology and Immunology,* 24**,** 469-477.

CAMPOCCIA, D., MIRZAEI, R., MONTANARO, L. & ARCIOLA, C. R. 2019. Hijacking of immune defences by biofilms: a multifront strategy. *Biofouling,* 35**,** 1055-1074.

CANABARRO, A., VALLE, C., FARIAS, M. R., SANTOS, F. B., LAZERA, M. & WANKE, B. 2013. Association of subgingival colonization of Candida albicans and other yeasts with severity of chronic periodontitis. *Journal of Periodontal Research,* 48**,** 428-432.

CASTILLO PEDRAZA, M. C., DE OLIVEIRA FRATUCELLI, E. D., RIBEIRO, S. M., FLOREZ SALAMANCA, E. J., DA SILVA COLIN, J. & KLEIN, M. I. 2020a. Modulation of Lipoteichoic Acids and Exopolysaccharides Prevents Streptococcus mutans Biofilm Accumulation. *Molecules (Basel, Switzerland)* [Online], 25. Available: http://europepmc.org/abstract/MED/32397430

https://doi.org/10.3390/molecules25092232

https://europepmc.org/articles/PMC7249192

https://europepmc.org/articles/PMC7249192?pdf=render [Accessed 2020/05//].

CASTILLO PEDRAZA, M. C., DE OLIVEIRA FRATUCELLI, E. D., RIBEIRO, S. M., FLOREZ SALAMANCA, E. J., DA SILVA COLIN, J. & KLEIN, M. I. 2020b. Modulation of Lipoteichoic Acids and Exopolysaccharides Prevents Streptococcus mutans Biofilm Accumulation. *Molecules (Basel, Switzerland),* 25**,** 2232.

CASTILLO PEDRAZA, M. C., NOVAIS, T. F., FAUSTOFERRI, R. C., QUIVEY, R. G., TEREKHOV, A., HAMAKER, B. R. & KLEIN, M. I. 2017. Extracellular DNA and lipoteichoic acids interact with exopolysaccharides in the extracellular matrix of Streptococcus mutans biofilms. *Biofouling,* 33**,** 722-740.

CAVALIERE, R., BALL, J. L., TURNBULL, L. & 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**,** 557-567.

CERI, H., OLSON, M. E., STREMICK, C., READ, R. R., MORCK, D. & BURET, A. 1999. The Calgary Biofilm Device: New Technology for Rapid Determination of Antibiotic Susceptibilities of Bacterial Biofilms. *Journal of Clinical Microbiology,* 37**,** 1771.

CHEN, D., CAO, Y., YU, L., TAO, Y., ZHOU, Y., ZHI, Q. & LIN, H. 2019a. Characteristics and influencing factors of amyloid fibers in S. mutans biofilm. *AMB Express,* 9**,** 31.

CHEN, I. & DUBNAU, D. 2004. DNA uptake during bacterial transformation. *Nature Reviews Microbiology,* 2**,** 241-249.

CHEN, L., CHAKRABORTY, B., ZOU, J., BURNE, R. A. & ZENG, L. 2019b. Amino Sugars Modify Antagonistic Interactions between Commensal Oral Streptococci and <em>Streptococcus mutans</em&gt. *Applied and Environmental Microbiology,* 85**,** e00370-19.

CHI, F., NOLTE, O., BERGMANN, C., IP, M. & HAKENBECK, R. 2007. Crossing the barrier: Evolution and spread of a major class of mosaic pbp2x in Streptococcus pneumoniae, S. mitis and S. oralis. *International Journal of Medical Microbiology,* 297**,** 503-512.

CHO, C., CHANDE, A., GAKHAR, L., BAKALETZ, L. O., JURCISEK, J. A., KETTERER, M., SHAO, J., GOTOH, K., FOSTER, E., HUNT, J., BRIEN, E. & APICELLA, M. A. 2015. Role of the Nuclease of Nontypeable Haemophilus influenzae in Dispersal of Organisms from Biofilms. *Infection and Immunity,* 83**,** 950.

CIARDI, J. E., RÖLLA, G., BOWEN, W. H. & REILLY, J. A. 1977. Adsorption of Streptococcus mutans lipoteichoic acid to hydroxyapatite. *European Journal of Oral Sciences,* 85**,** 387-391.

CISAR, J. O., KOLENBRANDER, P. E. & MCINTIRE, F. C. 1979. Specificity of coaggregation reactions between human oral streptococci and strains of Actinomyces viscosus or Actinomyces naeslundii. *Infection and Immunity,* 24**,** 742.

COSTA, R. C., SOUZA, J. G. S., BERTOLINI, M., RETAMAL-VALDES, B., FERES, M. & BARÃO, V. A. R. 2020. Extracellular biofilm matrix leads to microbial dysbiosis and reduces biofilm susceptibility to antimicrobials on titanium biomaterial: An in vitro and in situ study. *Clinical Oral Implants Research,* n/a.

COWDERY, J. S., CHACE, J. H., YI, A. K. & KRIEG, A. M. 1996. Bacterial DNA induces NK cells to produce IFN-gamma in vivo and increases the toxicity of lipopolysaccharides. *The Journal of Immunology,* 156**,** 4570.

CRUMP, K. E. & SAHINGUR, S. E. 2015. Microbial Nucleic Acid Sensing in Oral and Systemic Diseases. *Journal of Dental Research,* 95**,** 17-25.

CUGINI, C., SHANMUGAM, M., LANDGE, N. & RAMASUBBU, N. 2019. The Role of Exopolysaccharides in Oral Biofilms. *Journal of Dental Research,* 98**,** 739-745.

CURTIS, M. A., DIAZ, P. I. & VAN DYKE, T. E. 2020. The role of the microbiota in periodontal disease. *Periodontology 2000,* 83**,** 14-25.

DABDOUB, S. M., GANESAN, S. M. & KUMAR, P. S. 2016. Comparative metagenomics reveals taxonomically idiosyncratic yet functionally congruent communities in periodontitis. *Scientific Reports,* 6**,** 38993.

DAEP, C. A., LAMONT, R. J. & DEMUTH, D. R. 2008. Interaction of Porphyromonas gingivalis with Oral Streptococci Requires a Motif That Resembles the Eukaryotic Nuclear Receptor Box Protein-Protein Interaction Domain. *Infection and Immunity,* 76**,** 3273.

DAHLÉN, G. & WIKSTRÖM, M. 1995. Occurrence of enteric rods, staphylococci and Candida in subgingival samples. *Oral Microbiology and Immunology,* 10**,** 42-46.

DAS, T., SHARMA, P. K., BUSSCHER, H. J., VAN DER MEI, H. C. & KROM, B. P. 2010. Role of Extracellular DNA in Initial Bacterial Adhesion and Surface Aggregation. *Applied and Environmental Microbiology,* 76**,** 3405.

DAS, T., SHARMA, P. K., KROM, B. P., VAN DER MEI, H. C. & BUSSCHER, H. J. 2011. Role of eDNA on the Adhesion Forces between Streptococcus mutans and Substratum Surfaces: Influence of Ionic Strength and Substratum Hydrophobicity. *Langmuir,* 27**,** 10113-10118.

DE BUHR, N., NEUMANN, A., JERJOMICEVA, N., VON KÖCKRITZ-BLICKWEDE, M. & BAUMS, C. G. 2014. Streptococcus suis DNase SsnA contributes to degradation of neutrophil extracellular traps (NETs) and evasion of NET-mediated antimicrobial activity. *Microbiology,* 160**,** 385-395.

DEAS, D. E., MORITZ, A. J., SAGUN JR., R. S., GRUWELL, S. F. & POWELL, C. A. 2016. Scaling and root planing vs. conservative surgery in the treatment of chronic periodontitis. *Periodontology 2000,* 71**,** 128-139.

DEMUTH, D. R., IRVINE, D. C., COSTERTON, J. W., COOK, G. S. & LAMONT, R. J. 2001. Discrete Protein Determinant Directs the Species-Specific Adherence of Porphyromonas gingivalis to Oral Streptococci. *Infection and Immunity,* 69**,** 5736.

DERRÉ-BOBILLOT, A., CORTES-PEREZ, N. G., YAMAMOTO, Y., KHARRAT, P., COUVÉ, E., DA CUNHA, V., DECKER, P., BOISSIER, M.-C., ESCARTIN, F., CESSELIN, B., LANGELLA, P., BERMÚDEZ-HUMARÁN, L. G. & GAUDU, P. 2013. Nuclease A (Gbs0661), an extracellular nuclease of Streptococcus agalactiae, attacks the neutrophil extracellular traps and is needed for full virulence. *Molecular Microbiology,* 89**,** 518-531.

DEVARAJ, A., BUZZO, J., ROCCO, C. J., BAKALETZ, L. O. & GOODMAN, S. D. 2018. The DNABII family of proteins is comprised of the only nucleoid associated proteins required for nontypeable Haemophilus influenzae biofilm structure. *MicrobiologyOpen,* 7**,** e00563.

DEVARAJ, A., BUZZO, J. R., MASHBURN-WARREN, L., GLOAG, E. S., NOVOTNY, L. A., STOODLEY, P., BAKALETZ, L. O. & GOODMAN, S. D. 2019. The extracellular DNA lattice of bacterial biofilms is structurally related to Holliday junction recombination intermediates. *Proceedings of the National Academy of Sciences,* 116**,** 25068.

DEVARAJ, A., JUSTICE, S. S., BAKALETZ, L. O. & GOODMAN, S. D. 2015. DNABII proteins play a central role in UPEC biofilm structure. *Molecular Microbiology,* 96**,** 1119-1135.

DIAZ, P. I., CHALMERS, N. I., RICKARD, A. H., KONG, C., MILBURN, C. L., PALMER, R. J. & KOLENBRANDER, P. E. 2006. Molecular Characterization of Subject-Specific Oral Microflora during Initial Colonization of Enamel. *Applied and Environmental Microbiology,* 72**,** 2837.

DIAZ, P. I., ZILM, P. S. & ROGERS, A. H. 2002. Fusobacterium nucleatum supports the growth of Porphyromonas gingivalis in oxygenated and carbon-dioxide-depleted environments. *Microbiology,* 148**,** 467-472.

DIGE, I., NYENGAARD, J. R., KILIAN, M. & NYVAD, B. 2009. Application of stereological principles for quantification of bacteria in intact dental biofilms. *Oral Microbiology and Immunology,* 24**,** 69-75.

DO, T., GILBERT, S. C., KLEIN, J., WARREN, S., WADE, W. G. & BEIGHTON, D. 2011. Clonal structure of Streptococcus sanguinis strains isolated from endocarditis cases and the oral cavity. *Molecular Oral Microbiology,* 26**,** 291-302.

DOKE, M., FUKAMACHI, H., MORISAKI, H., ARIMOTO, T., KATAOKA, H. & KUWATA, H. 2017. Nucleases from Prevotella intermedia can degrade neutrophil extracellular traps. *Molecular Oral Microbiology,* 32**,** 288-300.

DORWARD, D. W. & GARON, C. F. 1990a. DNA Is Packaged within Membrane-Derived Vesicles of Gram-Negative but Not Gram-Positive Bacteria. *Applied and Environmental Microbiology,* 56**,** 1960.

DORWARD, D. W. & GARON, C. F. 1990b. DNA Is Packaged within Membrane-Derived Vesicles of Gram-Negative but Not Gram-Positive Bacteria. *Applied and environmental microbiology,* 56**,** 1960-1962.

DRAGAN, A. I., CASAS-FINET, J. R., BISHOP, E. S., STROUSE, R. J., SCHENERMAN, M. A. & GEDDES, C. D. 2010. Characterization of PicoGreen Interaction with dsDNA and the Origin of Its Fluorescence Enhancement upon Binding. *Biophysical Journal,* 99**,** 3010-3019.

DRAGOŠ, A. & KOVÁCS, Á. T. 2017. The Peculiar Functions of the Bacterial Extracellular Matrix. *Trends in Microbiology,* 25**,** 257-266.

EASTCOTT, A. D. & STALLARD, R. E. 1973. Sequential Changes in Developing Human Dental Plaque As Visualized by Scanning Electron Microscopy. *Journal of Periodontology,* 44**,** 218-224.

EGLAND, P. G., PALMER, R. J., JR. & KOLENBRANDER, P. E. 2004. Interspecies communication in Streptococcus gordonii-Veillonella atypica biofilms: signaling in flow conditions requires juxtaposition. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 16917-16922.

FIELD, C. A., GIDLEY, M. D., PRESHAW, P. M. & JAKUBOVICS, N. 2012. Investigation and quantification of key periodontal pathogens in patients with type 2 diabetes. *Journal of Periodontal Research,* 47**,** 470-478.

FLEMMING, H.-C. & WINGENDER, J. 2010. The biofilm matrix. *Nature Reviews Microbiology,* 8**,** 623-633.

FLEMMING, H.-C., WINGENDER, J., SZEWZYK, U., STEINBERG, P., RICE, S. A. & KJELLEBERG, S. 2016. Biofilms: an emergent form of bacterial life. *Nature reviews. Microbiology,* 14**,** 563-575.

FONTAINE, M. C., PEREZ-CASAL, J. & WILLSON, P. J. 2004. Investigation of a Novel DNase of Streptococcus suis Serotype 2. *Infection and Immunity,* 72**,** 774.

FRANK, R. M. 1970. An ultrastructural study of human supragingival dental plaque formation. *Dental plaque***,** 85-108.

FUXMAN BASS, J. I., RUSSO, D. M., GABELLONI, M. L., GEFFNER, J. R., GIORDANO, M., CATALANO, M., ZORREGUIETA, Á. & TREVANI, A. S. 2010. Extracellular DNA: A Major Proinflammatory Component of Pseudomonas aeruginosa Biofilms. *The Journal of Immunology,* 184**,** 6386.

GANESH, A., NAGENDRABABU, V., JOHN, A. & DEIVANAYAGAM, K. 2015. The Effect of Addition of an EPS Degrading Enzyme with and without Detergent to 2% Chlorhexidine on Disruption of Enterococcus faecalis Biofilm: A

Confocal Laser Scanning Microscopic Study. *Journal of clinical and diagnostic research : JCDR,* 9**,** ZC61-ZC65.

GAO, X. J., FAN, Y., KENT, R. L., VAN HOUTE, J. & MARGOLIS, H. C. 2001. Association of Caries Activity with the Composition of Dental Plaque Fluid. *Journal of Dental Research,* 80**,** 1834-1839.

GNANADHAS, D. P., ELANGO, M., DATEY, A. & CHAKRAVORTTY, D. 2015. Chronic lung infection by Pseudomonas aeruginosa biofilm is cured by L-Methionine in combination with antibiotic therapy. *Scientific Reports,* 5**,** 16043.

GÖDEKE, J., HEUN, M., BUBENDORFER, S., PAUL, K. & THORMANN, K. M. 2011. Roles of Two Shewanella oneidensis MR-1 Extracellular Endonucleases. *Applied and Environmental Microbiology,* 77**,** 5342.

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. & BAKALETZ, L. O. 2011. Biofilms can be dispersed by focusing the immune system on a common family of bacterial nucleoid-associated proteins. *Mucosal Immunology,* 4**,** 625-637.

GRAZIANI, F., KARAPETSA, D., ALONSO, B. & HERRERA, D. 2017. Nonsurgical and surgical treatment of periodontitis: how many options for one disease? *Periodontology 2000,* 75**,** 152-188.

GRENIER, D. 1992. Nutritional interactions between two suspected periodontopathogens, Treponema denticola and Porphyromonas gingivalis. *Infection and Immunity,* 60**,** 5298.

GUO, L., HE, X. & SHI, W. 2014. Intercellular communications in multispecies oral microbial communities. *Frontiers in Microbiology,* 5.

GUSTAVE, J. E., JURCISEK, J. A., MCCOY, K. S., GOODMAN, S. D. & BAKALETZ, L. O. 2013. Targeting bacterial integration host factor to disrupt biofilms associated with cystic fibrosis. *Journal of Cystic Fibrosis,* 12**,** 384-389.

HAFFAJEE, A. D. & SOCRANSKY, S. S. 1994. Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000,* 5**,** 78-111.

HAJISHENGALLIS, G. 2014. The inflammophilic character of the periodontitis-associated microbiota. *Molecular Oral Microbiology,* 29**,** 248-257.

HAJISHENGALLIS, G., DARVEAU, R. P. & CURTIS, M. A. 2012. The keystone-pathogen hypothesis. *Nature Reviews Microbiology,* 10**,** 717-725.

HAJISHENGALLIS, G. & LAMONT, R. J. 2012. Beyond the red complex and into more complexity: the polymicrobial synergy and dysbiosis (PSD) model of periodontal disease etiology. *Molecular Oral Microbiology,* 27**,** 409-419.

HAJISHENGALLIS, G. & LAMONT, R. J. 2016. Dancing with the Stars: How Choreographed Bacterial Interactions Dictate Nososymbiocity and Give Rise to Keystone Pathogens, Accessory Pathogens, and Pathobionts. *Trends in Microbiology,* 24**,** 477-489.

HAJISHENGALLIS, G., LIANG, S., PAYNE, MARK A., HASHIM, A., JOTWANI, R., ESKAN, MEHMET A., MCINTOSH, MEGAN L., ALSAM, A., KIRKWOOD,

KEITH L., LAMBRIS, JOHN D., DARVEAU, RICHARD P. & CURTIS, MICHAEL A. 2011. Low-Abundance Biofilm Species Orchestrates Inflammatory Periodontal Disease through the Commensal Microbiota and Complement. *Cell Host & Microbe,* 10**,** 497-506.

HALL-STOODLEY, L., NISTICO, L., SAMBANTHAMOORTHY, K., DICE, B., NGUYEN, D., MERSHON, W. J., JOHNSON, C., ZE HU, F., STOODLEY, P., EHRLICH, G. D. & POST, J. C. 2008. Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in Streptococcus pneumoniae clinical isolates. *BMC Microbiology,* 8**,** 173.

HALL, C. W. & MAH, T.-F. 2017. Molecular mechanisms of biofilm-based antibiotic resistance and tolerance in pathogenic bacteria. *FEMS Microbiology Reviews,* 41**,** 276-301.

HAMADY, M., WALKER, J. J., HARRIS, J. K., GOLD, N. J. & KNIGHT, R. 2008. Error-correcting barcoded primers for pyrosequencing hundreds of samples in multiplex. *Nature methods,* 5**,** 235-237.

HAMILTON, H. L., DOMÍNGUEZ, N. M., SCHWARTZ, K. J., HACKETT, K. T. & DILLARD, J. P. 2005. Neisseria gonorrhoeae secretes chromosomal DNA via a novel type IV secretion system. *Molecular Microbiology,* 55**,** 1704-1721.

HANNAN, S., READY, D., JASNI, A. S., ROGERS, M., PRATTEN, J. & ROBERTS, A. P. 2010. Transfer of antibiotic resistance by transformation with eDNA within oral biofilms. *FEMS Immunology & Medical Microbiology,* 59**,** 345-349.

HANNIG, C., HANNIG, M. & ATTIN, T. 2005. Enzymes in the acquired enamel pellicle. *European Journal of Oral Sciences,* 113**,** 2-13.

HASEGAWA, T., MINAMI, M., OKAMOTO, A., TATSUNO, I., ISAKA, M. & OHTA, M. 2010. Characterization of a virulence-associated and cell-wall-located DNase of Streptococcus pyogenes. *Microbiology,* 156**,** 184-190.

HATA, S. & MAYANAGI, H. 2003. Acid diffusion through extracellular polysaccharides produced by various mutants of Streptococcus mutans. *Archives of Oral Biology,* 48**,** 431-438.

HE, X., HU, W., KAPLAN, C. W., GUO, L., SHI, W. & LUX, R. 2012. Adherence to streptococci facilitates Fusobacterium nucleatum integration into an oral microbial community. *Microbial ecology,* 63**,** 532-542.

HERRERO, E. R., SLOMKA, V., BERNAERTS, K., BOON, N., HERNANDEZ-SANABRIA, E., PASSONI, B. B., QUIRYNEN, M. & TEUGHELS, W. 2016. Antimicrobial effects of commensal oral species are regulated by environmental factors. *Journal of Dentistry,* 47**,** 23-33.

HEUN, M., BINNENKADE, L., KREIENBAUM, M. & THORMANN, K. M. 2012. Functional Specificity of Extracellular Nucleases of Shewanella oneidensis MR-1. *Applied and Environmental Microbiology,* 78**,** 4400.

HO, M.-H., CHEN, C.-H., GOODWIN, J. S., WANG, B.-Y. & XIE, H. 2015. Functional Advantages of Porphyromonas gingivalis Vesicles. *PLOS ONE,* 10**,** e0123448.

HOFFMAN, H. & FRANK, M. 1966. Microbial burden of mucosal squamous epithelial cells. *Acta Cytologica,* 10**,** 272-&.

HOJO, K., NAGAOKA, S., OHSHIMA, T. & MAEDA, N. 2009. Bacterial Interactions in Dental Biofilm Development. *Journal of Dental Research,* 88**,** 982-990.

HOLLIDAY, R., PRESHAW, P. M., BOWEN, L. & JAKUBOVICS, N. S. 2015. The ultrastructure of subgingival dental plaque, revealed by high-resolution field emission scanning electron microscopy. *BDJ Open,* 1**,** 15003.

HOLMBERG, K. & HALLANDER, H. O. 1973. Production of bactericidal concentrations of hydrogen peroxide by Streptococcus sanguis. *Archives of Oral Biology,* 18**,** 423-IN7.

HOSHINO, T., FUJIWARA, T. & KILIAN, M. 2005. Use of Phylogenetic and Phenotypic Analyses To Identify Nonhemolytic Streptococci Isolated from Bacteremic Patients. *Journal of Clinical Microbiology,* 43**,** 6073.

HOSSAIN, M. S. & BISWAS, I. 2011. Mutacins from Streptococcus mutans are active against multiple Streptococcal Species. *Applied and Environmental Microbiology,* 77**,** 2428.

HYMES, S. R., RANDIS, T. M., SUN, T. Y. & RATNER, A. J. 2013. DNase Inhibits Gardnerella vaginalis Biofilms In Vitro and In Vivo. *The Journal of Infectious Diseases,* 207**,** 1491-1497.

IBÁÑEZ DE ALDECOA, A. L., ZAFRA, O. & GONZÁLEZ-PASTOR, J. E. 2017. Mechanisms and Regulation of Extracellular DNA Release and Its Biological Roles in Microbial Communities. *Frontiers in Microbiology,* 8.

ITZEK, A., ZHENG, L., CHEN, Z., MERRITT, J. & KRETH, J. 2011a. Hydrogen Peroxide-Dependent DNA Release and Transfer of Antibiotic Resistance Genes in genus-species ;Streptococcus gordonii. *Journal of Bacteriology,* 193**,** 6912.

ITZEK, A., ZHENG, L., CHEN, Z., MERRITT, J. & KRETH, J. 2011b. Hydrogen Peroxide-Dependent DNA Release and Transfer of Antibiotic Resistance Genes in Streptococcus gordonii. *Journal of Bacteriology,* 193**,** 6912.

IYER, V. S. & HANCOCK, L. E. 2012. Deletion of σ54 (rpoN) Alters the Rate of Autolysis and Biofilm Formation in Enterococcus faecalis. *Journal of Bacteriology,* 194**,** 368-375.

IZANO, E. A., AMARANTE, M. A., KHER, W. B. & KAPLAN, J. B. 2008a. Differential Roles of Poly-Acetylglucosamine Surface Polysaccharide and Extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis Biofilms. *Applied and Environmental Microbiology,* 74**,** 470.

IZANO, E. A., SADOVSKAYA, I., WANG, H., VINOGRADOV, E., RAGUNATH, C., RAMASUBBU, N., JABBOURI, S., PERRY, M. B. & KAPLAN, J. B. 2008b. Poly-N-acetylglucosamine mediates biofilm formation and detergent resistance in Aggregatibacter actinomycetemcomitans. *Microbial Pathogenesis,* 44**,** 52-60.

JAKUBOVICS, N. S. & BURGESS, G. J. 2015. Extracellular DNA in oral microbial biofilms. *Microbes and Infection,* 17**,** 531-537.

JAKUBOVICS, N. S., GILL, S. R., VICKERMAN, M. M. & KOLENBRANDER, P. E. 2008. Role of hydrogen peroxide in competition and cooperation between Streptococcus gordonii and Actinomyces naeslundii. *FEMS Microbiology Ecology,* 66**,** 637-644.

JAKUBOVICS, N. S. & KOLENBRANDER, P. E. 2010. The road to ruin: the formation of disease-associated oral biofilms. *Oral Diseases,* 16**,** 729-739.

JAKUBOVICS, N. S., SMITH, A. W. & JENKINSON, H. F. 2000. Expression of the virulence-related Sca (Mn2+) permease in Streptococcus gordonii is regulated by a diphtheria toxin metallorepressor-like protein ScaR. *Molecular Microbiology,* 38**,** 140-153.

JENKINSON, H. F. & LAMONT, R. J. 1997. Streptococcal Adhesion and Colonization. *Critical Reviews in Oral Biology & Medicine,* 8**,** 175-200.

JONES, E. A., MCGILLIVARY, G. & BAKALETZ, L. O. 2013. Extracellular DNA within a Nontypeable Haemophilus influenzae-Induced Biofilm Binds Human Beta Defensin-3 and Reduces Its Antimicrobial Activity. *Journal of Innate Immunity,* 5**,** 24-38.

KAPLAN, J. B., MLYNEK, K. D., HETTIARACHCHI, H., ALAMNEH, Y. A., BIGGEMANN, L., ZURAWSKI, D. V., BLACK, C. C., BANE, C. E., KIM, R. K. & GRANICK, M. S. 2018. Extracellular polymeric substance (EPS)-degrading enzymes reduce staphylococcal surface attachment and biocide resistance on pig skin in vivo. *PLOS ONE,* 13**,** e0205526.

KARYGIANNI, L., ATTIN, T. & THURNHEER, T. 2020. Combined DNase and Proteinase Treatment Interferes with Composition and Structural Integrity of Multispecies Oral Biofilms. *Journal of clinical medicine,* 9**,** 983.

KAWARAI, T., NARISAWA, N., SUZUKI, Y., NAGASAWA, R. & SENPUKU, H. 2016. Streptococcus mutans biofilm formation is dependent on extracellular DNA in primary low pH conditions. *Journal of Oral Biosciences,* 58**,** 55-61.

KEKE, Z., XUEDONG, Z. & XIN, X. 2017. [The origin of hydrogen peroxide in oral cavity and its role in oral microecology balance]. *Hua xi kou qiang yi xue za zhi = Huaxi kouqiang yixue zazhi = West China journal of stomatology,* 35**,** 215-220.

KIEDROWSKI, M. R., KAVANAUGH, J. S., MALONE, C. L., MOOTZ, J. M., VOYICH, J. M., SMELTZER, M. S., BAYLES, K. W. & HORSWILL, A. R. 2011. Nuclease modulates biofilm formation in community-associated methicillin-resistant Staphylococcus aureus. *PloS one,* 6**,** e26714.

KIM, M., JEON, J. & KIM, J. 2018. Streptococcus mutans extracellular DNA levels depend on the number of bacteria in a biofilm. *Scientific Reports,* 8**,** 13313.

KIM, Y., JO, A.-R., JANG, D. H., CHO, Y.-J., CHUN, J., MIN, B.-M. & CHOI, Y. 2012a. Toll-like receptor 9 mediates oral bacteria-induced IL-8 expression in gingival epithelial cells. *Immunology & Cell Biology,* 90**,** 655-663.

KIM, Y., JO, A.-R., JANG, D. H., CHO, Y.-J., CHUN, J., MIN, B.-M. & CHOI, Y. 2012b. Toll-like receptor 9 mediates oral bacteria-induced IL-8 expression in gingival epithelial cells. 90**,** 655-663.

KIN, L. X., BUTLER, C. A., SLAKESKI, N., HOFFMANN, B., DASHPER, S. G. & REYNOLDS, E. C. 2020. Metabolic cooperativity between Porphyromonas gingivalis and Treponema denticola. *Journal of Oral Microbiology,* 12**,** 1808750.

KLEIN, M. I., HWANG, G., SANTOS, P. H. S., CAMPANELLA, O. H. & KOO, H. 2015. Streptococcus mutans-derived extracellular matrix in cariogenic oral biofilms. *Frontiers in Cellular and Infection Microbiology,* 5.

KOLENBRANDER, P. E. 2000. Oral Microbial Communities: Biofilms, Interactions, and Genetic Systems. *Annual Review of Microbiology,* 54**,** 413-437.

KOLENBRANDER, P. E., ANDERSEN, R. N., KAZMERZAK, K. & PALMER, R., JR 2000. Coaggregation and coadhesion in oral biofilms. *SYMPOSIA-SOCIETY FOR GENERAL MICROBIOLOGY Cambridge; Cambridge University Press; 1999, 65-86.,* 1999**,** 65-86

KOLENBRANDER, P. E., PALMER JR, R. J., RICKARD, A. H., JAKUBOVICS, N. S., CHALMERS, N. I. & DIAZ, P. I. 2006. Bacterial interactions and successions during plaque development. *Periodontology 2000,* 42**,** 47-79.

KOLENBRANDER, P. E., PALMER, R. J., PERIASAMY, S. & JAKUBOVICS, N. S. 2010. Oral multispecies biofilm development and the key role of cell–cell distance. *Nature Reviews Microbiology,* 8**,** 471-480.

KÖNÖNEN, E., GURSOY, M. & GURSOY, U. K. 2019. Periodontitis: A Multifaceted Disease of Tooth-Supporting Tissues. *Journal of clinical medicine* [Online], 8. Available: http://europepmc.org/abstract/MED/31370168

https://doi.org/10.3390/jcm8081135

https://europepmc.org/articles/PMC6723779

https://europepmc.org/articles/PMC6723779?pdf=render [Accessed 2019/07//].

KONSTAN, M. W. & RATJEN, F. 2012. Effect of dornase alfa on inflammation and lung function: Potential role in the early treatment of cystic fibrosis. *Journal of Cystic Fibrosis,* 11**,** 78-83.

KOO, H., FALSETTA, M. L. & KLEIN, M. I. 2013. The Exopolysaccharide Matrix: A Virulence Determinant of Cariogenic Biofilm. *Journal of Dental Research,* 92**,** 1065-1073.

KRETH, J., MERRITT, J., ZHU, L., SHI, W. & QI, F. 2006. Cell density- and ComE-dependent expression of a group of mutacin and mutacin-like genes in Streptococcus mutans. *FEMS Microbiology Letters,* 265**,** 11-17.

KRETH, J., VU, H., ZHANG, Y. & HERZBERG, M. C. 2009a. Characterization of Hydrogen Peroxide-Induced DNA Release by Streptococcus sanguinis and <em>Streptococcus gordonii</em&gt. *Journal of Bacteriology,* 191**,** 6281.

KRETH, J., VU, H., ZHANG, Y. & HERZBERG, M. C. 2009b. Characterization of Hydrogen Peroxide-Induced DNA Release by Streptococcus sanguinis and Streptococcus gordonii. *Journal of Bacteriology,* 191**,** 6281.

KRETH, J., ZHANG, Y. & HERZBERG, M. C. 2008a. Streptococcal Antagonism in Oral Biofilms ;Streptococcus sanguinis and Streptococcus gordonii Interference with Streptococcus mutans. *Journal of Bacteriology,* 190**,** 4632.

KRETH, J., ZHANG, Y. & HERZBERG, M. C. 2008b. Streptococcal Antagonism in Oral Biofilms: Streptococcus sanguinis&Streptococcus gordonii Interference with Streptococcus mutans. *Journal of Bacteriology,* 190**,** 4632.

KRIEG, A. M. 2002. CpG motifs in bacterial DNA and their immune effects. *Annual review of immunology,* 20**,** 709-760.

KUBONIWA, M. & LAMONT, R. J. 2010. Subgingival biofilm formation. *Periodontology 2000,* 52**,** 38-52.

KUNITZ , M. 1950. CRYSTALLINE DESOXYRIBONUCLEASE : I. ISOLATION AND GENERAL PROPERTIES SPECTROPHOTOMETRIC METHOD FOR THE MEASUREMENT OF DESOXYRIBONUCLEASE ACTIVITY. *Journal of General Physiology,* 33**,** 349-362.

LACKS, S. & GREENBERG, B. 1976. Single-strand breakage on binding of DNA to cells in the genetic transformation of Diplococcus pneumoniae. *Journal of Molecular Biology,* 101**,** 255-275.

LACKS, S., GREENBERG, B. & NEUBERGER, M. 1974. Role of a Deoxyribonuclease in the Genetic Transformation of Diplococcus pneumoniae. *Proceedings of the National Academy of Sciences,* 71**,** 2305.

LACKS, S., GREENBERG, B. & NEUBERGER, M. 1975. Identification of a deoxyribonuclease implicated in genetic transformation of Diplococcus pneumoniae. *Journal of Bacteriology,* 123**,** 222.

LACKS, S. & NEUBERGER, M. 1975. Membrane location of a deoxyribonuclease implicated in the genetic transformation of Diplococcus pneumoniae. *Journal of Bacteriology,* 124**,** 1321.

LAMONT, R. J., EL-SABAENY, A., PARK, Y., COOK, G. S., COSTERTON, J. W. & DEMUTH, D. R. 2002. Role of the Streptococcus gordonii SspB protein in the development of Porphyromonas gingivalis biofilms on streptococcal substrates. *Microbiology,* 148**,** 1627-1636.

LAMONT, R. J., KOO, H. & HAJISHENGALLIS, G. 2018. The oral microbiota: dynamic communities and host interactions. *Nature Reviews Microbiology,* 16**,** 745-759.

LETHEM, M. I., JAMES, S. L., MARRIOTT, C. & BURKE, J. F. 1990. The origin of DNA associated with mucus glycoproteins in cystic fibrosis sputum. *European Respiratory Journal,* 3**,** 19.

LEWENZA, S., JOHNSON, L., CHARRON-MAZENOD, L., HONG, M., MULCAHY-O, APOS & GRADY, H. 2020. Extracellular DNA controls expression of Pseudomonas aeruginosa genes involved in nutrient utilization, metal homeostasis, acid pH tolerance and virulence. *Journal of Medical Microbiology,* 69**,** 895-905.

LI, T., KHAH, M. K., SLAVNIC, S., JOHANSSON, I. & STRÖMBERG, N. 2001. Different Type 1 Fimbrial Genes and Tropisms of Commensal and Potentially Pathogenic Actinomyces spp. with Different Salivary Acidic Proline-Rich Protein and Statherin Ligand Specificities. *Infection and Immunity,* 69**,** 7224.

LI, W., LIU, H. & XU, Q. 2012. Extracellular Dextran and DNA Affect the Formation of Enterococcus faecalis Biofilms and Their Susceptibility to 2% Chlorhexidine. *Journal of Endodontics,* 38**,** 894-898.

LIAO, S., KLEIN, M. I., HEIM, K. P., FAN, Y., BITOUN, J. P., AHN, S.-J., BURNE, R. A., KOO, H., BRADY, L. J. & WEN, Z. T. 2014. Streptococcus mutans Extracellular DNA Is Upregulated during Growth in Biofilms, Actively Released via Membrane Vesicles, and Influenced by Components of the Protein Secretion Machinery. *Journal of Bacteriology,* 196**,** 2355.

LIE, T. 1977. Early dental plaque morphogenesis. *Journal of Periodontal Research,* 12**,** 73-89.

LILJESTRAND, J. M., PAJU, S., PIETIÄINEN, M., BUHLIN, K., PERSSON, G. R., NIEMINEN, M. S., SINISALO, J., MÄNTYLÄ, P. & PUSSINEN, P. J. 2018. Immunologic burden links periodontitis to acute coronary syndrome. *Atherosclerosis,* 268**,** 177-184.

LIPFORD, G. B., SPARWASSER, T., BAUER, M., ZIMMERMANN, S., KOCH, E.-S., HEEG, K. & WAGNER, H. 1997. Immunostimulatory DNA: Sequence-dependent production of potentially harmful or useful cytokines. *European Journal of Immunology,* 27**,** 3420-3426.

LISTGARTEN, M. A. 1976. Structure of the Microbial Flora Associated with Periodontal Health and Disease in Man: A Light and Electron Microscopic Study. *Journal of Periodontology,* 47**,** 1-18.

LISTGARTEN, M. A., MAYO, H. E. & TREMBLAY, R. 1975. Development of Dental Plaque on Epoxy Resin Crowns in Man: A Light and Electron Microscopic Study. *Journal of Periodontology,* 46**,** 10-26.

LIU, J., CHEN, J., DU, X., HU, L. & CHEN, L. 2014. The expression of hBDs in the gingival tissue and keratinocytes from healthy subjects and periodontitis patients. *Archives of Oral Biology,* 59**,** 193-198.

LIU, J., SUN, L., LIU, W., GUO, L., LIU, Z., WEI, X. & LING, J. 2017. A Nuclease from Streptococcus mutans Facilitates Biofilm Dispersal and Escape from Killing by Neutrophil Extracellular Traps. *Frontiers in Cellular and Infection Microbiology,* 7.

LIU, Y. & BURNE, R. A. 2011. The Major Autolysin of Streptococcus gordonii is Subject to Complex Regulation and Modulates Stress Tolerance, Biofilm Formation, and Extracellular-DNA Release. *Journal of Bacteriology,* 193**,** 2826.

LUO, T. L., EISENBERG, M. C., HAYASHI, M. A. L., GONZALEZ-CABEZAS, C., FOXMAN, B., MARRS, C. F. & RICKARD, A. H. 2018. A Sensitive Thresholding Method for Confocal Laser Scanning Microscope Image Stacks of Microbial Biofilms. *Scientific Reports,* 8**,** 13013.

LUO, T. L., HAYASHI, M., ZSISKA, M., CIRCELLO, B., EISENBERG, M., GONZALEZ-CABEZAS, C., FOXMAN, B., MARRS, C. F. & RICKARD, A. H. 2019. Introducing BAIT (Biofilm Architecture Inference Tool): a software program to evaluate the architecture of oral multi-species biofilms. *Microbiology,* 165**,** 527-537.

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. & BAYLES, K. W. 2009. Modulation of eDNA release and degradation affects Staphylococcus aureus biofilm maturation. *PloS one,* 4**,** e5822-e5822.

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. & HARTL, D. 2012. Ultrastructural characterization of cystic fibrosis sputum using atomic force and scanning electron microscopy. *Journal of Cystic Fibrosis,* 11**,** 84-92.

MARSH, P. D. 1989. Host defenses and microbial homeostasis : role of microbial interactions. *J Dent Res,* 68**,** 1567-1575.

MARSH, P. D. 2003. Are dental diseases examples of ecological catastrophes? *Microbiology,* 149**,** 279-294.

MARSH, P. D., LEWIS , M., ROGERS, H., WILLIAMS, D. & WILSON, M. 2016. Marsh and Martin`s Oral Microbiology,. *Elsevier Ltd.*

MARSH, P. D., MOTER, A. & DEVINE, D. A. 2011. Dental plaque biofilms: communities, conflict and control. *Periodontology 2000,* 55**,** 16-35.

MARSH, P. D. & ZAURA, E. 2017. Dental biofilm: ecological interactions in health and disease. *Journal of Clinical Periodontology,* 44**,** S12-S22.

MATTOS-GRANER, R. O., SMITH, D. J., KING, W. F. & MAYER, M. P. A. 2000. Water-insoluble Glucan Synthesis by Mutans Streptococcal Strains Correlates with Caries Incidence in 12- to 30-month-old Children. *Journal of Dental Research,* 79**,** 1371-1377.

MEI, C.-C., LEE, F.-Y. & YEH, H.-C. 2016. Assessment of pain perception following periodontal and implant surgeries. *Journal of Clinical Periodontology,* 43**,** 1151-1159.

MERRITT, J. & QI, F. 2012. The mutacins of Streptococcus mutans: regulation and ecology. *Molecular Oral Microbiology,* 27**,** 57-69.

MESSIAEN, A.-S., NELIS, H. & COENYE, T. 2014. Investigating the role of matrix components in protection of Burkholderia cepacia complex biofilms against tobramycin. *Journal of Cystic Fibrosis,* 13**,** 56-62.

MILES, A. A., MISRA, S. S. & IRWIN, J. O. 1938. The estimation of the bactericidal power of the blood. *Epidemiology and Infection,* 38**,** 732-749.

MILLER, D. P., FITZSIMONDS, Z. R. & LAMONT, R. J. 2019. Metabolic Signaling and Spatial Interactions in the Oral Polymicrobial Community. *Journal of Dental Research,* 98**,** 1308-1314.

MIRA, A., SIMON-SORO, A. & CURTIS, M. A. 2017. Role of microbial communities in the pathogenesis of periodontal diseases and caries. *Journal of clinical periodontology,* 44 Suppl 18**,** S23-S38.

MORITA, C., SUMIOKA, R., NAKATA, M., OKAHASHI, N., WADA, S., YAMASHIRO, T., HAYASHI, M., HAMADA, S., SUMITOMO, T. & KAWABATA, S. 2014. Cell Wall-Anchored Nuclease of Streptococcus sanguinis Contributes to Escape from Neutrophil Extracellular Trap-Mediated Bacteriocidal Activity. *PLOS ONE,* 9**,** e103125.

MORRISON, D. A. & GUILD, W. R. 1973. Breakage prior to entry of donor DNA in Pneumococcus transformation. *Biochimica et Biophysica Acta (BBA) - Nucleic Acids and Protein Synthesis,* 299**,** 545-556.

MULCAHY, H., CHARRON-MAZENOD, L. & LEWENZA, S. 2010. Pseudomonas aeruginosa produces an extracellular deoxyribonuclease that is required for utilization of DNA as a nutrient source. *Environmental Microbiology,* 12**,** 1621-1629.

NAGASAWA, R., SATO, T., NOMURA, N., NAKAMURA, T. & SENPUKU, H. 2020a. Potential Risk of Spreading Resistance Genes within Extracellular-DNA-Dependent Biofilms of Streptococcus mutans in Response to Cell Envelope Stress Induced by Sub-MICs of Bacitracin. *Applied and Environmental Microbiology,* 86**,** e00770-20.

NAGASAWA, R., YAMAMOTO, T., UTADA, A. S., NOMURA, N. & OBANA, N. 2020b. Competence-Stimulating-Peptide-Dependent Localized Cell Death and Extracellular DNA Production in Streptococcus mutans Biofilms. *Applied and Environmental Microbiology,* 86**,** e02080-20.

NANCE, W. C., DOWD, S. E., SAMARIAN, D., CHLUDZINSKI, J., DELLI, J., BATTISTA, J. & RICKARD, A. H. 2013. A high-throughput microfluidic dental plaque biofilm system to visualize and quantify the effect of antimicrobials. *Journal of Antimicrobial Chemotherapy,* 68**,** 2550-2560.

NEU, T. R. & LAWRENCE, J. R. 1997. Development and structure of microbial biofilms in river water studied by confocal laser scanning microscopy. *FEMS Microbiology Ecology,* 24**,** 11-25.

NIJLAND, R., HALL, M. J. & BURGESS, J. G. 2010. Dispersal of biofilms by secreted, matrix degrading, bacterial DNase. *PloS one* [Online], 5. [Accessed 2010/12//].

NISHIKAWA, K. & TANAKA, Y. 2013. A simple mutagenesis using natural competence in Tannerella forsythia. *Journal of Microbiological Methods,* 94**,** 378-380.

NOBBS, A. H., JENKINSON, H. F. & JAKUBOVICS, N. S. 2011. Stick to Your Gums: Mechanisms of Oral Microbial Adherence. *Journal of Dental Research,* 90**,** 1271-1278.

NONNENMACHER, C., DALPKE, A., ZIMMERMANN, S., FLORES-DE-JACOBY, L., MUTTERS, R. & HEEG, K. 2003a. DNA from periodontopathogenic bacteria is immunostimulatory for mouse and human immune cells. *Infection and immunity,* 71**,** 850-856.

NONNENMACHER, C., DALPKE, A., ZIMMERMANN, S., FLORES-DE-JACOBY, L., MUTTERS, R. & HEEG, K. 2003b. DNA from Periodontopathogenic Bacteria Is Immunostimulatory for Mouse and Human Immune Cells. *Infection and Immunity,* 71**,** 850.

NOVOTNY, L. A., AMER, A. O., BROCKSON, M. E., GOODMAN, S. D. & BAKALETZ, L. O. 2013. Structural stability of Burkholderia cenocepacia biofilms is reliant on eDNA structure and presence of a bacterial nucleic acid binding protein. *PloS one,* 8**,** e67629-e67629.

NUR, A., HIROTA, K., YUMOTO, H., HIRAO, K., LIU, D., TAKAHASHI, K., MURAKAMI, K., MATSUO, T., SHU, R. & MIYAKE, Y. 2013. Effects of extracellular DNA and DNA-binding protein on the development of a Streptococcus intermedius biofilm. *Journal of Applied Microbiology,* 115**,** 260-270.

NYVAD, B. & KILIAN, M. 1987. Microbiology of the early colonization of human enamel and root surfaces in vivo. *Scand J Dent Res,* 95**,** 369-80.

NYVAD, B. & KILIAN, M. 1990. Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. *Caries Res,* 24**,** 267-72.

OHTA, H., KATO, K., KOKEGUCHI, S., HARA, H., FUKUI, K. & MURAYAMA, Y. 1991. Nuclease-sensitive binding of an Actinobacillus actinomycetemcomitans leukotoxin to the bacterial cell surface. *Infection and Immunity,* 59**,** 4599.

OKABE, S., HIRATIA, K., OZAWA, Y. & WATANABE, Y. 1996. Spatial microbial distributions of nitrifiers and heterotrophs in mixed-population biofilms. *Biotechnology and Bioengineering,* 50**,** 24-35.

OKSHEVSKY, M. & MEYER, R. L. 2015. The role of extracellular DNA in the establishment, maintenance and perpetuation of bacterial biofilms. *Critical Reviews in Microbiology,* 41**,** 341-352.

OKSHEVSKY, M., REGINA, V. R. & MEYER, R. L. 2015. Extracellular DNA as a target for biofilm control. *Current Opinion in Biotechnology,* 33**,** 73-80.

OLI, M. W., OTOO, H. N., CROWLEY, P. J., HEIM, K. P., NASCIMENTO, M. M., RAMSOOK, C. B., LIPKE, P. N. & BRADY, L. J. 2012. Functional amyloid formation by Streptococcus mutans. *Microbiology (Reading, England),* 158**,** 2903-2916.

PAES LEME, A. F., BELLATO, C. M., BEDI, G., DEL BEL CURY, A. A., KOO, H. & CURY, J. A. 2008. Effects of Sucrose on the Extracellular Matrix of Plaque-Like Biofilm Formed in vivo, Studied by Proteomic Analysis. *Caries Research,* 42**,** 435-443.

PALCHEVSKIY, V. & FINKEL, S. E. 2006. Competence Gene Homologs Are Essential for Competitive Fitness and the Use of DNA as a Nutrient. *Journal of Bacteriology,* 188**,** 3902.

PALMER, L. J., CHAPPLE, I. L. C., WRIGHT, H. J., ROBERTS, A. & COOPER, P. R. 2012. Extracellular deoxyribonuclease production by periodontal bacteria. *Journal of Periodontal Research,* 47**,** 439-445.

PALMER, R. J., GORDON, S. M., CISAR, J. O. & KOLENBRANDER, P. E. 2003. Coaggregation-Mediated Interactions of Streptococci and Actinomyces Detected in Initial Human Dental Plaque. *Journal of Bacteriology,* 185**,** 3400.

PARK, Y., SIMIONATO, M. R., SEKIYA, K., MURAKAMI, Y., JAMES, D., CHEN, W., HACKETT, M., YOSHIMURA, F., DEMUTH, D. R. & LAMONT, R. J. 2005. Short Fimbriae of Porphyromonas gingivalis and Their Role in Coadhesion with Streptococcus gordonii</em&gt. *Infection and Immunity,* 73**,** 3983.

PENG, N., CAI, P., MORTIMER, M., WU, Y., GAO, C. & HUANG, Q. 2020a. The exopolysaccharide–eDNA interaction modulates 3D architecture of Bacillus subtilis biofilm. *BMC Microbiology,* 20**,** 115.

PENG, N., CAI, P., MORTIMER, M., WU, Y., GAO, C. & HUANG, Q. 2020b. The exopolysaccharide–eDNA interaction modulates 3D architecture of Bacillus subtilis biofilm. Research Square.

PÉREZ-CRUZ, C., DELGADO, L., LÓPEZ-IGLESIAS, C. & MERCADE, E. 2015. Outer-Inner Membrane Vesicles Naturally Secreted by Gram-Negative Pathogenic Bacteria. *PLOS ONE,* 10**,** e0116896.

PERIASAMY, S. & KOLENBRANDER, P. E. 2010. Central Role of the Early Colonizer Veillonella sp. in Establishing Multispecies Biofilm Communities with Initial, Middle, and Late Colonizers of Enamel. *Journal of Bacteriology,* 192**,** 2965.

PERRY, J. A., CVITKOVITCH, D. G. & LÉVESQUE, C. M. 2009a. Cell death in Streptococcus mutans biofilms: a link between CSP and extracellular DNA. *FEMS Microbiology Letters,* 299**,** 261-266.

PERRY, J. A., JONES, M. B., PETERSON, S. N., CVITKOVITCH, D. G. & LÉVESQUE, C. M. 2009b. Peptide alarmone signalling triggers an auto-active bacteriocin necessary for genetic competence. *Molecular Microbiology,* 72**,** 905-917.

PETERSEN, F. C., TAO, L. & SCHEIE, A. A. 2005. DNA Binding-Uptake System: a Link between Cell-to-Cell Communication and Biofilm Formation. *Journal of Bacteriology,* 187**,** 4392.

PINCHUK, G. E., AMMONS, C., CULLEY, D. E., LI, S.-M. W., MCLEAN, J. S., ROMINE, M. F., NEALSON, K. H., FREDRICKSON, J. K. & BELIAEV, A. S. 2008. Utilization of DNA as a Sole Source of Phosphorus, Carbon, and Energy by Shewanella spp.: Ecological and Physiological Implications for Dissimilatory Metal Reduction. *Applied and Environmental Microbiology,* 74**,** 1198.

PINGEL, L. C., KOHLGRAF, K. G., HANSEN, C. J., EASTMAN, C. G., DIETRICH, D. E., BURNELL, K. K., SRIKANTHA, R. N., XIAO, X., BÉLANGER, M., PROGULSKE-FOX, A., CAVANAUGH, J. E., GUTHMILLER, J. M., JOHNSON, G. K., JOLY, S., KURAGO, Z. B., DAWSON, D. V. & BROGDEN, K. A. 2008. Human β-defensin 3 binds to hemagglutinin B (rHagB), a non-fimbrial adhesin from Porphyromonas gingivalis, and attenuates a pro-inflammatory cytokine response. *Immunology & Cell Biology,* 86**,** 643-649.

PRATTEN, J. 2007. Growing Oral Biofilms in a Constant Depth Film Fermentor (CDFF). *Current Protocols in Microbiology,* 6**,** 1B.5.1-1B.5.18.

PRETZL, B., SÄLZER, S., EHMKE, B., SCHLAGENHAUF, U., DANNEWITZ, B., DOMMISCH, H., EICKHOLZ, P. & JOCKEL-SCHNEIDER, Y. 2019. Administration of systemic antibiotics during non-surgical periodontal therapy—a consensus report. *Clinical Oral Investigations,* 23**,** 3073-3085.

PROVVEDI, R., CHEN, I. & DUBNAU, D. 2001. NucA is required for DNA cleavage during transformation of Bacillus subtilis. *Molecular Microbiology,* 40**,** 634-644.

PUYET, A., GREENBERG, B. & LACKS, S. A. 1990. Genetic and structural characterization of endA. A membrane-bound nuclease required for transformation of Streptococcus pneumoniae. *J Mol Biol,* 213**,** 727-38.

QIN, Z., OU, Y., YANG, L., ZHU, Y., TOLKER-NIELSEN, T., MOLIN, S. & QU, D. 2007. Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. *Microbiology,* 153**,** 2083-2092.

RAMIREZ, T., SHRESTHA, A. & KISHEN, A. 2019. Inflammatory potential of monospecies biofilm matrix components. *International Endodontic Journal,* 52**,** 1020-1027.

RANI, S. A., PITTS, B., BEYENAL, H., VELUCHAMY, R. A., LEWANDOWSKI, Z., DAVISON, W. M., BUCKINGHAM-MEYER, K. & STEWART, P. S. 2007. Spatial Patterns of DNA Replication, Protein Synthesis, and Oxygen Concentration within Bacterial Biofilms Reveal Diverse Physiological States. *Journal of Bacteriology,* 189**,** 4223.

REDANZ, S., CHENG, X., GIACAMAN, R. A., PFEIFER, C. S., MERRITT, J. & KRETH, J. 2018. Live and let die: Hydrogen peroxide production by the commensal flora and its role in maintaining a symbiotic microbiome. *Molecular Oral Microbiology,* 33**,** 337-352.

REESE, S. & GUGGENHEIM, B. 2007. A novel TEM contrasting technique for extracellular polysaccharides in in vitro biofilms. *Microscopy Research and Technique,* 70**,** 816-822.

ROBERTS, A. P. & KRETH, J. 2014. The impact of horizontal gene transfer on the adaptive ability of the human oral microbiome. *Frontiers in Cellular and Infection Microbiology,* 4.

ROBINSON, C. 2011. Mass transfer of therapeutics through natural human plaque biofilms: A model for therapeutic delivery to pathological bacterial biofilms. *Archives of Oral Biology,* 56**,** 829-836.

ROBINSON, C., STRAFFORD, S., REES, G., BROOKES, S. J., KIRKHAM, J., SHORE, R. C., WATSON, P. S. & WOOD, S. 2006. Plaque biofilms: The effect of chemical environment on natural human plaque biofilm architecture. *Archives of Oral Biology,* 51**,** 1006-1014.

ROCCO, C. J., BAKALETZ, L. O. & GOODMAN, S. D. 2018. Targeting the HUβ Protein Prevents Porphyromonas gingivalis from Entering into Preexisting Biofilms. *Journal of Bacteriology,* 200**,** e00790-17.

ROCCO, C. J., DAVEY, M. E., BAKALETZ, L. O. & GOODMAN, S. D. 2017. Natural antigenic differences in the functionally equivalent extracellular DNABII proteins of bacterial biofilms provide a means for targeted biofilm therapeutics. *Molecular Oral Microbiology,* 32**,** 118-130.

ROGERS, A. H., VAN DER HOEVEN, J. S. & MIKX, F. H. M. 1979. Effect of Bacteriocin Production by Streptococcus mutans on the Plaque of Gnotobiotic Rats. *Infection and Immunity,* 23**,** 571.

ROGERS, J. D., PALMER, R. J., JR., KOLENBRANDER, P. E. & SCANNAPIECO, F. A. 2001. Role of Streptococcus gordonii amylase-binding protein A in adhesion to hydroxyapatite, starch metabolism, and biofilm formation. *Infect Immun,* 69**,** 7046-56.

ROKY, M., TRENT, J. O. & DEMUTH, D. R. 2020. Identification of functional domains of the minor fimbrial antigen involved in the interaction of Porphyromonas gingivalis with oral streptococci. *Molecular Oral Microbiology,* 35**,** 66-77.

RØLLA, G., OPPERMANN, R. V., BOWEN, W. H., CIARDI, J. E. & KNOX, K. W. 1980. High Amounts of Lipoteichoic Acid in Sucrose-Induced Plaque in vivo. *Caries Research,* 14**,** 235-238.

ROSTAMI, N., SHIELDS, R. C., YASSIN, S. A., HAWKINS, A. R., BOWEN, L., LUO, T. L., RICKARD, A. H., HOLLIDAY, R., PRESHAW, P. M. & JAKUBOVICS, N. S. 2017. A Critical Role for Extracellular DNA in Dental Plaque Formation. *Journal of dental research,* 96**,** 208-216.

RUHL, S., SANDBERG, A. L. & CISAR, J. O. 2004. Salivary Receptors for the Proline-rich Protein-binding and Lectin-like Adhesins of Oral Actinomyces and Streptococci. *Journal of Dental Research,* 83**,** 505-510.

SAHINGUR, S. E., XIA, X.-J., ALAMGIR, S., HONMA, K., SHARMA, A. & SCHENKEIN, H. A. 2010. DNA from Porphyromonas gingivalis and Tannerella forsythia induce cytokine production in human monocytic cell lines. *Molecular Oral Microbiology,* 25**,** 123-135.

SAHINGUR, S. E., XIA, X.-J. & SCHIFFERLE, R. E. 2012. Oral Bacterial DNA Differ in Their Ability to Induce Inflammatory Responses in Human Monocytic Cell Lines. *Journal of Periodontology,* 83**,** 1069-1077.

SAHU, P. K., IYER, P. S., OAK, A. M., PARDESI, K. R. & CHOPADE, B. A. 2012. Characterization of eDNA from the Clinical Strain Acinetobacter baumannii AIIMS 7 and Its Role in Biofilm Formation. *The Scientific World Journal,* 2012**,** 973436.

SANZ, M., BEIGHTON, D., CURTIS, M. A., CURY, J. A., DIGE, I., DOMMISCH, H., ELLWOOD, R., GIACAMAN, R. A., HERRERA, D., HERZBERG, M. C., KÖNÖNEN, E., MARSH, P. D., MEYLE, J., MIRA, A., MOLINA, A., MOMBELLI, A., QUIRYNEN, M., REYNOLDS, E. C., SHAPIRA, L. & ZAURA, E. 2017. Role of microbial biofilms in the maintenance of oral health and in the development of dental caries and periodontal diseases. Consensus report of group 1 of the Joint EFP/ORCA workshop on the boundaries between caries and periodontal disease. *Journal of Clinical Periodontology,* 44**,** S5-S11.

SCHILLING, K. M. & BOWEN, W. H. 1992. Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for Streptococcus mutans. *Infection and Immunity,* 60**,** 284.

SCHLAFER, S., GARCIA, J., MEYER, R. L., VAETH, M. & NEUHAUS, K. W. 2018a. Effect of DNase treatment on adhesion and early biofilm formation of Enterococcus faecalis. *European Endodontic Journal,* 3**,** 82+.

SCHLAFER, S., GARCIA, J., MEYER, R. L., VAETH, M. & NEUHAUS, K. W. 2018b. Effect of DNase Treatment on Adhesion and Early Biofilm Formation of Enterococcus Faecalis. *European endodontic journal,* 3**,** 82-86.

SCHLAFER, S., MEYER, R. L., DIGE, I. & REGINA, V. R. 2017. Extracellular DNA Contributes to Dental Biofilm Stability. *Caries Research,* 51**,** 436-442.

SCHOOLING, S. R., HUBLEY, A. & BEVERIDGE, T. J. 2009. Interactions of DNA with Biofilm-Derived Membrane Vesicles. *Journal of Bacteriology,* 191**,** 4097.

SEPER, A., FENGLER, V. H. I., ROIER, S., WOLINSKI, H., KOHLWEIN, S. D., BISHOP, A. L., CAMILLI, A., REIDL, J. & SCHILD, S. 2011. Extracellular nucleases and extracellular DNA play important roles in Vibrio cholerae biofilm formation. *Molecular Microbiology,* 82**,** 1015-1037.

SHAKIR, A., ELBADAWEY, M. R., SHIELDS, R. C., JAKUBOVICS, N. S. & BURGESS, J. G. 2012. Removal of Biofilms from Tracheoesophageal Speech Valves Using a Novel Marine Microbial Deoxyribonuclease. *Otolaryngology–Head and Neck Surgery,* 147**,** 509-514.

SHARMA, P., GARG, N., SHARMA, A., CAPALASH, N. & SINGH, R. 2019. Nucleases of bacterial pathogens as virulence factors, therapeutic targets and diagnostic markers. *International Journal of Medical Microbiology,* 309**,** 151354.

SHIELDS, R. C., MOKHTAR, N., FORD, M., HALL, M. J., BURGESS, J. G., ELBADAWEY, M. R. & JAKUBOVICS, N. S. 2013. Efficacy of a marine bacterial nuclease against biofilm forming microorganisms isolated from chronic rhinosinusitis. *PloS one,* 8**,** e55339-e55339.

SINGER, V. L., JONES, L. J., YUE, S. T. & HAUGLAND, R. P. 1997. Characterization of PicoGreen reagent and development of a fluorescence-based solution assay for double-stranded DNA quantitation. *Analytical biochemistry,* 249**,** 228-238.

SIQUEIRA, W. L., CUSTODIO, W. & MCDONALD, E. E. 2012. New Insights into the Composition and Functions of the Acquired Enamel Pellicle. *Journal of Dental Research,* 91**,** 1110-1118.

SOCRANSKY, S. S., HAFFAJEE, A. D., CUGINI, M. A., SMITH, C. & KENT JR., R. L. 1998. Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology,* 25**,** 134-144.

SOTO-BARRERAS, U., CORTÉS-SANDOVAL, G., DOMINGUEZ-PEREZ, R., LOYOLA-LEYVA, A., MARTINEZ-RODRIGUEZ, P.-R. & LOYOLA-RODRIGUEZ, J. P. 2017. Stimulatory effect of Aggregatibacter actinomycetemcomitans DNA on proinflammatory cytokine expression by human gingival fibroblasts. *Archives of Oral Biology,* 82**,** 127-133.

SPARWASSER, T., MIETHKE, T., LIPFORD, G., ERDMANN, A., HÄCKER, H., HEEG, K. & WAGNER, H. 1997. Macrophages sense pathogens via DNA motifs: induction of tumor necrosis factor-α-mediated shock. *European Journal of Immunology,* 27**,** 1671-1679.

STACEY, K. J., SWEET, M. J. & HUME, D. A. 1996. Macrophages ingest and are activated by bacterial DNA. *The Journal of Immunology,* 157**,** 2116.

STEICHEN, C. T., CHO, C., SHAO, J. Q. & APICELLA, M. A. 2011. The Neisseria gonorrhoeae Biofilm Matrix Contains DNA, and an Endogenous Nuclease Controls Its Incorporation. *Infection and Immunity,* 79**,** 1504.

STEINBERGER, R. E. & HOLDEN, P. A. 2005. Extracellular DNA in Single- and Multiple-Species Unsaturated Biofilms. *Applied and Environmental Microbiology,* 71**,** 5404.

STEWART, P. S. & COSTERTON, J. 2001. Antibiotic resistance of bacteria in biofilms. *The Lancet,* 358**,** 135-138.

SUCK, D. 1994. DNA recognition by DNase I. *Journal of Molecular Recognition,* 7**,** 65-70.

SULLIVAN, D., ROSS, R. P., FITZGERALD, G. F. & COFFEY, A. 2000. Investigation of the Relationship between Lysogeny and Lysis of Lactococcus lactis in Cheese Using Prophage-Targeted PCR. *Applied and Environmental Microbiology,* 66**,** 2192.

SUZUKI, N., NAKANO, Y., YOSHIDA, A., YAMASHITA, Y. & KIYOURA, Y. 2004. Real-Time TaqMan PCR for Quantifying Oral Bacteria during Biofilm Formation. *Journal of Clinical Microbiology,* 42**,** 3827.

SWARTJES, J. J. T. M., DAS, T., SHARIFI, S., SUBBIAHDOSS, G., SHARMA, P. K., KROM, B. P., BUSSCHER, H. J. & VAN DER MEI, H. C. 2013. A Functional DNase I Coating to Prevent Adhesion of Bacteria and the Formation of Biofilm. *Advanced Functional Materials,* 23**,** 2843-2849.

TAKAHASHI, N. 2015. Oral Microbiome Metabolism: From “Who Are They?” to “What Are They Doing?”. *Journal of Dental Research,* 94**,** 1628-1637.

TAN, K. H., SEERS, C. A., DASHPER, S. G., MITCHELL, H. L., PYKE, J. S., MEURIC, V., SLAKESKI, N., CLEAL, S. M., CHAMBERS, J. L., MCCONVILLE, M. J. & REYNOLDS, E. C. 2014. Porphyromonas gingivalis and Treponema denticola Exhibit Metabolic Symbioses: e1003955. *PLoS Pathogens,* 10**,** e1003955.

TAN, Y., MA, S., LEONHARD, M., MOSER, D., LUDWIG, R. & SCHNEIDER-STICKLER, B. 2020. Co-immobilization of cellobiose dehydrogenase and deoxyribonuclease I on chitosan nanoparticles against fungal/bacterial polymicrobial biofilms targeting both biofilm matrix and microorganisms. *Materials Science and Engineering: C,* 108**,** 110499.

TATEVOSSIAN, A. 1985. The effects of heat inactivation, tortuosity, extracellular polyglucan and ion-exchange sites on the diffusion of [14C]-sucrose in human dental plaque residue in vitro. *Archives of Oral Biology,* 30**,** 365-371.

THOMAS, V. C., HIROMASA, Y., HARMS, N., THURLOW, L., TOMICH, J. & HANCOCK, L. E. 2009. A fratricidal mechanism is responsible for eDNA

release and contributes to biofilm development of Enterococcus faecalis. *Molecular Microbiology,* 72**,** 1022-1036.

THURNHEER, T., BAO, K. & BELIBASAKIS, G. N. 2018. Subgingival Biofilms as Etiological Factors of Periodontal Disease. *In:* BOSTANCI, N. & BELIBASAKIS, G. N. (eds.) *Pathogenesis of Periodontal Diseases: Biological Concepts for Clinicians.* Cham: Springer International Publishing.

THURNHEER, T. & BELIBASAKIS, G. N. 2018. Streptococcus oralis maintains homeostasis in oral biofilms by antagonizing the cariogenic pathogen Streptococcus mutans. *Molecular Oral Microbiology,* 33**,** 234-239.

THURNHEER, T., BOSTANCI, N. & BELIBASAKIS, G. N. 2016. Microbial dynamics during conversion from supragingival to subgingival biofilms in an in vitro model. *Mol Oral Microbiol,* 31**,** 125-35.

THURNHEER, T., GMÜR, R., SHAPIRO, S. & GUGGENHEIM, B. 2003. Mass Transport of Macromolecules within an In Vitro Model of Supragingival Plaque. *Applied and Environmental Microbiology,* 69**,** 1702.

TINANOFF, N. & GROSS, A. 1976. Epithelial Cells Associated with the Development of Dental Plaque. *Journal of Dental Research,* 55**,** 580-583.

TOKUNAGA, T., YAMAMOTO, H., SHIMADA, S., ABE, H., FUKUDA, T., FUJISAWA, Y., FURUTANI, Y., YANO, O., KATAOKA, T., SUDO, T., MAKIGUCHI, N. & SUGANUMA, T. 1984. Antitumor Activity of Deoxyribonucleic Acid Fraction From Mycobacterium bovis BCG. I. Isolation, Physicochemical Characterization, and Antitumor Activity2. *JNCI: Journal of the National Cancer Institute,* 72**,** 955-962.

TONETTI, M. S., EICKHOLZ, P., LOOS, B. G., PAPAPANOU, P., VAN DER VELDEN, U., ARMITAGE, G., BOUCHARD, P., DEINZER, R., DIETRICH, T., HUGHES, F., KOCHER, T., LANG, N. P., LOPEZ, R., NEEDLEMAN, I., NEWTON, T., NIBALI, L., PRETZL, B., RAMSEIER, C., SANZ-SANCHEZ, I., SCHLAGENHAUF, U. & SUVAN, J. E. 2015. Principles in prevention of periodontal diseases. *Journal of Clinical Periodontology,* 42**,** S5-S11.

TRIBBLE, G. D., RIGNEY, T. W., DAO, D.-H. V., WONG, C. T., KERR, J. E., TAYLOR, B. E., PACHA, S. & KAPLAN, H. B. 2012. Natural Competence Is a Major Mechanism for Horizontal DNA Transfer in the Oral Pathogen Porphyromonas gingivalis. *mBio,* 3**,** e00231-11.

TSENG, B. S., ZHANG, W., HARRISON, J. J., QUACH, T. P., SONG, J. L., PENTERMAN, J., SINGH, P. K., CHOPP, D. L., PACKMAN, A. I. & PARSEK, M. R. 2013. The extracellular matrix protects Pseudomonas aeruginosa biofilms by limiting the penetration of tobramycin. *Environmental Microbiology,* 15**,** 2865-2878.

VALM, A. M. 2019. The Structure of Dental Plaque Microbial Communities in the Transition from Health to Dental Caries and Periodontal Disease. *Journal of Molecular Biology,* 431**,** 2957-2969.

VAN DER PLOEG, J. R. 2005. Regulation of Bacteriocin Production in Streptococcus mutans by the Quorum-Sensing System Required for Development of Genetic Competence. *Journal of Bacteriology,* 187**,** 3980.

VAN WINKELHOFF, A. J., LOOS, B. G., VAN DER REIJDEN, W. A. & VAN DER VELDEN, U. 2002. Porphyromonas gingivalis, Bacteroides forsythus and other putative periodontal pathogens in subjects with and without periodontal destruction. *Journal of Clinical Periodontology,* 29**,** 1023-1028.

VELUSAMY, S. K., SAMPATHKUMAR, V., RAMASUBBU, N., PASTER, B. J. & FINE, D. H. 2019. Aggregatibacter actinomycetemcomitans colonization and persistence in a primate model. *Proceedings of the National Academy of Sciences,* 116**,** 22307.

VITKOV, L., KLAPPACHER, M., HANNIG, M. & KRAUTGARTNER, W. D. 2009. Extracellular neutrophil traps in periodontitis. *Journal of Periodontal Research,* 44**,** 664-672.

VITKOV, L., KLAPPACHER, M., HANNIG, M. & KRAUTGARTNER, W. D. 2010. Neutrophil Fate in Gingival Crevicular Fluid. *Ultrastructural Pathology,* 34**,** 25-30.

WANG, B.-Y. & KURAMITSU, H. K. 2005. Interactions between Oral Bacteria: Inhibition of Streptococcus mutans Bacteriocin Production by Streptococcus gordonii. *Applied and Environmental Microbiology,* 71**,** 354.

WANG, S., LIU, X., LIU, H., ZHANG, L., GUO, Y., YU, S., WOZNIAK, D. J. & MA, L. Z. 2015. The exopolysaccharide Psl–eDNA interaction enables the formation of a biofilm skeleton in Pseudomonas aeruginosa. *Environmental Microbiology Reports,* 7**,** 330-340.

WARYAH, C. B., WELLS, K., ULLUWISHEWA, D., CHEN-TAN, N., GOGOI-TIWARI, J., RAVENSDALE, J., COSTANTINO, P., GÖKÇEN, A., VILCINSKAS, A., WIESNER, J. & MUKKUR, T. 2017. In Vitro Antimicrobial Efficacy of Tobramycin Against Staphylococcus aureus Biofilms in Combination With or Without DNase I and/or Dispersin B: A Preliminary Investigation. *Microb Drug Resist,* 23**,** 384-390.

WATTERS, C., FLEMING, D., BISHOP, D. & RUMBAUGH, K. P. 2016. Chapter Seven - Host Responses to Biofilm. *In:* SAN FRANCISCO, M. & SAN FRANCISCO, B. (eds.) *Progress in Molecular Biology and Translational Science.* Academic Press.

WECKE, J., KERSTEN, T., MADELA, K., MOTER, A., GÖBEL, U. B., FRIEDMANN, A. & BERNIMOULIN, J.-P. 2000. A novel technique for monitoring the development of bacterial biofilms in human periodontal pockets. *FEMS Microbiology Letters,* 191**,** 95-101.

WERNER, E., ROE, F., BUGNICOURT, A., FRANKLIN, M. J., HEYDORN, A., MOLIN, S., PITTS, B. & STEWART, P. S. 2004. Stratified Growth in Pseudomonas aeruginosa Biofilms. *Applied and Environmental Microbiology,* 70**,** 6188.

WHITCHURCH, C. B., TOLKER-NIELSEN, T., RAGAS, P. C. & MATTICK, J. S. 2002. Extracellular DNA Required for Bacterial Biofilm Formation. *Science,* 295**,** 1487.

WHITE, P. C., CHICCA, I. J., COOPER, P. R., MILWARD, M. R. & CHAPPLE, I. L. C. 2015. Neutrophil Extracellular Traps in Periodontitis: A Web of Intrigue. *Journal of Dental Research,* 95**,** 26-34.

WHITTAKER, CHRISTIANE M. KLIER, A. & KOLENBRANDER, P. E. 1996. MECHANISMS OF ADHESION BY ORAL BACTERIA. *Annual Review of Microbiology,* 50**,** 513-552.

WICKSTRÖM, C., HERZBERG, M. C., BEIGHTON, D. & SVENSÄTER, G. 2009. Proteolytic degradation of human salivary MUC5B by dental biofilms. *Microbiology (Reading, England),* 155**,** 2866-2872.

WILTON, M., CHARRON-MAZENOD, L., MOORE, R. & LEWENZA, S. 2016. Extracellular DNA Acidifies Biofilms and Induces Aminoglycoside Resistance in Pseudomonas aeruginosa. *Antimicrobial agents and chemotherapy,* 60**,** 544-553.

WITTENBERGER, C. L. & ANGELO, N. 1970. Purification and Properties of a Fructose-1,6-Diphosphate-Activated Lactate Dehydrogenase from <em>Streptococcus faecalis</em&gt. *Journal of Bacteriology,* 101**,** 717.

WOOD, S. R., KIRKHAM, J., MARSH, P. D., SHORE, R. C., NATTRESS, B. & ROBINSON, C. 2000. Architecture of Intact Natural Human Plaque Biofilms Studied by Confocal Laser Scanning Microscopy. *Journal of Dental Research,* 79**,** 21-27.

WRIGHT, C. J., BURNS, L. H., JACK, A. A., BACK, C. R., DUTTON, L. C., NOBBS, A. H., LAMONT, R. J. & JENKINSON, H. F. 2013. Microbial interactions in building of communities. *Molecular Oral Microbiology,* 28**,** 83-101.

XU, Y. & KRETH, J. 2013a. Role of LytF and AtlS in eDNA release by Streptococcus gordonii. *PloS one,* 8**,** e62339.

XU, Y. & KRETH, J. 2013b. Role of LytF and AtlS in eDNA release by Streptococcus gordonii. *PloS one,* 8**,** e62339-e62339.

YAMANAKA, T., YAMANE, K., FURUKAWA, T., MATSUMOTO-MASHIMO, C., SUGIMORI, C., NAMBU, T., OBATA, N., WALKER, C. B., LEUNG, K.-P. & FUKUSHIMA, H. 2011. Comparison of the virulence of exopolysaccharide-producing Prevotella intermedia to exopolysaccharide non-producing periodontopathic organisms. *BMC Infectious Diseases,* 11**,** 228.

YAMASHITA, Y., BOWEN, W. H., BURNE, R. A. & KURAMITSU, H. K. 1993. Role of the Streptococcus mutans gtf genes in caries induction in the specific-pathogen-free rat model. *Infection and Immunity,* 61**,** 3811.

YAN, J. & BASSLER, B. L. 2019. Surviving as a Community: Antibiotic Tolerance and Persistence in Bacterial Biofilms. *Cell Host & Microbe,* 26**,** 15-21.

YANG, X., BEYENAL, H., HARKIN, G. & LEWANDOWSKI, Z. 2000. Quantifying biofilm structure using image analysis. *Journal of Microbiological Methods,* 39**,** 109-119.

YASUNAGA, A., YOSHIDA, A., MORIKAWA, K., MAKI, K., NAKAMURA, S., SOH, I., AWANO, S. & ANSAI, T. 2013. Monitoring the prevalence of viable and dead cariogenic bacteria in oral specimens and in vitro biofilms by qPCR combined with propidium monoazide. *BMC Microbiology,* 13**,** 157.

YE, J., SHAO, C., ZHANG, X., GUO, X., GAO, P., CEN, Y., MA, S. & LIU, Y. 2017. Effects of DNase I coating of titanium on bacteria adhesion and biofilm formation. *Materials Science and Engineering: C,* 78**,** 738-747.

YU, M.-K., KIM, M.-A., ROSA, V., HWANG , Y.-C., DEL FABBRO, M., SOHN, W.-J. & MIN, K.-S. 2019. Role of extracellular DNA in Enterococcus faecalis biofilm formation and its susceptibility to sodium hypochlorite. *Journal of Applied Oral Science,* 27.

YU, W., HALLINEN, K. M. & WOOD, K. B. 2018. Interplay between Antibiotic Efficacy and Drug-Induced Lysis Underlies Enhanced Biofilm Formation at Subinhibitory Drug Concentrations. *Antimicrobial Agents and Chemotherapy,* 62**,** e01603-17.

YUAN, Q.-B., HUANG, Y.-M., WU, W.-B., ZUO, P., HU, N., ZHOU, Y.-Z. & ALVAREZ, P. J. J. 2019. Redistribution of intracellular and extracellular free & adsorbed antibiotic resistance genes through a wastewater treatment plant by an enhanced extracellular DNA extraction method with magnetic beads. *Environment International,* 131**,** 104986.

ZEE, K.-Y., SAMARANAYAKE, L. P. & ATTSTRÖM, R. 1997. Scanning electron microscopy of microbial colonization of 'rapid' and 'slow' dental-plaque formers in vivo. *Archives of Oral Biology,* 42**,** 735-742.

ZHANG, Y., SHI, W., SONG, Y. & WANG, J. 2019. Metatranscriptomic analysis of an in vitro biofilm model reveals strain-specific interactions among multiple bacterial species. *Journal of oral microbiology* [Online], 11. Available: http://europepmc.org/abstract/MED/31007867

https://doi.org/10.1080/20002297.2019.1599670

https://europepmc.org/articles/PMC6461087

https://europepmc.org/articles/PMC6461087?pdf=render [Accessed 2019].

ZHAO, Z., DING, C., WANG, Y., TAN, H. & LI, J. 2019. pH-Responsive polymeric nanocarriers for efficient killing of cariogenic bacteria in biofilms. *Biomaterials science,* 7**,** 1643-1651.

ZHENG, L., CHEN, Z., ITZEK, A., HERZBERG, M. C. & KRETH, J. 2012. CcpA regulates biofilm formation and competence in Streptococcus gordonii. *Molecular Oral Microbiology,* 27**,** 83-94.

ZHOU, Y., YANG, J., ZHANG, L., ZHOU, X., CISAR, J. O. & PALMER, R. J. 2016. Differential Utilization of Basic Proline-Rich Glycoproteins during Growth of Oral Bacteria in Saliva. *Applied and Environmental Microbiology,* 82**,** 5249.

ZHOU, Z., HU, F., HU, S., KONG, M., FENG, C., LIU, Y., CHENG, X., JI, Q. & CHEN, X. 2018. pH-Activated nanoparticles with targeting for the treatment of oral plaque biofilm. *Journal of materials chemistry. B,* 6**,** 586-592.

ZHU, B., MACLEOD, L. C., KITTEN, T. & XU, P. 2018. Streptococcus sanguinis biofilm formation & interaction with oral pathogens. *Future Microbiology,* 13**,** 915-932.

ZHUANG, Z., YOSHIZAWA-SMITH, S., GLOWACKI, A., MALTOS, K., PACHECO, C., SHEHABELDIN, M., MULKEEN, M., MYERS, N., CHONG, R., VERDELIS, K., GARLET, G. P., LITTLE, S. & SFEIR, C. 2018. Induction of

M2 Macrophages Prevents Bone Loss in Murine Periodontitis Models. *Journal of Dental Research,* 98**,** 200-208.

ZIJNGE, V., LEEUWEN, M. B. M. V., DEGENER, J. E., ABBAS, F., THURNHEER, T., GMÜR, R. & HARMSEN, H. J. M. 2010. Oral Biofilm Architecture on Natural Teeth. *PLoS One,* 5.

ZWEIG, M., SCHORK, S., KOERDT, A., SIEWERING, K., STERNBERG, C., THORMANN, K., ALBERS, S.-V., MOLIN, S. & VAN DER DOES, C. 2014. Secreted single-stranded DNA is involved in the initial phase of biofilm formation by Neisseria gonorrhoeae. *Environmental Microbiology,* 16**,** 1040-1052.