Investigation of biofilms associated with chronic otitis media with effusion and adenoids hypertrophy

Ali Mostafa Wayes

A thesis submitted for the degree of Doctor of Philosophy

Institute of Cellular Medicine

Centre of Oral Health Science, School of Medical Sciences

Faculty of Medical Sciences

December 2019

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

Chronic otitis media with effusion (COME) is the most common cause of acquired hearing loss in young children. Bacterial biofilm is an important contributor to the aetiopathogenesis of COME, although conventional culture generally recovers few microorganisms from the middle ear. Extracellular DNA (eDNA) is a key structural component within the matrix of many microbial biofilms including those associated with COME. The aims of this study were to characterise microbial populations associated with COME, and to explore the efficacy of the DNase, NucB, to control *in vitro* biofilms associated with COME.

Methods were established to culture biofilms *in vitro* and to challenge with NucB. NucB efficiently disrupted biofilms of clinically isolated *Staphylococcus aureus* strains either alone or in combination with the antibiotic Co-amoxiclav. Concentrations of NucB 100-fold higher than those required for biofilm inhibition had no toxic effects on human epithelial cells.

Twenty-seven bacterial species were isolated from middle ear effusion fluids (MEEFs) of 34 patients with COME. Culture-positive MEEFs were increased two-fold following optimisation of the culture methods. Microbiome analysis of MEEFs by 16S rDNA sequencing identified the majority of the cultured species and several additional species. Similar species were also detected by 16S rRNA gene sequencing of microbial DNA from adenoids.

The ability of 23 bacterial isolates from MEEF to form biofilm was assessed. Twenty strains formed biofilms, and 16 of these were sensitive to NucB. Imaging analysis showed significant structural alterations in biofilms of the selected COME isolates after NucB treatment.

In conclusion, this study has provided further insights into the microbiology of middle ear infections and has shown that many bacteria from this environment are capable of forming biofilms. NucB alone or in combination with antibiotics may potentially be a potent and safe agent to control biofilm-associated conditions including COME.

Acknowledgements

First and foremost, my most grateful thanks go to my supervisors Dr Nick Jakubovics, Mr. Mohamed Reda ElBadawey, and Prof Grant Burgess for their continuous support, and encouragement during my PhD study. They inspired me greatly to work in this project and in particular Dr Nick, who is an exceptional scientist and I have learnt so much from him.

I would like to acknowledge Iraqi Ministry of Higher Education and Scientific Research and Kirkuk University for granting my study leave and financially funding my PhD. I would like also to acknowledge Dr. Michael Ford, Department of Pathology, Freeman Hospital in Newcastle upon Tyne for microbial identification using MALDI-TOF analysis. My special thank you and acknowledgement go to Dr Jason Powell and Chris Ward for their collaboration in NucB safety studies. I would like to make a special thank you to Dr Alex Lude and Dr Trevor Booth for their training and dedicated guidance to get high quality microscopy images for my thesis. My special thank you to surgeons, theatre team, and nurses at the Department of ENT, Freeman Hospital, Newcastle upon Tyne for their outstanding help and support in clinical samples collection. I would like also to acknowledge all children and their families for participating in this project. My special thanks to Dr John Tylor and Prof Janet Wilson for the many discussions, contribution, and their fantastic suggestions in our meetings throughout my PhD journey. I would also like to thank the Society of Applied Microbiology and Newcastle University who co-funded conference travel grant.

I would like to thank all friends and staff in Oral Biology Lab and level 7 in Dental School, Newcastle University including Nadia, Becky, Waleed, Suhair, Nicola, Katya, Nisreen, Joy, Sandra, Mark, and Chris. Special thanks go to Mrs. Lesley Old for teaching me how to do many basic Microbiology techniques and for being very kind friend.

Most importantly, a massive THANK YOU goes to my family, mom, father (in heaven), and my caring, loving, supportive wife, Shaymaa: your exceptional support and encouragement when the times got rough are greatly appreciated. Last but not least, I would like to express my thanks and gratitude to the almighty God, Allah, for answering my praying and giving me the strength and the patience to overcome all the difficulties that I have faced during my study.

Abbreviations

°C	Degrees Celsius
µl	Microlitre
µg	Microgram
µM	Micromolar
µm	Micrometre
A. odontolyticus	*Actinomyces odontolyticus*
A. otitidis	*Alloiococcus otitidis*
AO	Acridine orange
AOM	Acute otitis media
B. licheniformis	*Bacillus licheniformis*
BEGM	Bronchial epithelial growth medium
BHYE	Brain heart infusion with yeast extract
CAMHB	Cation adjusted Mueller Hinton broth
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
COME	Chronic otitis media with effusion
CRS	Chronic rhinosinusitis
CSOM	Chronic suppurative otitis media
CTDNA	Calf thymus DNA
CV	Crystal violet
dB	Decibel
DNase	Deoxyribonuclease
EB	Ethidium bromide

ECM	Extracellular matrix
eDNA	Extracellular deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EEC	External ear canal
ELISA	Enzyme-linked immunosorbent assay
ENT	Ear, nose, and throat
EPS	Extracellular polymeric substances
EPS	Extracellular polymeric substances
ET	Eustachian tube
FAA	Fastidious anaerobe agar
FISH	Fluorescence *in situ* hybridization
g	Gram
GIT	Gastrointestinal tract
h	Hour
H. influenzae	*Haemophilus influenzae*
hBD-3	Host β-defensin-3
HCl	Hydrochloric acid
HMP	Human microbiome project
HMW	High molecular weight
HSD	Honestly Significant Difference
HU	Histone-like protein
IHF	Integration host factor
IRAS	Integrated Research Application System
L	Litre

LMW	Low molecular weight
LPR	Laryngopharyngeal reflux
M. catarrhalis	*Moraxella catarrhalis*
MALDI-TOF MS	Matrix assisted laser desorption/ionization time-of-flight mass spectroscopy
MBC	Minimum bactericidal concentration
MBEC	Minimum biofilm inhibitory concentration
MBF	Moderate biofilm formation
Mg	Magnesium
mg	Milligram
MIC	Minimum inhibitory concentration
min	Minute
ml	Millilitre
mM	Milimolar
MRSA	Methicillin resistant *S. aureus*
MTP	Microtitre plate
NA	Not applicable
NBF	No biofilm formation
NET	Neutrophil extracellular trap
ng	Nanogram
NICE	National Institute for Health and Care Excellence
NucB	Nuclease B
OD	Optical density
OSA	Obstructive sleep apnoea

OTU	Operational taxonomic unit
P. acnes	*Propionibacterium acnes*
P. aeruginosa	*Pseudomonas aeruginosa*
PAS	Polyanethole sulfonate
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Propidium iodide
PMMA	Polymethylmethacrylate
PNAG	Poly-N-acetylglucosamine
QC	Quality control
R&D	Research and Development
REC	Research ethic committee
rhDNase I	Recombinant human DNase I
rhDNase I	Recombinant human DNase I
$RLU.min^{-1}$	Relative Light Units per min
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RTF	Reduced transport fluid
S. aureus	*Staphylococcus aureus*
S. auricularis	*Staphylococcus auricularis*
S. mitis	*Streptococcus mitis*
S. oralis	*Streptococcus oralis*
S. parasanguinis	*Streptococcus parasanguinis*

S. pneumoniae	*Streptococcus pneumonia*
S. pyogenes	*Streptococcus pyogenes*
SBF	Strong biofilm formation
SD	Standard deviation
SE	Standard error
SEM	Scanning electron microscopy
SNPs	Single nucleotide polymorphisms
T. otitidis	*Turicella otitidis*
TESV	Tracheoesophageal speech valves
TLR	Toll-like receptor
TM	Tympanic membrane
TSB	Tryptone soya broth
TYEG	Tryptone yeast extract glucose
UK	United Kingdom
UKSMI	UK Standards for Microbiology Investigations
URTI	Upper respiratory tract infection
URTI	Upper respiratory tract infection
USA	United states of America
UV	Ultraviolet
VT	Ventilation tube
WBF	Weak biofilm formation

Table of contents

List of Figures

List of Tables

Chapter 1. Review of the literature

1.1 Chronic otitis media with effusion

Chronic otitis media with effusion (COME) is defined as the persistence of non-purulent middle ear fluid behind the intact ear drum (tympanic membrane) beyond 12 weeks in the absence of signs and symptoms of acute inflammation. It is distinct from persistent effusion after acute otitis media, which resolves spontaneously after two months in 90% of cases (Blanc et al., 2018). Hearing impairment is the most common presenting symptom of COME. The effusion fluid may be thin (serous) or thick (mucoid). When it is tenacious and very thick, it is also known as "glue ear" (Robb and Williamson, 2012). Histologically, COME is characterised by chronic inflammation in the middle ear mucosa which leads to overproduction of mucin-predominant middle ear effusion. Other components including bacteria have been identified in the middle ear effusion fluid (Kubba et al., 2000).

Chronic otitis media with effusion is the most common childhood ear problem and the commonest cause of hearing loss in children in the developed world. COME is commonly asymptomatic, and therefore accurate determination of its prevalence and incidence in children is difficult. Nevertheless, estimates demonstrate that children between one and six years old are most likely to be affected. The prevalence of COME in children around 2 years of age is approximately 20% (Atkinson et al., 2015). There are two peaks in the prevalence of COME, an initial one at the age of 2 years, and a second at the age of 5 years. The incidence rate of COME then decreases to around 8% at 8 years of age (Atkinson et al., 2015, Zielhuis et al., 1989). It is estimated that COME affects about 80% of children at some stage in their development (Kubba et al., 2000, NICE, 2016).

Although COME resolves spontaneously in most cases, complications are not uncommon. A persistence of hearing loss particularly in younger children may negatively impact speech, language development, behaviour, social skills, and school performance (Hall et al., 2009, Qureishi et al., 2014, Rosenfeld et al., 2016). In cases where COME persists for over 3 months of watchful waiting and has considerable effects on the child's quality of life, the insertion of ventilation tubes (VTs, grommets) may be indicated (Lous et al., 2005, NICE, 2016). Currently, tympanotomy and insertion of VT is the gold standard for surgical management of COME, and in some cases may be combined with an adenoidectomy (Qureishi et al., 2014, NICE, 2016). COME is the leading cause of elective surgery in children in the developed world (Rovers et al., 2004), and throughout 2018/2019, 22,000 operations of myringotomy with the insertion of VT were performed in England (HES, 2019).

COME should be differentiated from other closely related middle ear infections such as acute otitis media (AOM) and recurrent AOM (rAOM). Acute otitis media (AOM) involves a rapid onset of inflammation that may be caused by bacteria or viruses (Kubba et al., 2000, Schilder et al., 2016). It is characterised by otalgia and fever in a generally ill child. By contrast, COME is a chronic inflammatory condition with an absence of pain and fever that can persist for several months. Within 7 days of AOM, a proportion of cases may be complicated by tympanic membrane perforation which is associated with drainage of the purulent discharge out of the middle ear and subsequent improvement of the symptoms. Acute otitis media may also affect patients with existing VTs resulting in purulent ear discharge (otorrhoea) which is also classified as AOM with tympanic membrane perforation. Persistence of purulent otorrhoea for more than 2 weeks is described as chronic suppurative otitis media. Recurrent AOM is characterised by 3 or more episodes of AOM in 6 months or more than 4 episodes within 12 months (Berman, 1995, Schilder et al., 2016). The clinical types of otitis media are shown in Figure 1. 1 with an illustration of ear anatomy in Figure 1. 2.

The diagnostic criteria for COME include a history of hearing impairment, poor attention, delayed speech and language development, and abnormal behaviour (SIGN, 2003, Rosenfeld et al., 2016). Furthermore, COME is the leading cause of balance problems in childhood that are observed in approximately 50% of children with COME (Golz et al., 1998). The most important diagnostic method is pneumatic otoscopic examination of the tympanic membrane, which combines visual inspection of the tympanic membrane with assessment of its mobility, and has an estimated sensitivity and specificity of 94% and 80%, respectively (Shekelle et al., 2002). Other clinical findings are less clear, and include changes in the colour (yellow, amber, blue), shape (retracted or concave) of the ear drum, and ear-fluid levels. The gold standard for diagnosing COME is confirmation of middle ear effusion via needle aspiration of middle ear effusion fluid (tympanocentesis); however, tympanocentesis is an invasive procedure and difficult to perform in outpatient clinic. Further adjunctive confirmation of the diagnosis is obtained via tympanogram and tone audiometric measurement. The tympanogram quantitatively measures both tympanic membrane mobility and middle ear function, and usually demonstrates a flat curve. Many studies have demonstrated high reliability of the tympanogram for the diagnosis of COME (NICE 2008). The sensitivity and specificity are approximately 93% and 70%, respectively (NICE, 2008, Sassen et al., 1994, Shekelle et al., 2002, Watters et al., 1997). Audiometric examination can be applied on children around the age of 4 years. Most children with COME show a hearing impairment and can only detect sounds above 20-30 dB (normal hearing is between 0 and 20

dB). The extent of impairment is dependent on the amount of the middle ear fluid, but not the viscosity. In contrast to the tympanogram, the reliability of audiometry is not entirely clear and studies on this topic are rare. The estimated sensitivity and specificity are in the region of 52-88% and 53-92%, respectively (Haapaniemi, 1997, Mitchell et al., 1990, Vaughan-Jones and Mills, 1992).

Otitis Media

Acute otitis media (AOM): Acute onset of middle ear space infection.

Recurrent AOM (rAOM): 3 or more attacks of AOM within 6 months or more than 4 episodes within 12 months.

Chronic otitis media with effusion (COME): Accumulation of fluid in middle ear without signs and symptoms of acute infection beyond 12 weeks.

Chronic suppurative otitis media: Persistent middle ear inflammation usually associated with a perforated ear drum and persistent drainage from middle ear for more than 2-6 weeks.

Cholesteatoma: Abnormal skin cells accumulation in middle ear predisposing to chronic erosive inflammation.

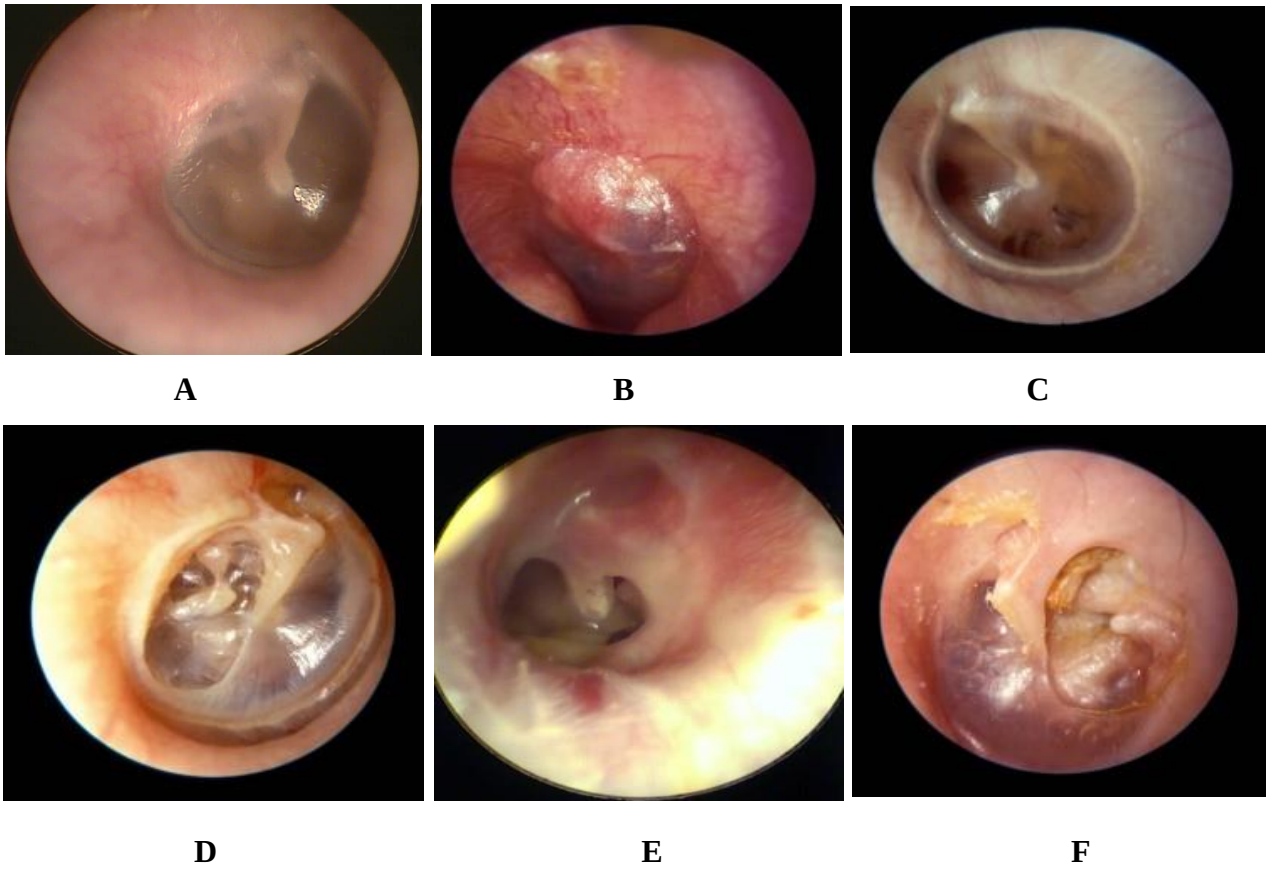


Figure 1. 1 Types of otitis media and clinical images

List of otitis media types (upper box). Clinical images show normal tympanic membrane (TM); pale, grey semi-translucent membrane (A), AOM; congested and bulged TM (B), COME; effusion fluid behind dull and retracted TM (C), severe retraction of TM (Atelectasis) (D), CSOM; perforated TM with purulent middle ear discharge (E), Cholesteatoma; whitish-yellow cystic skin lesion can be seen in the middle ear (F). A-D and F (Hawke, 2004), E (Rudyard, 2012).

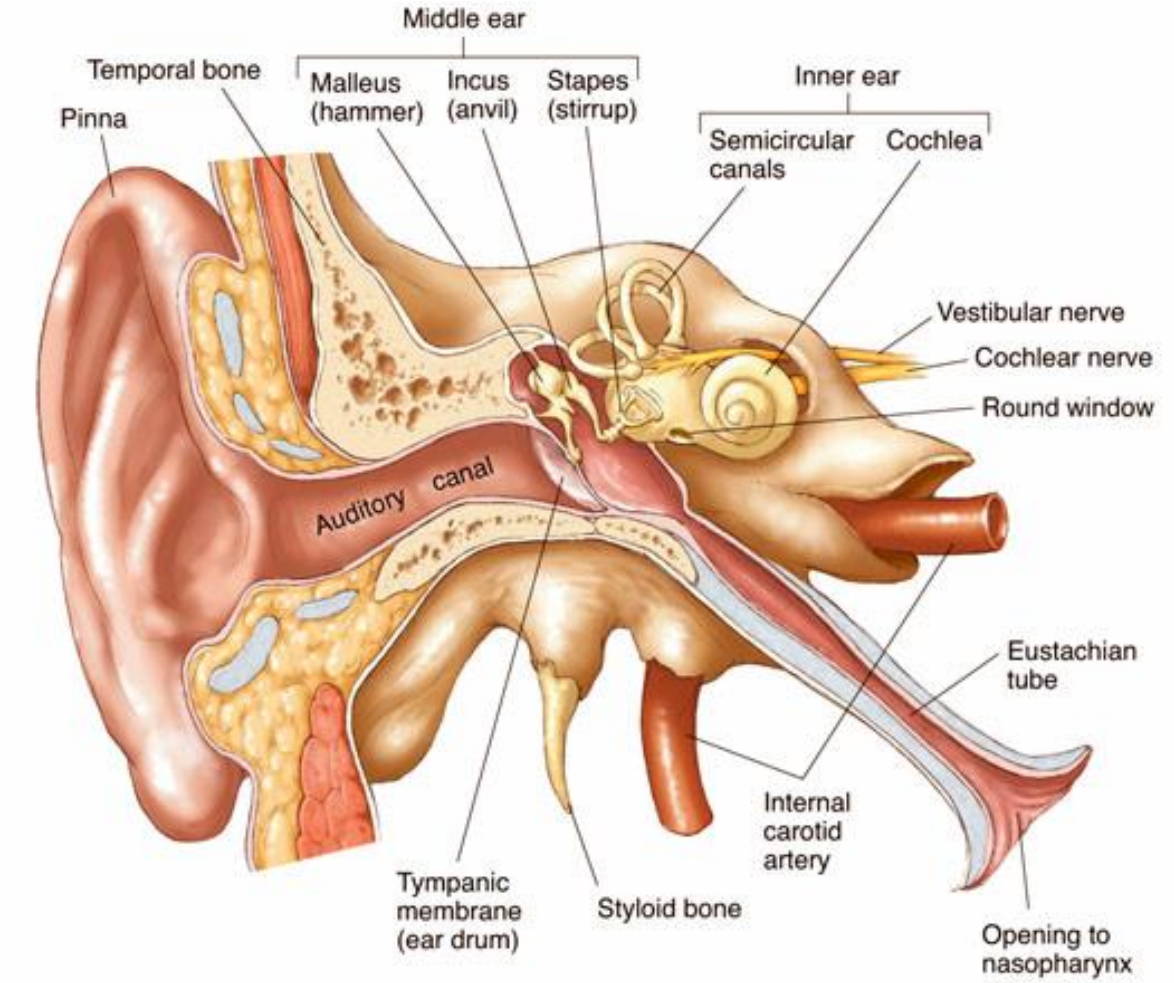


Figure 1. 2 Anatomy of the ear (Biographix, 2006)

The external ear formed by the pinna and external auditory canal is separated by the TM from the middle ear cavity that houses auditory ossicles (Malleus, Incus and Stapes). This in turn is connected to the nasopharynx via the Eustachian tube and is medially related to the inner ear that consists of semi-circular canals and the cochlea.

1.2 Current therapeutic options for COME

Children with COME should undergo an age-specific hearing test to establish their baseline hearing level. Visual response audiometry is commonly used to assess hearing in children aged 8 months to 2.5 years and can provide reliable results in infants as young as 6 months when performed by an experienced audiologist (Widen et al., 2000). Play audiometry is the most suitable hearing test to assess hearing in children aged 2.5-4 years. This involves children carrying out a task such as placing a toy in a container in response to an auditory stimulus (Rosenfeld et al., 2016). For children older than 4, formal audiometric examination using a pure tone audiogram is the gold standard test to assess hearing (Harlor and Bower, 2009). The current NICE guidelines recommend a 3-month period of active "watchful waiting" in children presenting with persistent bilateral (affecting both ears) COME unless the hearing loss is judged to have a significant impact on the child's developmental, social and educational status (NICE, 2016). During this "watchful waiting", all children with COME undergo frequent evaluations of their hearing loss and the impact it has on their developmental status prior to indicating the need for surgical intervention. However, there is frequent non- compliance with these guidelines (NICE 2008).

One reason for the lack of compliance with guidelines is that COME resolves spontaneously in the majority of cases without treatment and the possibility of the spontaneous resolution depends on the cause and duration of COME (Rosenfeld and Kay, 2003). Approximately 75-90% of children who develop COME following an episode of AOM show spontaneous resolution of the symptoms within 3 months (Teele et al., 1989). However, in children with COME of unknown duration, the rate of spontaneous resolution is estimated to be as low as 28% after 3 months, 42% at 6 months, and 59% by 9 months as determined by tympanometric criteria (Rosenfeld and Kay, 2003). Therefore, the decision between conservative treatment and surgical intervention should be weighed cautiously.

Many other medical treatments have been trialled for COME, such as antibiotics, steroids, and antihistamines. The use of antibiotics for the treatment of COME is controversial (Venekamp et al., 2016). Although short term benefits of antibiotic administration have been reported, there was no significant long-term benefit after discontinuation of the treatment (Venekamp et al., 2016, Rosenfeld et al., 2016). Furthermore, there are adverse effects, elevated costs, and a risk of bacterial resistance (van Zon et al., 2012, Venekamp et al., 2016).

A meta-analysis studied the effects of topical or oral steroids in conjunction with or without antibiotics for the treatment of COME (Simpson et al., 2011b). Although there was a

short-term benefit consistent with increasing COME resolution rate, there was no evidence of long-term advantages. Specifically, oral steroids did not significantly resolve the effusion or reduce the level of hearing loss in children with COME. However, steroids were associated with significant adverse effects including behavioural disturbances, weight gain, and idiosyncratic reactions (Berkman et al., 2013). The application of antibiotics, steroids, antihistamines, and decongestants either alone or in combination are not recommended in the UK (NICE, 2016). Additional conservative strategies include the Politzer manoeuvre to relieve Eustachian tube dysfunction by blowing air up the nostril using a balloon or other devices. However, these approaches, in order to be effective, should be carried out frequently and it is difficult for the younger children to comply with them (Bidarian-Moniri et al., 2014, Robb, 2006).

According to the current NICE guidelines, the only efficient surgical treatment to improve hearing is myringotomy with or without ventilation tube placement (NICE, 2016). This approach is recommended only in children with bilateral and persistent COME beyond 3 months with a degree of hearing loss degree in the better ear of 25-30 dB or more. According to the guidelines, surgical intervention is not recommended in children with short-term COME or when COME has a minimal or negligible impact on the child's quality of life and development. Different types of ventilation tubes have been used over the last decades. These are either short-term tubes such as Shepard, Shah, Armstrong and Reuter Bobine devices that last from 6 months to 24 months and are indicated for most children who undergo primary VT insertion, or long-term tubes including T-tubes and Permavents that last for several years and are generally used in cases where multiple sets of short acting VTs fail to resolve the disease (Lindstrom et al., 2004, Yaman et al., 2010).

The placement of VTs has been shown to reduce the duration of middle ear effusion symptoms by 32% and is also associated with short-term mild improvement of hearing loss by 4.2-10 dB at 6-9 months when compared with the conservative therapy. However, this improvement in hearing level was lost after 1 year (Berkman et al., 2013, Browning et al., 2010). Furthermore, the impact of VTs insertion on children's language development remains inconclusive. No improvement in language development was found in surgical treatment compared with conservative management of COME in several trials (Berkman et al., 2013, Browning et al., 2010). Nevertheless, the insertion of VTs has been shown to improve speech and language development in COME patients with cleft palate (Kuo et al., 2014).

Several short- and long-term complications have been associated with the insertion of VTs including purulent ear discharge (10%-26%), early expulsion of VTs (4%), displacement of VT into middle ear cavity (0.5%), tympanosclerosis (39%-65%), permanent ear drum perforation (3% overall, and around 24% of patients with long-term T-tubes), and retraction pockets (21%) (Kay et al., 2001, Rosenfeld et al., 2013). Furthermore, a trial reported that 20%-25 % of the cases treated with short-lasting tubes required further surgical intervention within 2 years, and on average more than two operations per case were performed (Daniel et al., 2012a, Vlastarakos et al., 2007).

Another surgical treatment which is considered effective in preventing the recurrence of COME is adenoidectomy as an adjunct treatment to VT placement. Adenoidectomy is not recommended in children younger than 4 years undergoing a primary grommet insertion because of COME unless there is a strong indication such as nasal obstruction or chronic adenoiditis that exists other than COME (NICE, 2016, Rosenfeld et al., 2016). This is because the surgical risks of adenoidectomy do not outweigh the limited, short-term advantage in young children without previous history of VT placement (Paradise et al., 1999). Risks associated with adenoidectomy include longer duration of anaesthesia, small risk of post-surgical bleeding and a prolonged recovery period (24-48 h). Therefore, adenoidectomy is usually preserved for children older than 4 years of age with recurrent or chronic upper respiratory tract infections (van den Aardweg et al., 2010, NICE, 2016).

1.3 Pathogenesis of COME

The pathogenesis of COME is still not well understood. There is a general agreement that COME is a multifactorial disease and a combination of several host and environmental factors are thought to be involved in the development of COME (Blanc et al., 2018, Qureishi et al., 2014). Eustachian tube dysfunction, laryngopharyngeal reflux, cleft palate, and several genetic factors are important host factors that predispose for the development of COME. Furthermore, there are a number of environmental factors that have been implicated in increasing the risk of AOM and COME such as bacterial infection, allergy, childcare attendance, exposure to tobacco smoke, and lack of breastfeeding. Many of these environmental factors also have an impact on general health in the population and they have been the subject of interest for policy makers and public health authorities. Regardless of the mechanism involved in pathogenesis of COME, the resulting middle ear effusion fluids generally consist of water, host cells and cell debris, bacterial cells and products, and many

other components that may be produced secondary to bacterial inflammatory reactions such as immunoglobulins, complement, cytokines, and others. In addition many other components such as minerals and macromolecules including proteins, lipids, mucins and DNA have been detected in middle ear effusions, indicating that middle ear fluid is an exudate (Kubba et al., 2000, Smirnova et al., 2002).

Eustachian tube (ET) dysfunction has been considered an important underlying pathophysiological factor implicated in the pathogenesis of COME. The ET is a muscular tube that provides a connection between the middle ear cavity and the nasopharynx (Figure 1. 2). It is lined by ciliated respiratory epithelium and it is involved in maintaining normal middle ear function through three essential roles: (i) pressure balance between the outer and middle ear, (ii) drainage of middle ear fluid, and (iii) prevention of retrograde ascension of pathogens and other external insults from the upper respiratory and gastrointestinal tracts into the middle ear (Rovers et al., 2004). In children, the ET is shorter, more horizontal, and less rigid than in adults, which is thought to increase the risk of otitis media (Minovi and Dazert, 2014). For this reason, the prevalence of COME decreases with age (approximately 3-7 years) in correlation with the normalisation of ET function. The potential role of ET dysfunction in the pathogenesis of COME has been studied extensively for decades. Traditionally, it has been thought that mechanical obstruction of the ET in the nasopharynx due to enlarged adenoids, nasopharyngeal mass or other factors leads to negative pressure within the middle ear cavity which ultimately results in transudation of fluid from the epithelial mucosa into middle ear space (Coticchia et al., 2013, Minovi and Dazert, 2014). This concept has some limitations including that middle ear effusion fluid in COME is an actively secreted exudate and it does not adequately explain the chronic inflammatory process and metaplasia associated with COME (Sade and Weissman, 1977).

The aetiopathogenesis of COME is much more complicated than can be explained by ET obstruction only. Evidence is accumulating that ET dysfunction, rather than ET obstruction, is the major contributor to the pathogenesis of COME. Children with premature, horizontally oriented malfunctioning ET are more likely to develop COME (Iwano et al., 1993). However, there is still some controversy regarding whether ET dysfunction is the primary cause of COME or whether it is the result of a prolonged chronic inflammatory reaction. In experimental animal models, it has been shown that creation of negative pressure within the middle ear cavity by ET occlusion did not lead to the accumulation of effusion fluid unless bacterial infection was present (Ovesen and Borglum, 1998). Furthermore, it is

still debated whether the observed therapeutic efficacy of adenoidectomy in children with recurrent COME is due to removal of the potential physical obstruction, or due to the removal of the reservoir that allows direct ascension of pathogens from the nasopharynx into the middle ear (Hoa et al., 2010, Saafan et al., 2013). A combination of ET dysfunction and retrograde ascending infection is perhaps the most likely mechanism that leads to the development of COME.

Pathologically, COME is a chronic inflammatory condition of the middle ear. The inflammatory irritation of the middle ear mucosa leads to the production of cytokines and secretion of an exudate rich in proteins and other inflammatory mediators. Furthermore, the associated vasodilatation enhances absorption of the air and ultimately induces negative pressure within the middle ear space (Li et al., 2013). Prolonged inflammation of the middle ear mucosa leads to epithelial metaplasia and an increase in the number of mucous secreting cells which leads to over-secretion of mucous and the production of altered mucin types (Kubba et al., 2000). These changes in the mucous result in the impairment of middle ear mucosal ciliary clearance action and ET dysfunction with subsequent retention of thick, tenacious, mucin-enriched middle ear fluid (Rovers et al., 2004). Although COME is clearly a chronic inflammatory condition, the source of the persistent inflammatory stimulus has been difficult to establish (Kubba et al., 2000). Chronic otitis media with effusion is hypothesized to be initiated by inflammation and immune reaction against ascending infections from the adenoid and nasopharynx (Vanneste and Page, 2019).

1.3.1 The role of bacteria in COME

Bacterial infections have been implicated to play a key role in the pathogenesis of AOM and COME. Lines of evidence that support an "infective aetiology" of COME include a strong association between AOM and COME: more than 50% of cases of COME are preceded by an episode of AOM (Kubba et al., 2000). Moreover, some of the high-risk groups of children such as younger siblings, nursery children, and absence of breast feeding, are those that are susceptible to infections, also supporting an infective aetiology of COME (Robb, 2006). In addition, as noted above, the nature of the effusion fluid in COME is an exudate not transudate, which is again consistent with a localised infection. However, lack of the typical signs and symptoms of acute bacterial infections such as ear pain, fever, and other constitutional symptoms is more difficult to rationalise with an infective aetiology.

COME has been considered by some researchers to be a non-infective inflammatory condition because of the failure to culture bacteria from middle ear effusion in approximately half of samples (21%-70%) (Bluestone et al., 1992, Gok et al., 2001, Hall-Stoodley et al., 2006). However, additional studies that examined middle ear effusions using polymerase chain reaction (PCR) found pathogenic bacterial DNA in more than 80% of culture-negative effusions (Kubba et al., 2000, Post et al., 1995, Rayner et al., 1998). Furthermore, this DNA is simultaneously present with a short half-life bacterial-specific mRNA, indicating the presence of metabolically active bacteria in culture-negative COME (Rayner et al., 1998). One hypothesis behind this inconsistency between the high negative cultures and high PCR-positive rate is that the majority of bacteria within the middle ear of patients with COME exist within a biofilm form and are not easily recovered and isolated (Fergie et al., 2004). Previous studies using experimental chinchilla otitis media models have demonstrated biofilm formation on the middle ear mucosa using confocal laser microscopy and/or scanning electron microscopy (SEM) (Ehrlich et al., 2002, Post, 2001).

Strong evidence for the role of biofilms in COME was obtained though the direct detection of bacterial biofilms by confocal laser microscopy on the middle ear mucosa of children with COME and rAOM in up to 92% of cases, but not in any studied control samples, strongly supporting the role of bacterial biofilms in the pathogenesis of COME and rAOM (Hall-Stoodley et al., 2006). Similarly, polymicrobial biofilms have been demonstrated on 65% of middle ear mucosal samples collected from 20 children with both COME and rAOM (Thornton et al., 2011). In addition to the polymicrobial biofilms, intracellular bacteria resembling cocci were also observed within epithelial cells of the middle ear mucosa using transmission electron microscopy. Using both universal and species-specific fluorescence *in situ* hybridization (FISH) probes, they were able to visualise unidentified bacterial species in addition to the three common otopathogens (*Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*) within these samples, suggesting that previously unidentified bacterial species are also involved in biofilm formation within the middle ear of COME and rAOM patients.

Furthermore, middle ear bacterial biofilms have also been demonstrated in patients with chronic suppurative OM (CSOM) (Homoe et al., 2009, Jensen et al., 2017) and with cholesteatoma (Chole and Faddis, 2002, Park et al., 2009). Recently, biofilms have also been shown on the surface of ventilation tubes removed from children with COME (Barakate et al.,

2007, Wang et al., 2014) or free floating within the middle ear effusion fluids recovered from patients with COME (Daniel et al., 2012b, Van Hoecke et al., 2016)

The most common bacterial species identified in patients with AOM are *S. pneumoniae* (27.8%), *H. influenzae* (23.1), and *M. catarrhalis* (7%). *H. influenzae* is more frequently isolated from patients with rAOM and AOM with a perforated tympanic membrane (*H. influenzae* 22.8% versus 18.6% for *S. pneumoniae* and 4.1% *M. catarrhalis* (Ngo et al., 2016). However, in patients with COME, the most commonly identified bacterial pathogen is *H. influenzae* (11.6%), followed by *S. pneumoniae* (6.5%), although bacteria are less likely to be identified compared to AOM (Ngo et al., 2016). All the three most commonly recognised otopathogens have been shown to have the ability to form biofilms *in vitro* and *in vivo* (Bakaletz, 2012, Hall-Stoodley and Stoodley, 2009, Silva and Sillankorva, 2019). *Staphylococcus aureus*, coagulase-negative staphylococci, *Veillonella* species, and numerous other bacterial pathogens are also often isolated from middle ear effusions (Bluestone et al., 1992, Hyden et al., 2006, Daniel et al., 2012b, Papp et al., 2016).

Studies suggest that the existence of biofilms on adenoids may be an essential pre-requisite for the initiation and development of biofilms within the middle ear, and that the adenoids potentially act as a reservoir for biofilm-forming otopathogens (Hoa et al., 2009, Hoa et al., 2010, Zuliani et al., 2009, Van Hoecke et al., 2016). With this in mind, it is believed that the therapeutic advantages of adenoidectomy are related to the debulking of nasopharyngeal biofilms, changing the microbial community in the nasopharynx, and minimizing the rate of *H. influenzae* and *S. pneumoniae* colonization (Aarts et al., 2010). Significantly higher biofilm formation on the adenoid mucosa was reported in children with rAOM and/or COME in comparison with children with adenoid hypertrophy only (Hoa et al., 2010, Saafan et al., 2013, Zuliani et al., 2009). The adenoids play key roles in regulating systemic and local immunity in children. However, evidence confirming their role in the pathogenesis of COME is scarce and further investigations are still required.

1.3.2 Other etiological theories for the pathogenesis of COME

A range of hypotheses have been proposed to explain the role of underlying pathophysiological factors that are thought to have a contribution in the pathogenesis of COME. A common process unifying these hypotheses is inflammation resulting in over-secretion and accumulation of thick and viscous mucus in the middle ear cavity combined

with impaired mucociliary clearance because of high mucous viscosity. The complex interaction of different risk factors predisposing to COME is illustrated in Figure 1. 3.

The potential role of ET dysfunction whether as a primary cause of inflammation or as a sequela of prolong inflammation that ultimately leads to development of COME are described in detail above. Several other host and environmental factors are also thought to have a role in COME including laryngopharyngeal reflux (LPR), respiratory allergy, genetic factors and exposure to tobacco smoking.

The link between LPR and COME has been suspected ever since digestive enzymes of the stomach such as pepsin and its proenzyme form, pepsinogen, were detected in high concentrations in middle ear aspirates of patients with COME (Miura et al., 2012, Formánek et al., 2015, Doğru et al., 2015). Furthermore, *Helicobacter pylori* DNA, retrieved from bacteria that primarily colonise the stomach and duodenum, have been detected in 6-18% of middle ear effusion samples using PCR and enzyme-linked immunosorbent assay (ELISA) (Boronat-Echeverría et al., 2016). However, despite these findings, a direct causal relationship between LPR and COME has not been determined (Miura et al., 2012, Kariya et al., 2014). Similarly, several published studies have suggested an association between respiratory allergy, specifically allergic rhinitis, and COME (Kreiner‐Møller et al., 2012, Pau and Ng, 2016). Again, a causal relationship has been difficult to prove, and no significant alteration in COME progression was demonstrated when anti-allergy treatments such as topical and oral steroids were used (Simpson et al., 2011a).

Chronic otitis media with effusion might also be initiated by the activation of certain inherited genes regulating mucin expression, mucin production, and host response against bacteria in the middle ear. Twelve such genes have been identified to date (Kubba et al., 2000, Vanneste and Page, 2019). Higher concentrations of mucins have been found to correlate with increased viscosity of middle ear effusions (Carrie et al., 1992), which can subsequently result in impaired mucociliary clearance of middle ear effusion. MUC5AC and MUC5B that are encoded at chromosome 11p15 are examples of respiratory mucin molecules that are thought to be involved in the accumulation of mucous in the middle ear cavity (Tsuboi et al., 2001, Kubba et al., 2000). Furthermore, hereditary diseases such as primary ciliary dyskinesia that affect mucociliary clearance and congenital malformations including cleft palate, Down syndrome and immune deficiency have also been associated with ET dysfunction followed by COME (Flynn et al., 2009, Hoffman et al., 2013, Qureishi et al., 2014). The association between COME and exposure to cigarette smoke has been highlighted in many studies,

however, a causal relationship has not yet been established. The presence of parental smoking has been shown to increase the risk of COME and rAOM by 66% (Jacoby et al., 2008), and the risk associated with passive smoking increases with age (Mansour et al., 2018). One potential mechanism is due to overexpression of mucin genes in response to passive smoking, which impairs the normal mucociliary function of the middle ear and leads to COME (Preciado et al., 2010).

Several clinical features of COME are consistent with biofilm infection including frequent spontaneous resolution, but higher rates of disease recurrence and the failure of antibiotic treatment to eradicate bacteria present in the middle ear (Harimaya et al., 2006). In addition, bacteria within biofilms may initiate low grade subacute inflammation and thus ultimately prolong the presence of middle ear effusion. However, dispersal of planktonic bacteria from the biofilm can result in an episode of acute infection with an associated intense inflammatory reaction. Therefore, children with COME are 5 times more likely to develop AOM compared to those without COME (Teele et al., 1989). A better understanding of the role of bacteria and biofilms in the pathogenesis of COME requires further investigation.

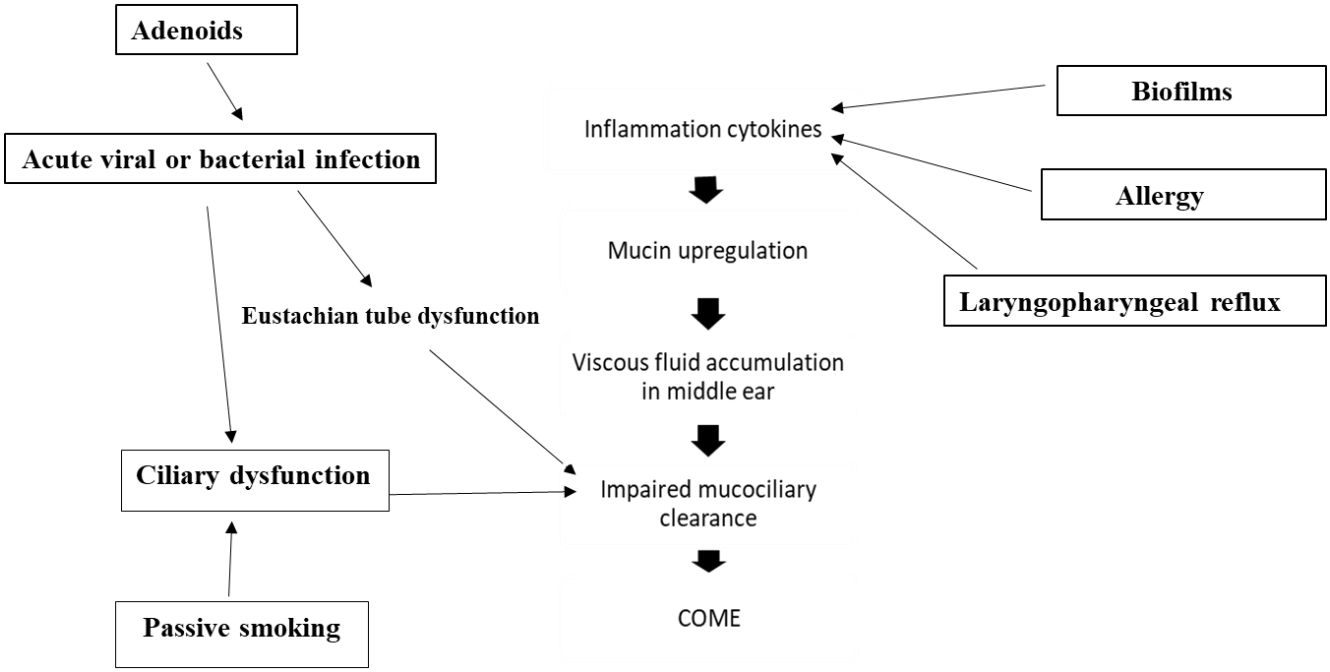


Figure 1. 3 Pathogenesis of COME, adapted from (Qureishi et al., 2014).

The figure shows the interaction of different host and environmental risk factors that are thought to have role in the pathogenesis of COME. A common mechanism unifying these factors is the inflammatory irritation of the middle ear mucosa leading to the production of cytokines, over-secretion and accumulation of thick and viscous mucus in the middle ear cavity combined with impaired mucociliary clearance that ultimately leads to development of COME. Eustachian tube dysfunction, either caused by an enlarged adenoid or as a sequela of acute viral or bacterial infection, leads to impaired mucociliary clearance that eventually result in COME. Prolonged inflammatory irritation induced by biofilms, allergy, and LPR can result in COME as described above. Exposure to cigarettes smoke or activation of certain inherited genes may lead to overexpression of mucin, which impairs the normal mucociliary function of the middle ear and leads to COME.

1.4 Bacterial biofilms and their formation

It has been estimated that approximately 40-80% of bacteria on Earth tend to aggregate on surfaces within biofilms rather than living in a planktonic (free-living) state (Flemming and Wuertz, 2019). The term ‘biofilm’ refers to an aggregation of microbial cells enclosed in a self-produced extracellular polymeric matrix that is irreversibly attached to an inert or living surface or interface (Costerton et al., 1999, Fergie et al., 2004, Yang et al., 2008). Parsek and Singh (2003) have proposed additional criteria to define medically relevant biofilm infections. These criteria include tolerance of biofilm cells to antibiotic treatment, despite the sensitivity of their planktonic counterparts to the antibiotic, and that the infection must be localised to a particular site. The protective structure of biofilms facilitates bacterial survival in hostile environments, the dissemination of genetic material, and resistance to the host's immune system (Hoiby et al., 2011, de la Fuente-Nunez et al., 2013).

The mechanism of biofilm formation and development has been extensively investigated in model microorganisms such as *Pseudomonas aeruginosa* (Sauer et al., 2002, Reichhardt and Parsek, 2019) and *Bacillus subtilis* (Vlamakis et al., 2013, Kovács and Dragoš, 2019). Basically, biofilm formation involves several phases initiated by reversible attachment of microorganisms to the surface which occurs when the attractive forces (Lifshitz-Van der Waals forces) overcome the repulsive forces (electrostatic forces on the microbial cell surface and substratum). This early phase is facilitated by the pre-coating of the substratum by a conditioning film, commonly consisting of proteins or glycoproteins, that forms on surfaces when they are exposed to a fluid or gas. Furthermore, complex interactions between other forces such as gravity, surface hydrophobicity, steering tensions, and Brownian motion can also influence initial microbial adhesion. Following the establishment of a stable attachment to the surface, the ability of microorganisms to stay irreversibly adherent is determined by a critical interaction between bacterial surface adhesins and specific receptors in the conditioning film (Gupta et al., 2016).

Following irreversible adhesion, microbes multiply, form micro-colonies and initiate production of extracellular matrix which enhances microbial adhesion to the surface, cell to cell cohesion and has many other functions. The mechanism of synthesis of the components of the biofilm matrix is not well understood. It has been proposed that quorum-sensing signals participate in *P. aeruginosa* and *Vibrio cholerae* polymeric matrix synthesis (Gupta et al., 2016, Bridges and Bassler, 2019). When the essential structure of the biofilms has

established, the biofilm enters the maturation phase in which the microorganisms multiply and aggregate, in some cases forming three-dimensional towers. Eventually, the bacterial cells in the outer layers will be detached and dispersed either individually or in aggregates to start a new cycle of surface colonisation and establishment of new biofilms (Otto, 2013). Bacterial dispersal and seeding from the biofilm surface is thought to be important for the dissemination of infections to the other parts of the host like a septic embolus (Fleming and Rumbaugh, 2018) and is driven by external forces such as the shearing force of high fluid flow (Oder et al., 2018) or by self-induced processes of dispersal such as enzymatic degradation of the extracellular polymeric matrix (Cho et al., 2015, Guilhen et al., 2017). Increasingly, these bacterial enzymes have been investigated for their potential to disperse pathogenic biofilms. Examples of these biofilm degrading microbial enzymes include the glycosidases dispersin B and alginate lyase, and deoxyribonucleases such as NucB of *Bacillus licheniformis* (Nijland et al., 2010, Fleming and Rumbaugh, 2017, Bou Haidar et al., 2020).

1.5 Biofilms and their clinical importance

Bacteria residing in biofilms display complex biological characteristics and activities. For example, cell-to-cell communication through quorum sensing involves dissemination of signalling molecules and genetic material among microbes which impacts on microbial virulence, co-aggregation, biofilm structure, and responses to environmental stresses (Hall-Stoodley and Stoodley, 2009, Wolska et al., 2016). Furthermore, the high tolerance of the biofilm to antibiotic treatment and host immune actions is linked to complex microbial communities within biofilms and the protective functions of the biofilm matrix (Hoiby et al., 2011, Flemming et al., 2016).

The potential role of bacterial biofilms in chronic infections was first described when *P. aeruginosa* was found colonizing the bronchial tree of patients with cystic fibrosis (Hoiby et al., 1977, Lam et al., 1980). Subsequently, biofilms were shown to contribute to the pathogenesis of chronic human infections such as chronic rhinosinusitis (CRS), otitis media, dental caries and others (Jakubovics, 2015, Van Hoecke et al., 2016, Rostami et al., 2017, Di Luca et al., 2017). Moreover, the increasing application of different kinds of medical implants poses challenges for controlling biofilm infections since artificial surfaces within the body provide new sites for microbial attachment and colonisation resulting in biofilm formation on almost all kinds of indwelling medical implants and devices (Donlan and Costerton, 2002, Arciola et al., 2018, Zatorska et al., 2017).

The US National Institutes of Health have estimated that biofilms account for over 80% of chronic infections and 65% of all microbial infections in the human body (Jamal et al., 2018). The role of bacterial biofilms in the pathogenesis of chronic infectious diseases can be difficult to establish because microorganisms residing within biofilms tend to resist traditional culture methods (Hall-Stoodley et al., 2006). Parsek and Singh (2003) have proposed a number of specific criteria that should be present in a medically relevant infection in order to be considered as a biofilm infection. These criteria include (i) bacterial attachment to a surface or substratum, (ii) bacterial aggregates or micro-colonies should be embedded in extracellular matrix, (iii) the infection is generally localised to one site, and (iv) biofilm cells are resistant to eradication by antibiotics, despite the sensitivity of planktonic counterparts to the same antibiotic. Later on, an additional feature was suggested to be added to the diagnostic criteria of biofilm infections by Hall-Stoodley and Stoodley (2009): evidence of impaired host immunity against bacterial aggregates in culture-negative cases with documented signs and symptoms of bacterial infection. Visualisation of localised bacterial aggregates surrounded by immune cells such as neutrophils and macrophages significantly increases the suspicion of biofilm infection. Furthermore, additional culture-independent diagnostic tools such as PCR and FISH were also recommended in addition to traditional culture approaches to increase the detection rate of bacteria within biofilms (Hall-Stoodley et al., 2006). Increasingly, other culture-independent molecular based techniques such as high throughput 16S rRNA gene sequencing are being employed to identify bacteria which are fastidious, non-cultivable, and those that are living within biofilms (Di Luca et al., 2017). Culture-independent molecular analysis is discussed in more detail in sections 1.8 and 1.9.

Due to the intrinsic tolerance of bacteria within biofilms to antimicrobial therapy and the immune response, the treatment and prevention of biofilm-associated chronic infections have been difficult. The protective organisation of biofilms enhances bacterial survival against different external stresses such as UV light, heavy metals, and changes in temperature, moisture, acidity, and host phagocytic defences (Espeland and Wetzel, 2001, Le Magrex-Debar et al., 2000, Leid et al., 2002, Teitzel and Parsek, 2003, Flemming et al., 2016). Furthermore, bacteria in biofilms exhibit tolerance to high concentrations of antimicrobials, typically more than 1,000 times higher than their planktonic counterparts. Different possible mechanisms have been proposed to explain this characteristic (Singh et al., 2017, Flemming et al., 2016). These include: (i) the nature of biofilm matrix impairs the penetration of antibiotics to reach bacteria within biofilms, (ii) diverse physiochemical niches within biofilm structure may affect antimicrobial efficacy directly or indirectly by altering the growth of

microorganisms in the anaerobic environment within the core of the biofilm, (iii) surface-attached bacterial cells in biofilms exhibit phenotypic and genotypic alterations consistent with changes in gene transcription and physiologic parameters that may ultimately affect their sensitivity to antibiotics, (iv) the presence of metabolically dormant populations of bacterial cells known as persister cells which exhibit high tolerance to antimicrobial agents, (v) slow bacterial growth rate makes them less vulnerable to antibiotics. These mechanisms can act individually or in combination (Yan and Bassler, 2019).

Bacteria within biofilms behave differently from their planktonic counterparts. Therefore, treatment of biofilm infections using planktonic bacteria-based Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) is often not suitable (Andrews, 2001). The need for an assay that can provide relevant and effective antibiotic dose recommendations for the eradication of bacterial biofilms led to the development of Minimum Biofilm Eradication Concentration (MBEC) assay as an alternative to MIC (Ceri et al., 1999). Minimum Biofilm Eradication Concentration is up to 1,000 times greater than MIC. Unfortunately, it is often not possible to deliver such high effective concentrations of antimicrobial agents due to toxicity. Therefore, attention has been directed towards the development of anti-biofilm agents that can return bacteria to their susceptible planktonic state. Extracellular polymeric substances (EPS) in the matrix of biofilms provide bacteria within biofilms with a wide range of protective advantages that will be discussed in the next section. Several components of EPS, including proteins, extracellular DNA (eDNA), and exopolysaccharides can be involved in these protective functions (Fleming and Rumbaugh, 2017). Of these components, eDNA has increasingly been shown to be a key structural component within the biofilm matrix of many bacteria that plays a pivotal role in enhancing biofilm formation and maintaining the structural integrity of biofilms (Jakubovics et al., 2013, Jakubovics and Burgess, 2015, Okshevsky et al., 2015).

1.6 Extracellular DNA and its role in bacterial biofilms

The amount and the composition of EPS in biofilms differ greatly among different microbial species. It has been estimated that EPS constitutes around 90% of the overall biomass in many biofilms, whereas the microbial cells accounts for less than 10% (Flemming and Wingender, 2010). The EPS of microbial biofilms plays a key role in maintaining the stable adhesion of the bacterial colonies to the substratum, boosting cell to cell cohesion, conserving nutrients, and disseminating genetic material. Furthermore, EPS helps to protect the microorganisms from external stresses such as heavy metals, antibiotics, and host

phagocytic activity (Flemming et al., 2016). Originally, polysaccharides were considered ubiquitous and thought to be the key component of biofilm EPS, hence it was often known as "exopolysaccharides". Later on, further investigations demonstrated that there are numerous other macromolecules including eDNA, proteins, and lipids present in considerable amounts in the biofilm matrix (Flemming and Wingender, 2010, Fleming and Rumbaugh, 2017). The heterogeneous composition of biofilm EPS, in addition to the complexity of the mechanisms via which the EPS components are produced and act, make it difficult to target bacteria within biofilms using traditional antimicrobial agents and disinfection procedures. Therefore, some researchers have switched their focus to targeting the EPS of biofilms to interfere with its protective functions, and disperse the bacteria which are sheltered within it. In theory, the release of bacterial cells from biofilms will enhance their susceptibility to antimicrobial treatment and host defence actions. One approach to target extracellular matrices of biofilms is through the application of DNase enzymes to degrade eDNA in the matrix (Jakubovics et al., 2013, Okshevsky et al., 2015).

Initially, eDNA was described as a "slime" enclosing bacteria and predisposing to the formation of a thin film in broth cultures (Catlin, 1956, Catlin and Cunningham, 1958). This eDNA was observed in *Staphylococcus aureus* broth cultures in spite of the well-known ability of *S. aureus* to synthesize abundant amounts of DNase enzymes. Importantly, inhibition of DNase in *S. aureus*, using low calcium with low pH culture conditions, was associated with excess production of eDNA (Catlin and Cunningham, 1958) . The critical role of eDNA in maintaining the structural integrity of bacterial biofilms was first described by Whitchurch et al. (2002) when they found substantial dispersal of *P. aeruginosa* biofilms treated with DNase I enzyme. These observations have led researchers to focus on understanding the structural roles of eDNA with the EPS of biofilms formed by wide range of microorganisms. The exact role of eDNA within biofilm is not well-understood. However, eDNA appears to have several functions within the extracellular matrix of many bacterial biofilms. These include facilitating the initial adhesion of microbial cells, maintaining the structural stability of biofilm, promoting the exchange of genetic material, acting as a nutrient source, and providing protection against antimicrobial and host defence actions.

1.6.1 Extracellular DNA enhances biofilm formation and structural stability

As mentioned earlier, the first study that discovered the role of eDNA in supporting the structural stability of *P. aeruginosa* biofilm was performed by Whitchurch *et al*. using

DNase I. Subsequently, several other investigations have confirmed important roles of eDNA in enhancing bacterial adhesion and maintaining the structural stability of biofilm using DNase enzymes as a tool (Table 1. 1). The amount of eDNA present within biofilm matrix varies greatly among different bacterial strains and is not necessarily related to its role in maintaining the structural integrity of biofilms (Izano et al., 2008, Mann et al., 2009). Importantly, mature biofilms have been shown to be less sensitive to DNase treatment in comparison to newly formed biofilms. This finding has been found in biofilms formed by different bacterial species such as *Staphylococcus epidermidis* (susceptibility to DNase reduced at 12 hours) (Qin et al., 2007b), *P. aeruginosa* (after 80 hours) (Whitchurch et al., 2002), and for *Vibrio cholerae* (after 72 hours) (Seper et al., 2011b). In contrast, efficient disruption of young biofilms as a result of an exogenous addition of DNase suggests that the eDNA plays a key role in maintaining the structural stability of newly formed biofilms (Fredheim et al., 2009, Harmsen et al., 2010, Nijland et al., 2010, Tetz et al., 2009, Rose et al., 2015, Ibáñez de Aldecoa et al., 2017). The reasons for the observed reductions in the efficacy of DNases against mature biofilms are not well understood. However, the transient sensitivity of young biofilms to DNase treatment suggests either that other components of EPS such as exopolysaccharides or proteins are replacing or complementing the structural role of eDNA within mature biofilms or that eDNA is being protected from the enzymatic degradation of DNases by forming complexes with other components of the matrix (Okshevsky et al., 2014, Sugimoto et al., 2018, Zetzmann et al., 2015b). Alternatively, eDNA may form duplexes with RNA over time. It has recently been shown that eDNA in *Pseudomonas* spp. biofilms forms complexes with RNA including non-canonical base paired structures that contribute to the elasticity of the biofilm (Seviour et al., 2019).

Several studies have investigated the role of eDNA in enhancing bacterial adhesion in the early phase of biofilm formation. For instance, in the case of *Bacillus cereus*, eDNA surrounded bacterial cells and enhanced biofilm formation when cells reached the exponential phase of growth, whereas these cells did not exhibit the same behaviour during the stationary growth phase (Vilain et al., 2009). In contrast, other studies have shown that *Bacillus cereus* and *S. pneumoniae* produce eDNA in the stationary growth phase (Lorenz et al., 1991, Moscoso and Claverys, 2004). This difference may be due to different methods, growth conditions and bacterial species used in these studies.

Furthermore, other studies also have highlighted the important role of eDNA in facilitating the initial attachment of *S. epidermidis* and *Acidovorax temporans* cells to abiotic substrata such as glass or polystyrene and glass wool (Heijstra et al., 2009, Qin et al., 2007a).

However, subsequent investigations suggested that eDNA requires other components to enhance the early adhesion. This may explain why numerous DNase treated bacterial species cannot regain their biofilm formation capability by the exogenous addition of DNA (Lappann et al., 2010, Harmsen et al., 2010, Das et al., 2011). Dispersal of *Listeria monocytogenes* biofilms using exogenous DNase I demonstrated that eDNA is necessary for the initial attachment of young biofilm. However, in the absence of eDNA, it was shown that the addition of genomic DNA could not restore the ability of *L. monocytogenes* cells to adhere and form biofilm in the absence of other essential components, such as N-acetylglucosamine. These observations indicate that the interaction of other components with eDNA is necessary for eDNA-mediated biofilm formation (Harmsen *et al.*, 2010).

Indeed, it seems that eDNA enhances early microbial adhesion to the surface and boosts cell to cell cohesion in young biofilms. However, the role of eDNA in maintaining the structural integrity of mature biofilms and its interaction with other components in the matrix requires further investigation.

1.6.2 Extracellular DNA provides protection from antimicrobial and host defense action

Potential mechanisms contributing to the observed increased tolerance of biofilms bacteria to antimicrobials were described in section 1.5. Impaired penetration of antibiotics through EPS is suggested to be caused by interactions of matrix components such as proteins and exopolysaccharide with antibiotics (Flemming and Wingender, 2010). The protective role of eDNA against antibiotics and host immune action is linked to its ability to bind and chelate host-produced cationic antimicrobial peptides or cationic antimicrobials. For instance, eDNA has been shown to bind and inhibit the antimicrobial activity of the antimicrobial host defence protein β-defensin-3 (hBD-3) against biofilms formed by non-typeable *H. influenzae*, a pathogen commonly implicated in COME (Jones et al., 2013). Interestingly, exogenous addition of both DNase I and recombinant hBD-3 led to substantial decreases in biofilm formation and enhanced the antimicrobial action of hBD-3. Similarly, the susceptibility of *P. aeruginosa* biofilms to aminoglycoside antibiotics was reduced by exogenous addition of DNA through the same mechanism (Chiang et al., 2013).

Numerous other investigations have demonstrated that eDNA can indirectly enhance resistance of biofilms to antibiotics by chelating divalent cations such as Mg^{+2}, Mn^{+2}, Zn^{+2}, and Ca^{+2} which leads to expression of genes or other virulence factors that modify physiological parameters of bacterial cells and make them more tolerant to cationic antibiotics and antimicrobial peptides (Johnson et al., 2012, Mulcahy et al., 2008). Furthermore, acidification produced by eDNA within biofilms formed by *P. aeruginosa* is a stimulus to induce resistance against aminoglycosides and antimicrobial peptides (Mulcahy et al., 2008, Wilton et al., 2016, Lewenza, 2013). However, eDNA may also have a negative impact on the viability of bacterial cells. It has been shown that excess eDNA can significantly affect the integrity of bacterial cell membrane by chelating cations such as Mg^{+2} and Ca^{+2} and leading to bacterial cell lysis (Mulcahy et al., 2008). It is unknown whether excess eDNA will have same effect on pathogenic bacteria *in vivo*.

Moreover, bacterial eDNA is thought to play a role in stimulating immune inflammatory reactions in the lungs of patients with cystic fibrosis (Lipford et al., 1998). Human inflammatory cells, such as macrophages, lymphocytes and dendritic cells identify eDNA on the bacterial surface as a foreign antigen. This results in triggering host innate immune action by stimulating Toll-like receptors of host immune cells (Pietrocola et al., 2011, Dapunt et al., 2016). The inflammatory reaction associated with chronic biofilm-mediated diseases such as COME and CRS may also be attributed to the role of bacterial

surface eDNA in triggering the host immune system. Although, eDNA can play a role in stimulating host immunity against pathogenic bacteria, bacteria within biofilms can develop complex protective strategies against host immune action. A good example is that biofilm growth can increase *S. aureus* resistance to the phagocytic action of host macrophages (Thurlow et al., 2011, Herzog et al., 2019). There is mounting evidence that eDNA is a key component within biofilm matrix of a wide range of microorganisms, and its critical roles in enhancing biofilm formation and maintaining biofilm stability outweigh the disadvantage of triggering host immune responses (Jakubovics et al., 2013, Okshevsky et al., 2015).

1.6.3 eDNA origin and turnover

Extracellular DNA is a ubiquitous structural component with the EPS of biofilms formed by many bacteria. Several studies have investigated the origin (whether plasmid DNA, or genomic DNA, or something else) or production of eDNA in bacterial biofilms such as *L. monocytogenes*, *P. aeruginosa*, and *Aspergillus fumigatus* (Harmsen et al., 2010, Steinberger and Holden, 2005, Allesen-Holm et al., 2006, Rajendran et al., 2013). Most of these studies found that eDNA in the biofilm matrix is identical to genomic DNA in mono-species biofilms. However, in multispecies biofilms, one study found that eDNA within biofilm matrix formed by four different species was significantly different from either genomic DNA of cells or total DNA of the same multispecies biofilm (Steinberger and Holden, 2005). Furthermore, it has been shown that eDNA within biofilms present in the sputum of patients with cystic fibrosis mostly originates from the human body (Lethem et al., 1990, Gray et al., 2018). It was also shown that the presence of some cellular components of host neutrophils can serve as biological matrix to enhance biofilm formation by *P. aeruginosa* (Walker et al., 2005). The role of eDNA in enhancing the ability of *E. coli* to form biofilms has been shown to be unrelated to its origin. The exogenous addition of genomic, plasmid, and even mammalian DNA to *E. coli* cultures did not enhance the ability of *E. coli* to form biofilm (Liu et al., 2012). However, further investigations are still required to validate these findings.

Many studies have shown that cell lysis is the main source for the release of eDNA into the extracellular compartment. Cells lysis leading to eDNA release in bacterial biofilms is mediated by different mechanisms. For instance, in *P. aeruginosa* and *S. pneumoniae*, it was shown that bacteriophage-mediated lysis constitutes an important source of eDNA for the biofilm matrix (Carrolo et al., 2010, Webb et al., 2003). In several species of *Streptococcus*,

including *S. pneumoniae*, eDNA production is thought to mediated by production of hydrogen peroxide (Regev-Yochay et al., 2006). Autolysis is the most commonly described mechanism of cell lysis, which is similar in some regards to apoptosis or programmed cell death in eukaryotes (Rice and Bayles, 2003, Bao et al., 2015, Domenech and Garcia, 2018) . In microbial fratricide a proportion of cells in a microbial population trigger the lysis of their siblings. The eDNA released from damaged cells enhances biofilm formation and coaggregation of the rest of microbial population. Several studies have shown that autolysins play a key role in quorum sensing-based cell lysis. Deletion of genes encoding these autolysins in *S. aureus* resulted in mutants lacking biofilm formation capacity (Bao et al., 2015). Autolysins are surface-attached enzymes that can degrade peptidoglycan. Their roles in eDNA production and biofilm formation have been extensively studied in several bacteria including *S. epidermidis* (Qin et al., 2007b, Wu et al., 2018), *S. aureus* (Mann et al., 2009, Fernández et al., 2017), *Neisseria meningitidis* (Lappann et al., 2010, Sigurlasdottir et al., 2019), *S. warneri* (Yokoi et al., 2008), and *S. pneumoniae* (Havarstein et al., 2006, Domenech and Garcia, 2018).

Another form of fratricide-mediated cell lysis has been described in *E. faecalis* biofilms, and is linked to protease (gelatinase)-mediated cell lysis involving a minor proportion of bacterial cells lacking the quorum sensing and gelatinase production (Thomas et al., 2009). Production of gelatinase by the overwhelming predatory population causes fratricidal cell lysis of a minority prey subpopulation. Co-expression of an immunity protein by the predatory population protects them from the cell lysis activity of their gelatinase. Another suggested mechanism leading to eDNA release in bacterial cells is the formation of DNA-containing membrane vesicles (Dorward and Garon, 1990, Schooling et al., 2009, Grande et al., 2015, Puca et al., 2019) . These vesicles have been demonstrated in exponentially growing *Acinetobacter baunmannii* (Sahu et al., 2012), in single-species biofilms of *P. aeruginosa*, and in mixed-species biofilms of dental plaque (Schooling and Beveridge, 2006, Frank, 1970, Holliday et al., 2015). Furthermore, the formation of vesicles in *Pseudomonas* spp. has been associated with increased biofilm formation (Baumgarten et al., 2012). It has been shown that these membrane vesicles can fuse to the outer membrane of cells in Gram-negative bacteria and attach to the external cell wall of Gram-positive bacteria (Domenech and Garcia, 2018).

A genomic screen of 3,985 non-lethal mutations in *E. coli* has shown that the global regulator *hns* gene is required for eDNA production. Deletion of the *hns* gene was associated with a dramatic reduction in eDNA production, but only a mild decrease in cell lysis and a

three-fold increase in membrane vesicle production. Therefore, it appears that there is another mechanism for eDNA release in *E. coli* cells lacking *hns*, possibly the active secretion of eDNA from living bacterial cells (Sanchez-Torres et al., 2010). In *N. gonorrhoeae*, a genetic-based eDNA releasing system via the type IV secretion system was observed in 80% of bacterial cell genomes, and disruption of this secretion system led to a profound reduction in eDNA release (Hamilton et al., 2005). Recently, Jurcisek et al. (2017) have shown that eDNA and associated DNABII proteins can also be actively released to the extracellular matrix of *H. influenzae* biofilms. These components are translocated from the bacterial cytoplasm to the periplasm via an inner-membrane pore complex (TraC and TraG) with homology to the type IV secretion system. The exact contribution of each mechanism to the total eDNA present within the extracellular matrix still not well understood (Jakubovics et al., 2013).

Many microbial species have been shown to produce extracellular DNase enzymes that are either attached to the bacterial cell wall or released to the extracellular niche (Jakubovics et al., 2013). The secretion of DNases by various bacterial strains seem to be independent of their reliance on eDNA during biofilm formation (Shields et al., 2013). The release of extracellular DNase enzymes appears to be a common feature for wide range of microorganisms, including *Cryptococcus neoformans* (Almeida et al., 2015), many oral bacteria (Palmer et al., 2012, Jakubovics and Burgess, 2015), anaerobic bacteria such as *Fusobacterium* spp. (Porschen and Sonntag, 1974, Doke et al., 2017), *S. pneumoniae* (Zhu et al., 2013, Jhelum et al., 2018), *Streptococcus pyogenes* (Hasegawa et al., 2010, Chalmers et al., 2017), *H. influenzae* (Cho et al., 2015, Chan et al., 2018), *Streptococcus suis* (de Buhr et al., 2015), *S. aureus* (Tang et al., 2011, Herzog et al., 2019), and *P. aeruginosa* (Mulcahy et al., 2010, Cherny and Sauer, 2019).

Several functions have been linked to extracellular DNases. For example, many of these enzymes have been considered important for bacterial virulence through their ability to digest DNA-rich neutrophil extracellular traps (NETs), which are complex networks of DNA and extracellular proteins produced by neutrophils as a part of host immune action to kill bacteria (de Buhr et al., 2015, Herzog et al., 2019, Jhelum et al., 2018). Thus, DNases are particularly important for pathogenic microorganisms to digest NETs and escape their potential antimicrobial activity. It has been also shown that the ability of isogenic nuclease-deficient strains of *S. aureus* to digest and escape NETs was significantly reduced compared to the wild-type parent strain (Berends et al., 2010). Additionally, the efficacy of *Streptococcus agalactiae* NucA to digest NETs was disrupted by substituting alanine with histidine at the active site (Derre-Bobillot et al., 2013).

Extracellular DNases have been shown to play important roles in regulating microbial biofilm formation. For example, in *Shewanella oneidensis*, deletion of extracellular nuclease genes *exeM* and *exeS* was associated with a significant increase in biofilm formation and eDNA accumulation within the biofilm matrix (Godeke et al., 2011). Similar findings have been reported in strains of *Vibrio cholerae* disrupted in the *xds* gene encoding extracellular DNase (Seper et al., 2011a) and in *nuc1* and *nuc2*- deficient strains of *S. aureus* (Beenken et al., 2012, Kiedrowski et al., 2011). However, other extracellular DNases of *Vibrio cholerae* and *S. oneidensis* have been shown to have functions other than controlling biofilm formation. For instance, disruption of *endA* affected the ability of *S. oneidensis* to scavenge eDNA as a phosphorus source (Heun et al., 2012b). Extracellular nuclease Dns of *V. cholerae* has been associated with genetic transformation. In addition, extracellular nucleases, Dns and Xds of *V. cholerae*, have been shown to play important roles in the degradation of NETs (Seper et al., 2013). Extracellular DNA is considered an important nutrient source for microorganisms in hostile environments, and therefore its degradation by extracellular DNases can provide nucleotides to be reused by bacteria as a source for phosphorus, carbon, and nitrogen (Mulcahy et al., 2010, Pinchuk et al., 2008, McDonough et al., 2016) . Furthermore, eDNA digestion by extracellular DNase likely impairs chelation of cations such as Mg^{+2}, and Ca^{+2} by eDNA and enhances scavenging of these cations by bacterial cells within the biofilm while also protecting cells from chelation-mediated lysis (Mulcahy et al., 2010). There is a strong association between the roles of extracellular DNases in bacterial nutrient scavenging and genetic transformation. Cell wall-associated nucleases, such as EndA in *S. pneumoniae* (Lacks and Neuberger, 1975, Bergé et al., 2013) have been shown to degrade eDNA into single-stranded DNA, which is a substrate for cellular DNA uptake systems involved in genetic transformation. Membrane associated nucleases in *S. oneidensis* also allow other enzymes such as extracellular phosphatases to scavenge DNA as a phosphorous source (Mulcahy et al., 2010, Heun et al., 2012a).

In conclusion, bacterial extracellular nucleases play a potential role in several functions including enhancing NETs digestion, using DNA as a nutrient source, regulating biofilm formation and facilitating natural genetic transformation.

1.7 Extracellular DNase enzymes as a potential anti-biofilm approach

Since eDNA is a key structural component within the matrix of many microbial biofilms, it is a promising target for biofilm control by DNase enzymes. As previously described, DNases are released by wide range of biofilm-forming microbial species that can naturally inhibit or eradicate the biofilms of other microorganisms (Rendueles and Ghigo, 2012). A good illustration of this is the extracellular nuclease NucB, which is released by a strain of *B. licheniformis* isolated from the surface of seaweed. It has been proposed that *B. licheniformis* naturally uses NucB to eradicate the biofilms of its competitors. Interestingly, exogenous addition of NucB to pre-established biofilms formed by *E. coli*, *Micrococcus luteus* and *B. subtilis* led to almost complete dispersal of these biofilms (Nijland et al., 2010). Moreover, the addition of NucB to monospecies *in vitro* biofilms formed by a wide range of bacteria freshly isolated from patients with chronic rhinosinusitis resulted in significant disruption of biofilms formed by 50% of these clinical isolates (Shields et al., 2013). Extracellular nuclease, Nuc1, of *S. aureus* has also been shown to disrupt biofilms formed by *Haemophilus parasuis, Actinobacillus pleuropneumoniae*, and *P. aeruginosa* (Tang et al., 2011). Consistent with the critical roles of eDNA in biofilm formation and maintaining the structural stability of mature biofilm, the exogenous addition of DNases can potentially inhibit biofilm formation, disperse pre-established biofilms, or increase the susceptibility of biofilms to antibiotics (Okshevsky et al., 2015). Different species appear to have different requirements for eDNA in biofilm formation and stability, and DNases could potentially interfere with any of the key functions of eDNA. For example, the DNase could digest eDNA associated with the microbial cell wall that serves as an adhesin for initial adhesion of microbial cells to biotic or abiotic surfaces. This would inhibit the initial stages of biofilm formation. Alternatively, the DNase enzyme could hydrolyse eDNA within the matrix of established biofilms, and degrade the adhesive network holiding the biofilm together. This would compromise the structural role of eDNA and promote biofilm dispersal. Furthermore, DNases have also been shown to enhance the sensitivity of bacteria within established biofilms to the killing action by various antibiotics or biocides, possibly by preventing the eDNA from sequestering the antibiotics (Kaplan et al., 2012, Tetz et al., 2009). Therefore, the ability of these enzymes to degrade eDNA within the matrix of biofilms makes them an excellent strategy for biofilm control.

Different types of DNases have been used as a tool to assess the role of eDNA in biofilm development. *In vitro* biofilms of many bacterial species and fungi have been shown

to be sensitive to nucleases when they were included during the early stages of biofilm formation or when they were added to pre-established mature biofilms (Table 1. 1). A number of studies have investigated the potential clinical application of DNase enzymes in treating biofilm-associated human diseases. Exogenous addition of bovine DNase I to dual-species biofilms formed by *P. aeruginosa* and *S. aureus* resulted in a substantial decrease in biofilm thickness (Yang et al., 2011). In addition, *B. licheniformis* NucB efficiently promoted the release of microorganisms from mixed-species biofilms on tracheoesophageal speech valves (Shakir et al., 2012). Together these data indicate that DNase enzymes can potentially be applied to control clinically relevant polymicrobial biofilms. Furthermore, DNases such as recombinant human DNase I (rhDNase I), also known as Dornase alfa, and Varidase ® have been used clinically in treating patients with cystic fibrosis and chronic wound infections, respectively, before the structural roles of eDNA in microbial biofilms were discovered. The application of inhaled Dornase alfa in patients with cystic fibrosis has been associated with increased pulmonary function through reducing the viscosity of DNA-rich bronchial secretions (Shak et al., 1990, Yang and Montgomery, 2018). More recently, a study has demonstrated that Dornase alfa can be used as an adjuvant therapy to increase the susceptibility of pulmonary biofilms to antibiotic therapy (Manzenreiter et al., 2012, Frederiksen et al., 2006). Varidase ®, which is a combination of a DNase (streptodornase) and a tissue plasminogen activator (streptokinase), has previously been used *in vivo* to enhance wound healing (Smith et al., 2011). However, due to doubts about its purity and its activity, Varidase ® has been withdrawn from the market in most countries (Steed, 2004). Dornase alfa is clinically being applied as an inhaled mucolytic agent in patients with cystic fibrosis and there is some evidence that Dornase alfa efficiently disrupted *in vitro* biofilms formed by *S. pneumoniae* and *S. aureus* (Hall-Stoodley et al., 2008, Kaplan et al., 2012). However, data confirming its anti-biofilm activity *in vivo* are scarce. Recently, it has been shown that Dornase alfa is potentially non-ototoxic in experimental animal models, and in a clinical trial, Dornase alfa showed an ability to unclog tympanostomy tubes in 59% of children with clogged tympanostomy tubes. However, this effect was not significantly different when compared with the treatment with antibiotics (Chan et al., 2018). One of the critical disadvantages of mammalian DNases, such as Dornase alfa, is that they require glycosylation for full activity and thermal stability, and therefore, it is difficult to produce them cheaply at scale using bacterial expression systems (Fujihara et al., 2008). This significantly increases the costs of production and limits potential medical and biotechnological applications. Nevertheless, different recombinant DNases including glycosylated mammalian DNase and

DNase I have been produced in a modified yeast *Picia pastoris* expression system which may reduce the cost of producing mammalian DNases (Cho et al., 2012).

Purified bacterial nucleases such as NucB from a marine strain of *B. licheniformis* (Rajarajan et al., 2013) have been successfully produced in large quantities and have been shown to be more effective than bovine DNase I in dispersing and inhibiting monospecies bacterial biofilms (Nijland et al., 2010, Shields et al., 2013). Furthermore, the onset of biofilm dispersal by NucB was much faster than DNase I. Recently, Basle et al. (2018) analysed the biochemical properties of *B. licheniformis* NucB and determined its crystal structure. This study revealed that NucB belongs to a unique subfamily of ββα metal-dependent non-specific endonucleases and shares less than 12% amino acid sequence identity with its closest structural neighbour (endonucleases of His-Me finger family), such as *Smendo*, an endonuclease from *Serratia marcescens.* The NucB family of endonucleases is characterised by its small size. For example B. licheniformis NucB contains only 110 amino acid residues, approximately 50% shorter than the length of the crystallized secreted endonucleases from Anabaena and Serratia marcescens, and half the size of the Dornase alfa used for the treatment of patients with cystic fibrosis (Horton N.C.Rice PA, 2008). As previously described, NucB has shown a potent ability to disrupt bacterial biofilms by degradation of eDNA (Nijland et al., 2010). Consistent with this behaviour, it has been shown that NucB is a non-specific endonuclease that can hydrolyse both single and double stranded DNA, and structurally complex molecules, such as supercoiled plasmid DNA. Both single- and double-stranded DNA play roles in biofilm formation (Zweig et al., 2014), and therefore it is entirely consistent that the non-specific endonuclease activity of NucB will potentially enable the enzyme to be effective against different types of DNA in the biofilm matrix (Nijland et al., 2010, Shakir et al., 2012, Shields et al., 2013, Rostami et al., 2017). Furthermore, NucB has been shown to be thermally stable enzyme that can regain significant nuclease activity after a heat-inactivation and cooling cycle (Basle et al., 2018). In contrast, Dornase alfa does not refold passively after thermal denaturation (Chan et al., 1996). Furthermore, as previously described, Dornase alfa requires glycosylation for full activity, thermal stability, and protease resistance (Fujihara et al., 2008). No data are currently available about the biological half-life (t1/2) of NucB, and approaches to further enhance the in vivo biocompatibility, activity, and stability of NucB are required before it can be used for biofilm-associated health problems.

Other purified bacterial nucleases include *Nuc* isolated from *Neisseria gonorrhoeae* (Steichen et al., 2011), and extracellular nuclease, *Nuc2*, from *S. aureus* (Kiedrowski et al., 2014). An important step to increase the potential applications of nucleases is to scale up their

cost-effective laboratory production. A *B. subtilis* protein production system to improve the production of *B. licheniformis* NucB has been described by Rajarajan et al. (2013).

Another therapeutic potential of DNases is to enhance the susceptibility of bacterial biofilms to antibiotics (Hymes et al., 2013, Kaplan et al., 2012, Martins et al., 2012, Tetz et al., 2009). For example, degradation of eDNA by exogenous DNases can inhibit the chelating activity of DNA against cationic antimicrobials such as aminoglycosides which will increase their permeability through the biofilm matrix (Chiang et al., 2013). An alternative approach to control biofilms through DNases is to stimulate the production of natural bacterial nucleases within the biofilm by targeting control mechanisms of bacteria that are involved in suppressing nuclease production. This will stimulate the natural biofilm dispersal systems of different biofilm-producing microorganisms, as seen in *B. licheniformis* and *P. aeruginosa* (Blokesch and Schoolnik, 2008, Kiedrowski et al., 2011, Nijland et al., 2010, Cherny and Sauer, 2019) . However, this approach requires a full understanding of the control mechanisms for nuclease production in a wide range of bacteria. Moreover, stimulation of the endogenous nucleases can also serve a variety of purposes including genetic transformation, escaping from NETs, and microbial nutrient scavenging which increase the difficulty of establishing a universal regulatory mechanism for a wide range of microbial species relying on eDNA for biofilm formation (Okshevsky et al., 2014).

Table 1. 1 Examples of the effects of treatment with exogenous DNases on microbial biofilm formation adopted from (Okshevsky et al., 2014)

Microorganism	Inhibition of biofilm formation	Dispersion of pre-established biofilm	DNase enzyme	Reference
Gram-negative bacteria				
Acinetobacter baumannii	✓	✓	DNase I	(Tetz et al., 2009) (Sahu et al., 2012)
Actinobacillus actinomycetemcomitans		✓	DNase I	(Inoue et al., 2003)
Bdellovibrio bacteriovorus	✓	✓	DNase I	(Medina and Kadouri, 2009)
Bordetella pertussis	✓	✓	DNase I	(Conover et al., 2011)
Bordetella bronchiseptica	✓	✓	DNase I	(Conover et al., 2011)
Campylobacter jejuni	✓		DNase I	(Svensson et al., 2009)
Comamonas denitrificans	✓		DNase I	(Andersson et al., 2009)
Escherichia coli	✓	✓	DNase I, NucB	(Nijland et al., 2010, Tetz and Tetz, 2010)
Haemophilus influenzae	✓	✓	DNase I	(Izano et al., 2009)
Klebsiella pneumonia		✓	DNase I	(Tetz et al., 2009)
Neisseria meningitides	✓		DNase I	(Lappann et al., 2010)
Pseudomonas aeruginosa	✓	✓	DNase I, DNase 1L2, DNase EndA	(Eckhart et al., 2007, Whitchurch et al., 2002, Cherny and Sauer, 2019)
Shewanella oneidensis	✓		DNase I	(Godeke et al., 2011)
Vibrio cholera	✓	✓	Nuclease *Dns* and *Xds*	(Seper et al., 2011a)
Gram-positive bacteria				
Bacillus licheniformis		✓	NucB	(Nijland et al., 2010)
Bacillus subtilis		✓	NucB	(Nijland et al., 2010)
Enterococcus faecalis		✓	DNase I	(Thomas et al., 2008, Torelli et al., 2017)

Table 1.1 (continued)

Microorganism	Inhibition of biofilm formation	Dispersion of pre-established biofilm	DNase enzyme	Reference
Listeria monocytogenes	✓	✓	DNase I	(Harmsen et al., 2010, Zetzmann et al., 2015a)
Micrococcus luteus		✓	NucB	(Nijland et al., 2010)
Staphylococcus aureus	✓	✓	rhDNase I, NucB, DNase I	(Kaplan et al., 2012, Shields et al., 2013, Sugimoto et al., 2018)
Staphylococcus epidermidis			NucB	(Shields et al., 2013)
Staphylococcus haemolyticus			DNase I	(Fredheim et al., 2009)
Streptococcus anginosus		✓	NucB	(Shields et al., 2013)
Streptococcus constellatus		✓	NucB	(Shields et al., 2013)
Streptococcus salivarius		✓	NucB	(Shields et al., 2013)
Staphylococcus lugdunesis		✓	NucB	(Shields et al., 2013)
Streptococcus intermedius		✓	NucB	(Shields et al., 2013)
Streptococcus intermedius	✓		DNase I	(Petersen et al., 2004)
Streptococcus mutans	✓		DNase I	(Petersen et al., 2005)
Streptococcus pneumonae		✓	rhDNase I	(Hall-Stoodley et al., 2008)
Streptococcus pyogenes		✓	DNase I	(Tetz et al., 2009)
Fungi				
Aspergillus fumigatus		✓	DNase I	(Rajendran et al., 2013)
Candida albicans	✓	✓	DNase I	(Martins et al., 2010, Farisa Banu et al., 2019)

1.8 Human microbiome and its clinical significance

The full range of microorganisms that live on or inside the human body is called the microbiota, whereas, human microbiome comprises the overall collection of microbial genomes that contribute to the wider genetic profile or metagenome of a human body(Rogers, 2016). Recent analysis has shown that the number of microbial cells in the body is roughly of the same order as the number of human cells and their overall mass is approximately 0.2 Kg (Sender et al., 2016). Increasing evidence indicates that the composition of the human microbial population has an important contribution to human health and certain disease conditions (Janssens et al., 2018). Microbial communities play a number of key roles in human health. For example, gut microbiota were initially thought to have a role mainly in preventing overgrowth of pathogenic bacteria within gastrointestinal tract, however, there is increasing evidence indicating that gut microbiota play important roles in digestion, inflammation, maintaining intestinal integrity, modify the host immune response, and affecting the health status of human body(Mueller et al., 2015, Rowland et al., 2018).

Human microbiome analysis has been revolutionized by the advanced culture-independent molecular-based methods such as Illumina ® and Roche/454 pyrosequencing platforms that can employ complete or partial high throughput bacterial 16S or fungal 18S ribosomal RNA (rRNA) gene sequencing to characterise the microbial communities colonising different anatomical sites of the human body at a level of detail that far exceeds previous culture-dependent or other targeted-molecular based methods (Huttenhower et al., 2012, Pollock et al., 2018). The 16S rRNA gene is a universal gene among all bacteria that contains both conserved and hypervariable genetic regions, which can be employed for phylogenetic classification to the species level of many complex bacterial communities. The eukaryotic equivalent of the 16S rRNA is the 18S rRNA, and this has been commonly used for fungal population analysis (Begerow et al., 2010, Johnson et al., 2019). Various internal transcribed spacer regions are becoming more commonly used for fungal (mycobiome) analysis than 18S rRNA sequencing (McTaggart et al., 2019).

There are many advantages that favour the use of 16S rRNA (or 18S rRNA) gene next generation sequencing in microbiome analysis. Firstly, this method involves simultaneous targeted sequencing of a short region of DNA for numerous samples, therefore it is much cheaper than classical Sanger sequencing. Furthermore, this method can provide important species level information about microbial communities that may harbour a high proportion of fastidious and difficult to culture microorganisms without the need for cultivation (Ranjan et

al., 2016). Finally, culture-independent methods are also superior to culturing for microbes residing in biofilms (Swearingen et al., 2016).

Human Microbiome Project (HMP), which commenced in 2007 and was run by the National Institute of Health, is the most well-known microbiome survey in healthy humans (Turnbaugh et al., 2007).The HMP conducted by Huttenhower et al. (2012) aimed to characterise the ecology of human-associated microbial communities of 242 healthy young western individuals in five different anatomical regions (skin, nose, oral cavity, gastrointestinal tract (GIT), and vagina), and to determine site-specific environmental factors that may influence the composition of gut microbiome. Samples were collected from 18 body habitats in women and 15 in men (excluding three vaginal sites), distributed among five major anatomical regions which were mentioned above.

The HMP has shown that the microbiome in healthy young adults is individual-specific and site-specific over time. Interestingly, each anatomical site was dominated by a single or a small number of certain microbial taxa almost in all studied individuals. For example, in the GIT, Firmicutes and Bacteroides were the most abundant families, while, Proteobacteria and Actinobacteria were more abundant in skin. These differences in the microbial abundance support the notion of microbiome involvement in the physiological and molecular processes that are specific to the human body system (Huttenhower et al., 2012).

Whilst the HMP focussed on health, many studies have investigated links between shifts in the microbiome at specific body sites (‘dysbiosis’) and different diseases. There have been so many of these studies, that a database has been created to catalogue the associations (Janssens et al., 2018). Association surveys have provided evidence that alteration of the microbiota composition can be associated with a variety of local (including recurrent *Clostridium difficile* infections, Crohn’s disease, ulcerative colitis, necrotizing enterocolitis, irritable bowel syndrome) and systemic diseases (diabetes mellitus, obesity, malignancy, bacterial vaginosis, autism and coronary heart diseases).

In recent years a number of culture-independent studies have investigated the microbiome in various regions of the head and neck in health and disease. These culture-independent analyses would be of particular importance in understanding the role of bacteria and biofilms in aetiopathogenesis of chronic head and neck diseases including COME and CRS. Ultimately, this will greatly assist in treating these diseases effectively.

1.9 Microbiome of middle ear and pharynx

An early comparative microbiome study of the oropharynx and nares in seven individuals using 16S rRNA gene and PhyloChip sequencing analysis found that the oropharynx houses a much more diverse microbiota than the nose (Lemon et al., 2010). At the phylum level, Firmicutes were the most dominant microbes in both anatomical sites, whereas *Staphylococcaceae* and *Lachnospiraceae* were most abundant bacterial families in nostrils, and *Streptococcaceae*, *Lachnospiraceae* and an unclassified group of Clostridia were most dominant families in the oropharynx.

Recently, a population-based microbiome study has analysed anterior nasal and oropharyngeal swabs collected from 524 participants using 16S rRNA gene sequencing techniques (Akmatov et al., 2017). Again, oropharyngeal microbial communities have shown higher species richness and diversity than the anterior nasal communities. The most dominant phyla in the anterior nose were Actinobacteria, Firmicutes, and Proteobacteria. *Corynebacterium accolens/segmentosum*, *Propionibacterium acnes*, *Staphylococcus epidermidis*, and *Staphylococcus aureus* were the most abundant bacterial species within anterior nasal communities, which collectively formed an average abundance of 47%. In contrast, oropharyngeal communities were dominated by a variety of bacterial species (*Leptotrichia sp*., *Fusobacterium periodonticum*, *Streptococcus salivarius/vestibularis*, Veillonella atypical, Prevotella melaninogenica and Prevotella histicola) that were detected at an average abundance of only 3.3-5.1%. Despite the anatomical relation between the nose and oropharynx, these findings support the concept that each body site has its own unique microbiome

As noted in section 1.3.1, the ascension of bacteria from the adenoids to the middle ear through the ET is thought to be important factor in the pathogenesis of AOM and COME. The adenoids are submucosal mass of lymphoid tissue located at the nasopharynx adjacent to the opening of the ET. The mucosal surface of the adenoids shows numerous depressions called crypts and elevations called folds which are known to house a diverse normal bacterial flora (Winther et al., 2009). Although adenoids are thought to play an important role in the pathogenesis of COME by acting as a reservoir for otopathogens, it has proved difficult to characterise the microbial population in enough detail to demonstrate clearly whether it is the primary source of bacteria that reach the middle ear space. Early surveys used traditional culture-based assays, which are relatively insensitive for identifying the total population of microorganisms, particularly fastidious, uncultivable microbes and bacteria residing in

biofilms (Swidsinski et al., 2007). Therefore, these studies potentially delivered partial or biased characterisation of the composition of the microbial communities colonising these anatomical niches (Ren et al., 2013).

When the work on this project started, there were only two published studies that had utilised culture-independent techniques to characterise microbial communities present in the middle ear effusion and adenoids of COME patients (Liu et al., 2011, Stol et al., 2013). Stol et al. (2013) obtained microbial community profiles of nasopharyngeal swabs and middle ear effusion samples from patients with rAOM and those with COME using quantitative real-time PCR analysis. In both groups of patients, *H. influenzae* and rhinovirus were the most abundant microbial pathogens in nasopharyngeal and middle ear effusion samples, and the abundance of microorganisms in the middle ear effusions of these patients was not significantly different. This PCR-based study was targeted to three common otopathogens (*S. pneumoniae, H. influenzae, and M. catarrhalis*) and 15 different viruses, and therefore it is not clear which other species were present.

Liu et al. (2011) analysed the microbiome in middle ear effusion, adenoid and tonsil samples recovered from a child with COME using 16S rRNA gene-based pyrosequencing. *Pseudomonadaceae* was the most abundant microbial family in the MEEF sample. Families of the three major otopathogens: *Streptococcaceae, Pastuerellaceae* (*Haemophilus*) and *Moraxellaceae* were also detected. There was a difference in the microbial communities in the three set of samples and the results indicated that the adenoid may be a potential source for both the middle ear and tonsil microbiota. However, differentiation at genus or species level and relative abundance of microorganisms were not reported. Furthermore, this study was restricted to samples from a single patient and its findings cannot be generalized until reproduced in a larger study population.

During the course of this project, several studies utilising culture-independent 16S rRNA gene sequencing methods to characterise the members of the microbiota associated with MEEF, adenoid, nasopharynx of COME children, were published and these are summarised in Table 1. 2.

The three common otopathogens were the most frequently detected genera in the MEEFs of children with COME; however, *Alloiococcus otitis* was the most predominant bacterial species in several microbiome studies (Ari et al., 2019, Boers et al., 2018, Chan et al., 2017a, Chan et al., 2016, Jervis-Bardy et al., 2015). *A. otitis* was not frequently cultured from the MEEFs before the widespread utilisation of PCR due to its fastidious growth requirements. However, since the use of culture-independent methods including 16S rRNA

gene sequencing, it has been shown to be the most abundant organism detected in MEEFs of children with COME, but not in those with AOM (see Table 1. 3). *Turicella otitidis* has also been reported as a dominant bacteria within middle ear microbiota of children with COME (Ari et al., 2019, Boers et al., 2018, Krueger et al., 2017).

The roles of adenoids or the nasopharynx as a reservoir for bacterial pathogens implicated in the pathogenesis of COME and AOM has been investigated in several studies which are summarised in Table 1. 2 and Table 1. 3. Differences in the cohort of patients studied, methods of DNA extraction, the region of 16S rRNA gene sequenced, and bioinformatics analysis pipelines can make the comparison of these microbiome studies difficult. However, there are some common patterns in what have been found. Overall, it has been demonstrated that the nose and nasopharynx of children with otitis media are more commonly colonised by the three major otopathogens compared with healthy controls, supporting the hypothesis that otopathogen colonisation of the nasopharynx is an important risk factor for the development of otitis media. Some of these microbiome studies have also observed that commensal genera, particularly *Corynebacterium and Dolosigranulum*, are more prevalent in the nose or nasopharynx of healthy children than children with otitis media (Pettigrew et al., 2012a, Man et al., 2019). These observations suggest that an imbalance in the composition of the nasopharyngeal microbiota as a result of the arrival of new pathogens, concomitant viral infection or other factors such as antibiotic treatment or exposure to cigarettes smoke, could be critical for otitis media. These microbiome studies have also observed that the microbial profiles of the nose or nasopharynx in children with otitis media are less diverse than those of healthy controls, and that the microbiome of the nasopharynx is more diverse than that of MEEFs (Jervis-Bardy et al., 2015, Man et al., 2019).

An important challenge in microbiome studies involving low microbial biomass clinical samples such as MEEFs of patients with COME, is contamination with DNA from exogeneous sources such as nucleic acid extraction kits, laboratory reagents or the skin of people handling the samples. This contaminating DNA can obscure the signal from the bacterial population present in these samples during the sequencing process (Salter et al., 2014, Eisenhofer et al., 2019). Thus, it is of particular importance to include negative sequencing controls concurrently with samples for low biomass microbiome analyses. There are also several challenges in the analysis of 16S rRNA gene sequencing data. Techniques have been developed to uncover hidden biases in sequencing, annotation, and bioinformatics analysis to overcome these limitations (Tripathi et al., 2016, Eisenhofer et al., 2019).

Even the best currently available methods for culture-independent microbiome analysis have a number of limitations. One key issue is that the identification of microbial DNA sequences does not necessarily indicate the presence of viable bacteria. For detection of viable bacteria, it is necessary to employ improved culture techniques or use RNA analysis methods to identify short-lived microbial transcripts in clinical samples (Wolk, 2016). Therefore, in order to provide a robust characterisation of complex microbial communities such as those present in MEEFs and adenoids of patients with COME, it is necessary to employ a combination of culture-based and culture-independent techniques. A "microbial culturomics" approach has been developed by incorporating the use of several culture conditions and mass spectroscopy microbial identification with the culture-independent molecular methods that enabled species-level identification of rare and new microorganisms of gut microbiota (Lagier et al., 2012). The key goal of this project was to characterise both the culturable and the total population of microorganisms in MEEFs and adenoids of patients with COME in the North East of England. This would then provide insights and isolates that could be employed to understand biofilm formation mechanisms in more detail.

Table 1. 2 Studies utilising culture-independent 16S rRNA gene sequencing methods to characterise microbiome of middle ear effusions, adenoids and/or nasopharynx in children with COME.

Microbiome study	Type of sample	Age of patients	Number and type of cases	Controls*	Key findings
Jervis-Bardy et al. (2015)	MEEF, adenoid and nasopharyngeal swabs	3-9 y	11 indigenous Australian children with COME	NA	● MEEFs were dominated by *A. otitis*, followed by *H. influenzae*. ● *A. otitis* not detected in nasopharynx or adenoid. ● Microbiomes of MEEFs were less diverse than that of nasopharynx.
Chan et al. (2016)	MEEF, adenoid swabs	1-12 y	23 children with COME	10 children without ear diseases undergoing other forms of surgery	● MEEFs were dominated by *A. otitis*, followed by *H. influenzae*. ● *A. otitis* almost absent from adenoid. ● Microbiomes of adenoids were similar in patients and controls, microbiomes of MEEFs and adenoids in patients were dissimilar.
Chan et al. (2017b)	MEEF, external ear canal (EEC) lavages	1-14 y	18 children with COME	NA	● MEEFs were dominated by *A. otitis*, followed by *H. influenzae*. ● EEC dominated by A. otitis and *Staphylococcus*. Otopathogens were rare in EEC. ● Both adenoid and EEC may act as reservoirs for middle ear bacteria.

Table 1.1 (continued)

Microbiome study	Type of sample	Age of patients	Number and type of cases	Controls*	Key findings
Krueger et al. (2017)	MEEF	3 m-14.6 y	55 children with COME	NA	● MEEFs were dominated by *Haemophilus* followed by *Moraxella* and *Turicella* ● Higher abundance of *Turicella* in children aged >24months, less abundant in children with hearing loss. ● *Haemophilus* associated with increased mucin production.
Boers et al. (2018)	MEEF, nasopharyngeal swabs	< 12 y	9 children with gastroesophageal reflux-associated OM	21 children with OM only	● MEEFs were dominated by *Alloiococcus* and *Turicella* ● Gastroesophageal reflux had no obvious impact on middle ear and nasopharyngeal microbiomes
Ari et al. (2019)	MEEF, adenoid specimens	1.5-9 y	25 children with OME	NA	● MEEFs were dominated by *Alloiococcus*, followed by *Turicella* ● Abundance of predominant bacteria was significantly different between MEEFs and adenoids microbiome
Johnston et al. (2019)	MEEF, adenoid and tonsil swabs	3 m-14.6 y	50 children with COME	NA	● *Fusobacterium*, *Haemophilus*, *Neisseria*, and *Porphyromonas* were most abundant genera in all sites. ● *Haemophilus* and *Moraxella* were most abundant in adenoid than in MEEFs. ● Tonsil and adenoid microbiomes similar to one another, adenoid and MEEF microbiomes less similar.
Kolbe et al. (2019)	MEEF	3 m-14.6 y	50 children with COME (13 with lower respiratory illness and 37 without)	NA	● *Haemophilus*, *Moraxella*, and *Turicella* were most abundant genera. MEEF microbiome less diverse in COME patients with lower respiratory illness than in patients without.

*NA= not applicable.

Table 1. 3 Studies utilising culture-independent 16S rRNA gene sequencing methods to characterise microbiome of middle ear and/or nasopharynx in patients with AOM.

Microbiome study	Type of sample	Age of patients	Number and type of cases	Controls*	Key findings
Pettigrew et al. (2012a)	Anterior nasal swabs	< 3 y	72 children with AOM	95 children without AOM, but with URTI and 73 healthy controls	● Colonisation with otopathogens associated with lower diversity of URT commensals ● High abundance of *Lactococcus*, *Propionibacterium*, *Corynebacterium* and *Dolosigranulum* less likely to develop AOM.
Sillanpää et al. (2017)	MEEF	5 m-3.5 y	79 children with AOM	NA	● *S. pneumoniae* most dominant bacteria ● *A. otitis*, *T. otitidis* and *Staphylococcus auricularis* detected less frequently than classical otopathogens. ● No novel pathogens were detected.
Chonmaitree et al. (2017)	Nasopharyngeal swabs	< 1 y	65 children with AOM	47 children without AOM	● Colonisation of otopathogens positively associated with higher URTI frequencies ● High abundance of *Staphylococcus* and *Sphingobium* associated with reduced risk of AOM complicating URTI ● Abundance of *Corynebacterium* reduced in AOM but had no effect on UTRI/AOM progression.
Man et al. (2019)	Paired middle ear fluid (otorrhoea) and nasopharyngeal swabs	< 5 y	94 children with AOM and tympanostomy tubes	NA	● Paired nasopharynx and MEF greatly correlated ● Microbiome of nasopharynx much more diverse than that of MEF ● Abundance of *Corynebacterium and Dolosigranulum* associated with shorter duration of otorrhoea

*NA= not applicable; URTI= upper respiratory tract infection.

1.10 Aim and objectives of the study

Evidence from previously published studies points to a clear deficiency in the current treatment of COME. Many clinical criteria for the diagnosis of COME are consistent with biofilm infections. The complex microbial community associated with COME is not well understood and there is pressing need to develop novel therapeutic approaches to improve treatment outcomes of this disease. The main aim of this thesis was to investigate the potential of the bacterial nuclease, NucB from *Bacillus licheniformis*, to improve the diagnosis and/or management of COME.

The objectives of the study were as follows:

1. To optimise methods for quantification of biofilm formation and to evaluate the potential of NucB for biofilm control.
2. To characterise and isolate bacterial population present in MEEFs and adenoids of patients with COME by a combination of culturing and 16S rRNA gene sequencing.
3. To study the sensitivity of *in vitro* biofilms formed by representative COME isolates to NucB treatment.

Chapter 2. Materials and Methods

2.1 Reagents and equipment

2.1.1 Reagents

Reagents are described in the text with their manufacturers and are abbreviated subsequently if needed.

2.1.2 List of equipment used in the study

Table 2. 1 List of equipment.

Application	Device	Manufacturer
Centrifugation	SK10 J2-21	Sigma Centrifuges, Osterode am Harz, Germany. Beckman Coulter Ltd., High Wycombe, UK
Bench-top Centrifugation	MiniSpin®	Eppendorf UK Ltd., Stevenage, UK
Static Incubator	30°C /37°C	LTE Scientific Ltd., Oldham, UK
Anaerobic Incubator	Bug Box Plus	Ruskinn Technology Ltd., Bridgend,UK
Incubation	Water bath	Grant instruments, Cambridge, UK
Orbital shaker	IKA KS 4000i control	IKA® England LTD., Oxford, UK
Microplate reader	Synergy™ HT Microplate Reader	BioTek UK, Swindon, UK
Measuring DNA concentration	NanoDrop® ND-1000	Thermo Fisher Scientific Ltd, Loughborough, UK
Measuring optical density	Libra S11	Biochrom Ltd., Cambridge, UK
Light/ Phase contrast Microscopy	Leica DM 750	Leica Biosystems Newcastle Ltd, Newcastle upon Tyne, UK
Fluorescence microscopy	Eclipse Ti-E	Nikon, Surrey, UK.
Confocal Laser Scanning Microscopy	TCS SP2 A1R-HD (Invert)	Leica, UK. Nikon, Surrey, UK.
Bead beater	Tissuelyzer LT	Qiagen, Manchester, UK
Microfluidics Biofilm Culture	Bioflux™	Fluxion Biosciences Inc., San Francisco, USA

2.2 Ethical approval

Ethical approval for this project was sought from the NHS National Research Ethics Service Committee (North East Newcastle and North Tyneside 2). Initially an online application was prepared via the Integrated Research Application System (IRAS) and submitted to the Research Ethics Service Committee electronically. The application form and the supporting documents of the study were prepared by me with regular review and editing by the supervisors. These documents included the research protocol (Appendix A), five age-specific participant information sheets (Appendix B) (for parents/carers, for younger children aged 3-6 years, children aged 6-11 years, young persons aged 12-15 years, and young persons aged 16 years), two participant consent forms (for parents/carers, and for younger persons aged 16 years), two age-specific assent forms (children aged 6-11 years, and young persons aged 12-15 years) (Appendix C), and in addition to other documents including research project insurance (Appendix D), letter of sponsorship review (Appendix E), and a letter from the project funder (Appendix F). All documents that were submitted to Research ethic committee (REC) are listed in the REC checklist (Appendix G).

Two rounds of amendments and revisions were required by NHS Research Ethics Service Committee (North East- Newcastle and North Tyneside 2) before ethical approval was granted for this study (no.15/NE/0225) titled “Investigation of biofilms associated with chronic otitis media with effusion and adenoids hypertrophy” (Appendix H (1)). Prior to the start of the study, it was necessary to obtain management permission or approval from all NHS organisations involved in this study in accordance with NHS research governance arrangements. Therefore, another application was submitted to the NHS host organisation (Newcastle upon Tyne Hospitals NHS Foundation Trust Research and Development) to obtain their management approval. This application also involved completing the relevant online application forms such as NHS Research and Development (R&D) form and NHS site specific information (SSI) form from IRAS and providing the supporting documentation listed in R&D valid submission checklist (see Appendix I). A full Newcastle upon Tyne Hospitals NHS Foundation Trust R&D approval (no. 7514) for the present study was obtained (Appendix H (2)) and patient recruitment was started.

The recruitment target of this project for the middle ear effusion samples of patients with COME was at least 60, whereas the sample size for adenoids tissue was 60 in total divided into two groups: those with COME (n=30) and those without COME (controls) (n= 30).

During the collection of clinical samples, I approached patients fulfilling inclusion criteria of the study and their parents/carers on the surgery day. Relevant information about the aims of the research project, methodology, and potential outcomes were explained to patients and their parents/carers in simple language. Parents and patients were given plenty of time to read the study participant information sheet. Any questions or concerns from the patients or their parents were answered by me. Parents/carers and patients who agreed to participate in the study were asked to sign the relevant consent form prior to enrolment in the study.

2.3 Sample collection

2.3.1 Middle ear effusion fluids

A total of 59 middle ear effusion fluid (MEEF) samples were collected from 34 patients aged less than 16 years, who were recruited in the Freeman and Royal Victoria Infirmary Hospitals, Newcastle upon Tyne, during the period between November 2015 and December 2016. Patients were listed for myringotomy and grommet tube insertion according to the standard clinical practice, for the treatment of persistent symptomatic COME for at least 3 months duration. Patients with previous history of grommet insertion, middle ear surgery, congenital craniofacial malformations, recurrent AOM, or those who did not have any visible MEEF in both ears during the operation were excluded from the study. Detailed instructions for the collection of samples were given to the operating surgeon to minimise the risk of contaminating MEEF samples by external ear canal flora. During the operation, wax and other debris was atraumatically removed from the ear canal using crocodile forceps or a separate suction catheter. Myringotomy (a small surgical incision in the eardrum) was performed using standard aseptic technique without prior ear canal sterilisation, and the MEEF aspirated through the myringotomy using a sterile suction catheter. Stringent precautions were taken to avoid contact with the external ear canal, and a new sterile set of suction catheter was used to aspirate MEEF from each ear. The MEEF was collected into a sterile mucous trap by aspiration of 2 ml of 0.9% saline solution through the suction catheter and transported by me on ice to the Oral Microbiology laboratory, Newcastle University for immediate processing.

2.3.2 Adenoid tissue

A total of 10 adenoid tissue specimens were collected from patients who were listed for either adenoidectomy plus grommet tube insertion (5/10), or adenotonsilectomy (5/10) according to standard clinical practice, for the treatment of persistent symptomatic COME of at least 3 months duration, or obstructive sleep apnea, respectively. Adenoidectomy was performed using the standard curette technique. The surgically removed adenoids tissue was immediately placed into a sterile collection tube containing 20 ml of a sterile reduced transport fluid (RTF) (Syed and Loesche, 1972). RTF contained per litre the following mineral salt mix (0.6 g K_2HPO_4, 1.2 g NaCl, 1.2 g $(NH_4)_2SO_4$, 0.6 g KH_2PO_4 and 0.25 g $MgSO_4$), 0.1 M EDTA, 8 g Na_2CO_3 and 1 g dithiothreitol. Specimens were transported on ice to the Oral Microbiology laboratory, Newcastle University and stored at -20°C. All specimens were processed within 24 h.

2.3.3 Processing of middle ear effusion fluid samples

MEEF samples were divided into 3 equal portions using a pipette and 50 µl portions were inoculated on each blood, chocolate, and Fastidious anaerobe agar (FAA) agar plates (see section). Pipetting was not possible for some very thick tenacious mucoid MEEF samples (see Figure 2. 1). In these cases, the sample was placed in a sterile petri dish and divided by surgical scalpel and a pipette into 3 equal portions, in the biological safety cabinet. The first portion of the sample was treated with NucB (see section 2.8.3), Recombinant NucB from *B. licheniformis* DSM13 was produced and purified as described in section 2.6.4. The second portion of the sample was used as a negative control (incubation control) for NucB treated portion (section 2.8.3). The last portion of MEEF underwent microbial DNA extraction for 16S rRNA gene sequencing (see section 2.6.1).

2.3.4 Processing of adenoid tissue samples

Adenoid tissue samples were thawed on ice. After thawing, 300-500 mg of tissue for each sample was transferred to a sterile petri dish and diced with a sterile surgical scalpel in the biological safety cabinet. This was transferred to a 2 ml disposable tube containing 0.5 ml of sterile phosphate buffer saline (PBS) and homogenized for 2 min using a cordless plastic pistol motor (Sigma Aldrich, UK). An additional 1 ml of PBS was added to the homogenate, vortexed briefly at high speed and filtered through a 40 µm cell culture strainer to dissociate cells from tissue clumps. The residual tissue from the filtration was washed three times with 1 ml PBS and filtered again. In selected samples, the filtered tissue homogenate was divided

into 2 equal portions using a pipette. The first portion was used to culture and isolate bacteria present in adenoid samples (see section 2.4.3). Microbial DNA was extracted from the second portion of the filtered tissue homogenate for 16S rRNA gene sequencing (see section 2.6.2).

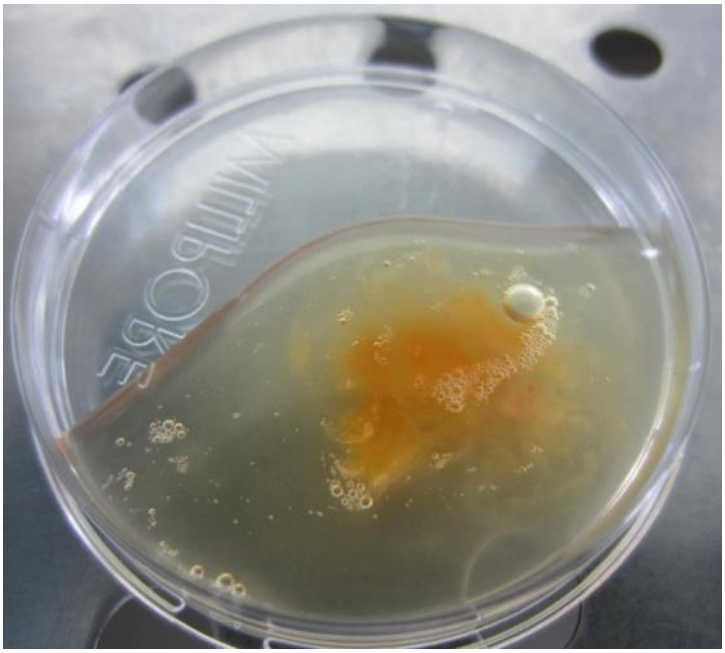


Figure 2. 1 A photograph of Middle ear effusion sample.

Shows yellowish orange coloured thick tenacious middle ear effusion fluid (21R) from patient number 21 placed in a sterile petri dish (47 mm in diameter) in a Class I biological safety cabinet before being divided by a surgical scalpel and a pipette into 3 portions for processing.

2.4 Microbiological methods

2.4.1 Culture media and chemicals

All growth media (broth or agar) were sterilised by autoclaving at 121˚C for 20 min. For the solidified media, 15 g/L of agar granules (Melford Laboratories Ltd, Suffolk, UK) were included prior to autoclaving. All growth media containing agar were allowed to cool in water bath at 45-50˚C before being poured into petri dishes. Chemicals were purchased from Sigma Aldrich, Dorset, UK unless stated otherwise.

Brain heart infusion with yeast extract (BHYE) was prepared by dissolving 37 g brain heart infusion (Melford, Suffolk, UK), and 5 g yeast extract (Melford, Suffolk, UK) in 1 L of distilled water (dH_2O).

Blood agar contained 5% (v/v) defibrinated horse blood (TCS Biosciences, Buckingham, UK) in pre-cooled BHYE agar.

Chocolate agar was prepared using the same ingredients as blood agar except that, following the addition of defibrinated horse blood, the medium was incubated in a water bath at 75-80˚C for 10-15 min until it turned a chocolate brown colour.

Fastidious anaerobe agar (FAA) was prepared by mixing 46 g of fastidious anaerobe broth (Lab-M, Lancashire, UK) and 15 g of agar granules in 1 L of dH_2O. Following autoclaving, 5% (v/v) defibrinated horse blood was added to the pre-cooled medium before being poured into petri dishes.

Tryptone soya broth (TSB) was used as a rich nutrient medium to culture *Staphylococcus aureus* strains. This was prepared by dissolving 30 g Trypto soya broth powder (Melford, Suffolk, UK) in one litre of dH_2O and autoclaved as described above.

Cation adjusted Mueller Hinton broth (CAMHB) was used for testing antibiotic sensitivity of both planktonic and biofilm cultures of *Staphylococcus aureus*. The medium was prepared by adding 17.3 g Casein acid hydrolysate (Oxoid, Hampshire, UK), 3 g Beef extract (Lab-Lemco broth, Oxoid, Hampshire, UK) and 1.5 g Soluble Starch to 1 L of dH_2O. The mixture was dissolved completely by heating in an oven at 70°C and, after cooling, the pH was adjusted to 7.3 +/- 0.2 before being autoclaved for sterilisation. Prior to use, Mg^{+2} stock solution (prepared by dissolving 8.36 g of $MgCl_2 \bullet 6H_2O$ in 100 ml of dH_2O; the final concentration is 10 mg ml^{-1} Mg^{+2}) was added to a final concentration of 10 mg L^{-1} Mg^{+2}. Calcium stock solution (prepared by dissolving 3.68 g of $CaCl_2 \bullet 2H_2O$ in 100 ml of dH_2O; the final concentration is 10 mg ml^{-1} Ca^{+2}) was also added to a final concentration of 20 mg L^{-1} Ca^{+2}.

Tryptone yeast extract glucose (TYEG) is an enriched growth medium used to culture streptococci and *Actinomyces odontolyticus*. TYEG was prepared by adding 10 g Bacto tryptone (Melford, UK), 5 g Yeast extract (Melford, UK), 3 g K_2HPO_4 (VWR International BVBA, Leuven, Belgium) and 2 g of glucose (Sigma, UK) to 1L of dH_2O. The pH was adjusted to 7.5 before autoclaving.

Todd Hewitt Yeast Extract (THYE) is a non-selective medium that was prepared by mixing 37 g of Todd Hewitt (Melford, UK) with 5 g of Yeast Extract (Melford, UK) per L of dH_2O before autoclaving.

Supplemented BHYE medium was used to culture *Haemophilus influenzae* strains. It was prepared by adding 1% (v/v) of Difco™ Supplement VX (Becton, Dickinson and Company, Sparks, USA) to pre-cooled sterile BHYE broth or agar.

2.4.2 Culture and isolation of chronic otitis media with effusion microbial population

To culture and isolate the microbial population present in MEEF samples, each sample was gently mixed and 50 µl was plated on blood agar, chocolate agar, and FAA in duplicate. Blood and chocolate agar plates were incubated at 37˚C with 5% CO_2 for 10-14 days and the cultures were checked for growth daily. Pre-reduced FAA plates were incubated anaerobically (Bug Box Plus, Ruskinn, Bridgend,UK) under a mixture of gases consisting of 10% CO_2, 10% H_2 and 80% N_2 for 7-14 days. The cultures were read at >40 hr, at 5 day, and at 14 day.

2.4.3 Culture and isolation of adenoid microbial population

Selected samples of adenoid tissue were cultured in an attempt to isolate and identify bacteria colonising paired adenoid and MEEF samples collected from the same patients. Ten microlitres of the filtered adenoid tissue homogenate was plated on non-selective blood agar, chocolate agar, and FAA in duplicate. Blood and chocolate agar plates were incubated at 37˚C with 5% CO_2 for up to 7 days and the cultures were checked for growth daily. Pre-reduced FAA plates were incubated anaerobically under a mixture of gases consisting of 10% CO_2, 10% H_2 and 80% N_2 for 7-14 days. The cultures were read at >40 hr, at 5 days, and at 14 days.

2.4.4 Glycerol stocks of bacteria

During examination of the agar plates, individual colonies were picked, and sub-cultured three times to establish a pure culture. Colonies were suspended in 20 ml BHYE broth and incubated for 24-48 h at 37˚C with 5% CO_2. The cultures were checked for contamination using a light microscope (Leica DM 750) prior to centrifugation (3,600 g) for

10 min at 4˚C. The pellet was suspended in 1 ml of BHYE medium and diluted by the addition of one volume of glycerol (Sigma Aldrich, UK) before being stored at -80˚C.

2.4.5 Routine culture of bacterial strains used in this study

Staphylococcus aureus SB14, SB17, NCTC 6571, 38, 35, and *Staphylococcus auricularis* 29, 33 were routinely grown in TSB medium overnight at 37˚C aerobically. *Turicella otitidis* 24, 28, 42, 43, 44, 45, 47 were grown in THYE medium for 48 h at 37˚C aerobically (see Table 2. 2 for more details about strains' names). *Haemophilus influenzae 38, 52,* SB11BBAII *were cultured in SBHYE medium overnight at* 37˚C with 5% CO_2. *Streptococcus pneumoniae* 28, 51, 11*, Streptococcus pyogenes* 28, *Streptococcus oralis* 31, 37, and *Streptococcus mitis* 37 were cultured in TYEG medium overnight *at* 37˚C with 5% CO_2. *Actinomyces odontolyticus* 31 was grown in TYEG medium for 48 h anaerobically at 37˚C.

2.4.6 Total viable count

The total viable count of bacterial cells was determined by preparing ten-fold serial dilutions of bacterial suspensions in PBS and placing triplicate 20 µl drops of each dilution (10^3 -10^6) on organism specific agar medium. The agar plates were incubated in air, 5% CO2, or anaerobically at 37˚C for various time periods. At the end of incubation, plates were photographed using a Canon IXUS 22HS camera and the number of colonies was counted using ImageJ (1.48v) computer software (http://imagej.nih.gov/ij/). Taking into account the dilutions, the colony forming units (CFU) per millilitre were then calculated.

2.4.7 Biofilm formation in a 96-well microtitre plate model

Bacterial stock cultures (5 µl) were added to triplicate wells of a sterile 96-well plate (Greiner Bio-One GmbH, Germany) containing 200 µl of organism specific culture media. The plate was covered, sealed with parafilm, and incubated statically for 24-48 h in appropriate growth conditions in a humid environment. At the end of incubation period, the OD_{600} was read in a microplate reader (BioTek Synergy HT) to quantify total growth. Biofilm formation was quantified using crystal violet (CV) staining assay (refer to section 2.8.1).

2.4.8 Bacterial strains used in this study

Table 2. 2 Bacterial strains used in this study

Strain	NU number*	Details	Source or Reference
Staphylococcus aureus			
SB14	NU118	Wild type	CRS project (Shields et al., 2013)
SB17	NU119	Wild type	CRS project
NCTC6571	N/A	Wild type	NCTC
18	NU85	Isolated from MEEF 18L	This project
21	NU84	Isolated from MEEF 21L	This project
Staphylococcus auricularis			
12	NU83	Isolated from MEEF 12L	This project
16	NU82	Isolated from MEEF 16L	This project
Turicella otitidis			
8	NU95	Isolated from MEEF 8R	This project
11	NU69	Isolated from MEEF 11R	This project
18	NU96	Isolated from MEEF 18L	This project
19	NU105	Isolated from MEEF 19L	This project
24	NU62	Isolated from MEEF 24L	This project
25	NU109	Isolated from MEEF 25R	This project
26	NU106	Isolated from MEEF 26R	This project
27	NU107	Isolated from MEEF 27R	This project
29	NU108	Isolated from MEEF 29R	This project
Haemophilus influenzae			
21	NU79	Isolated from MEEF 21L	This project
33	NU78	Isolated from MEEF 33L	This project
SB11BBAII	N/A	Wild type	CRS project
Streptococcus pneumoniae			
11	NU75	Isolated from MEEF 11R	This project
32	NU77	Isolated from MEEF 32R	This project
B10BBAI	N/A	Wild type	CRS project
SB11BBAI	N/A	Wild type	CRS project
Streptococcus pyogenes			
11	NU86	Isolated from MEEF 11R	This project
Streptococcus oralis			
14	NU88	Isolated from MEEF 14L	This project
20	NU39	Isolated from MEEF 20L	This project
Streptococcus mitis			
20	NU90	Isolated from MEEF 20L	This project
Actinomyces odontolyticus			
14	NU94	Isolated from MEEF 14L	This project

Table 2.2 (continued)

Strain	NU number*	Details	Source or Reference
Moraxella catarrhalis 22	NU74	Isolated from MEEF 22L	This project
SB11BBAIV	N/A	Wild type	CRS project

*A new nomenclature that recently has been adopted for the clinical isolates in Newcastle Oral Microbiology laboratory. These were included to avoid future confusion about the bacterial strains used in this thesis. 'N/A' – 'not applicable'.

2.5 Microbial identification

2.5.1 Conventional microbiological identification

During culturing of clinical isolates, the agar plates were examined for the presence of contaminant growth by employing conventional microbiological techniques such as checking microbial cell morphology under light microscope, Gram stain, and growth in anaerobic conditions. Furthermore, the characteristic morphology of colonies on agar medium was also utilised as a guide for isolation of the correct microorganism.

2.5.2 Microbial identification by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry ((MALDI-TOF MS)

The screened clinical isolates were further identified to species-level using MALDI-TOF MS, Bruker Daltonik MALDI Biotyper (Bruker UK Ltd., Coventry, UK). The clinical isolates were grown on blood or chocolate agar, incubated at appropriate growth conditions for various time periods and transferred to Dr Michael Ford at the Pathology Department in the Freeman Hospital, Newcastle upon Tyne for identification.

2.5.3 Microbial DNA next generation sequencing

The composition of microbial communities present in MEEF and adenoid samples was further characterized using culture-independent 16S rRNA gene sequencing. DNA was extracted from MEEFs and adenoids (see sections 2.6.1 and 2.6.2). The 16S rRNA gene V1-V3 variable region amplification, sequencing, and bioinformatics analysis were performed by Dr. Scot E. Dowd (www.mrdnalab.com, Shallowater, TX, USA) as previously described by Rostami et al. (2017) in the supplemental appendix of the paper.

2.5.4 Microbial full genome sequencing

To investigate whether similar bacterial strains colonising both adenoid and middle ear of COME patients are genetically identical, full genome sequencing of the similar bacterial species isolated from matched adenoid and MEEF samples of COME patient was performed by MicrobesNG in Birmingham University. Briefly, two putative *Streptococcus oralis* and *Streptococcus parasanguinis* strains (identified on the basis of MALDI-TOF) that were isolated from different sites of the same patient (patient 20) were grown overnight on blood agar *at* 37˚C with 5% CO_2. A single colony of the strain to be sequenced was taken from an overnight culture plate and mixed in 100 µl sterile PBS buffer. The strain was plated on blood agar from the 100 µl bacterial suspension. Around 1/3 of the plate was made as a lawn of bacteria and then the rest was streaked out to check that the culture was pure. A large sterile loop was used to harvest the bacterial culture from the plate and mix into the barcoded bead tube supplied by MicrobesNG. This included the 1/3 plate lawn of bacteria. The tube was mixed by inverting 10 times. The tube was sealed and sent at room temperature to MicrobesNG for DNA extraction and full genome sequencing (https://microbesng.uk/microbesng-faq/). The sequencing data of the similar bacterial species that were obtained from MicrobesNG were compared using Mauve multiple genome alignment software version 2.4.0 (http://darlinglab.org/mauve/download.html) and the numbers of different single nucleotide polymorphisms (SNPs) between the two similar bacterial genome were determined as one of the indications for genetic compatibility of these bacterial strains. Genome sequences were submitted to GenBank under BioProject ID PRJNA454893.

2.6 Molecular biology methods

2.6.1 Extraction of microbial DNA from middle ear effusion fluid samples

To characterise microbial community members present in MEEFs of COME patients, at least 250 µl of each sample underwent DNA extraction using QIAamp® DNA Microbiome Kit (Cat. No. 51704) (Qiagen,UK) according to the manufacturer's instructions for Next generation DNA sequencing. Initially the sample was centrifuged at 14,000 g for 10 minutes to pellet the cellular components. The pellet was resuspended in 500 µl of Buffer AHL in a 2 ml tube and incubated for 30 min at room temperature with end-over-end mixing at 18 rpm using Dynal sample mixer (Model MXICI, UK). The sample was then centrifuged at 10,000 x g for 10 min and supernatant was removed carefully. A 190 µl aliquot of Buffer RDD and 2.5

µl of Benzonase were added, mixed well and incubated at 37°C for 30 min at 600 rpm in a water bath to digest host DNA. The microbial genomic DNA was extracted and purified as per manufacturer's protocol (QIAamp® DNA Microbiome Handbook, version May 2014). The concentrations and purity of DNA were measured using a Nanodrop® ND-1000 spectrophotometer (ThermoFisher scientific Ltd, Loughborough, UK). The eluted DNA samples were stored in 1.5 ml microcentrifuge tubes at -80°C until they were sent for 16S rRNA next generation sequencing by Dr. Scot E. Dowd (www.mrdnalab.com, Shallowater, TX, and USA).

2.6.2 Extraction of microbial DNA from adenoid tissue samples

DNA extraction were performed using the PowerLyzer® PowerSoil® DNA Isolation Kit (Cat. No. 12855-50) (MO BIO Laboratories, Inc, Germany). The cellular pellet was re-suspended in 750 µl of Bead Solution, vortexed briefly, and transferred to the Power Bead Tubes before being vortexed briefly again. The tubes were placed in a Tissue Lyser LT **(**Qiagen, Manchester, UK) for 10 min at 50 Hz. Genomic DNA was extracted and purified as per the manufacturer's protocol (PowerLyzer® PowerSoil® DNA Isolation Kit instruction manual, version 07272016). The eluted DNA samples were stored in 2 ml microcentrifuge collection tubes at -80°C.

2.6.3 Agarose gel electrophoresis

The digestion products of NucB-treated calf thymus DNA samples were separated and visualised using 1% agarose gel electrophoresis. To prepare 1% agarose gels, 1 g of Molecular Biology Grade Agarose (Melford,UK) was dissolved by boiling in 100 ml of TAE buffer consisting of 40 mM Tris, 20 mM glacial acetic acid (Fisher Scientific), and 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma Aldrich), pH 8.0. The solution was allowed to cool for approximately 20 min before the addition of 5 µL of GelRed™ Nucleic Acid Gel Stain (10 000x in DMSO) (Cambridge Bioscience Ltd, UK). The solution was poured into a gel tray with comb and allowed to set. Solidified gel was then submerged in 1x TAE buffer in a gel tank. DNA samples were mixed with 5x DNA Loading Buffer (1:5 ratio) (Bioline Reagents Ltd., London, UK) before loading. To determine the molecular weight of DNA products, 5 µL of HyperLadder 1 kb Plus (250-12,007 bp) (Bioline, UK) was compared against DNA samples. Gel electrophoresis was undertaken for 90 min at 100 V using a Bio-Rad Power Pac 300 and then transferred to a G:BOX Transilluminator (Syngene, Cambridge, UK) to visualise DNA bands and capture images using GeneSnap software (Syngene, UK).

2.6.4 Production and purification of NucB

Recombinant NucB from *B. licheniformis* DSM13 was produced in *Bacillus subtilis* NZ8900 using the SURE expression system as previously described (Nijland et al., 2010) and purified as previously described (Rostami et al., 2017) by Professor Alastair Hawkins. Briefly, secreted NucB was precipitated from cell-free spent growth medium by making it 65% saturated with ammonium sulphate and incubating for 15 h at 6°C. NucB pellet was recovered by 4 serial centrifugation steps at 10,000 g for 60 min at 4°C. A minimal volume of 50 mM potassium phosphate pH 7.2, 1 mM dithiothreitol (buffer 1) was used to dissolve the NucB-containing pellet. All subsequent chromatography steps were performed at 6°C. Following clarification by centrifugation at 10,000 g for 60 min at 4°C, the soluble protein was dialyzed against buffer 1 at 6°C and loaded onto a Q sepharose column previously equilibrated with buffer 1. Following a wash with buffer 1, the sample flow through and column wash were collected as individual fractions and assayed by SDS PAGE (12% separating gel) for the presence of NucB. NucB-containing fractions were pooled appropriately and loaded onto a hydroxyapatite column previously equilibrated with buffer 1. The column was washed with buffer 1 and the sample flow through and column wash were collected as individual fractions. Following assay of individual fractions by SDS PAGE (12% separating gel) and UV absorbance spectroscopy, NucB-containing fractions were pooled appropriately. Using this procedure 50 mg of NucB at >95% purity was routinely recovered from a starting cell culture volume of 10 L. The purity of NucB was assessed using multiple independent and complementary methods. The presence of any nucleic acid contamination was assessed by measuring the UV absorption spectrum from 400 – 240 nm. Preparations had a typical A_{260}/A_{280} ratio of 0.57 (+/- 0.2) indicating an absence of nucleic acid contamination. Purity in terms of protein content was assessed by overloading an SDS PAGE gel and by differential scanning calorimetry analysis. A further indication of purity of NucB was provided by the observation that purified NucB crystallized under defined conditions thereby facilitating determination of its structure (Basle et al., 2018).

2.7 Measurement of NucB activity

2.7.1 Measurement of NucB nuclease activity in the optimal buffer solution

The main aim of this method was to monitor NucB DNase activity and ensure adding a consistent amount of active NucB in each experiment. The DNase activity of NucB was determined using the method developed by Kunitz (1950) with a slight modification. Each

assay contained the following: 25 µL of 50 mM Tris-HCl pH 8.0 (stock 500 mM), 12.5 µL of 5 mM $MnSO_4$ (stock 100 mM), 168.9 µL equivalent to 125 µg Calf thymus (CT) DNA (Sigma Aldrich D1501 - 1G, UK)(Stock 0.74 mg ml^{-1}), 2.5 µL equivalent to 10 ng NucB (Stock 1 µg ml^{-1}), and 41.1 µL of sterile dH_2O. The final reaction volume was 250 µL. The buffer and DNA mixture were incubated at 37°C for 10 min before the reaction was started by addition and gentle mixing of NucB followed by further incubation for 15 min, 30 min, and 60 min intervals at 37°C. A negative control made up with buffer and CTDNA with no enzyme was included.

For analysis by agarose gel (1% w/v) electrophoresis, 50µl of the reaction was halted by the addition and mixing of 50 µl of phenol/chloroform/isoamyl alcohol mixture (Sigma Aldrich, UK) for both enzyme and control. The mixture was shaken vigorously for 30 sec to form an emulsion. The sample was centrifuged at 13,000 rpm at 4°C for 3 min in a benchtop microcentrifuge (Prism R, Labnet International Inc, New Jersey, USA) forming two layers. The DNA was carefully removed from the upper layer and stored at 4°C until all time intervals were processed.

Gel electrophoresis was performed as described in section 2.6.3. For spectroscopic measurement, at the end of 60 min incubation, the reaction was stopped and the high molecular weight CTDNA was precipitated by the addition and mixing of 250 µl of cold (4°C) 4% (v/v) perchloric acid (Sigma Aldrich, UK). The mixture was incubated on ice for 40 min before being centrifuged at 13,000 rpm at 4°C for 3 min in a benchtop microcentrifuge to precipitate the residual high molecular weight CTDNA and to recover low molecular weight CTDNA in the supernatant. After this, 250 µl of the supernatant was transferred into a new microcentrifuge tube, and the amount of low molecular weight CTDNA produced by NucB was determined by reading the absorbance at 260 nm using Nanodrop® ND-1000 spectrophotometer.

In subsequent experiments, a range of NucB concentrations (0.25, 1, 5, 10, 25, 50, 100 ng) was used to generate a line graph for NucB activity which included the absorbance readings at 260 nm (A_{260}) against NucB concentration. This approach in conjunction with gel electrophoresis analysis enabled us to determine the optimal concentration of NucB to be used in measuring the unit of activity. A unit of NucB activity was defined as a production of perchloric acid soluble low molecular weight CTDNA that generated an absorbance of 1.0 at 260 nm, per hour at 37°C in 50 mM Tris pH 8.0, 5 mM $MnSO_4$ buffer. Since 1 ml of DNA with A_{260} of 1.0 contains 50 µg of DNA, one unit of activity is equivalent to the production of

50 μg of perchloric acid soluble low molecular weight CTDNA per hour at 37°C in 50 mM Tris pH 8.0, 5 mM $MnSO_4$ buffer.

2.7.2 Measurement of NucB nuclease activity in culture medium and saline solutions

Experiments investigating the anti-biofilm activity of NucB against different microbial biofilms involve addition of NucB into different culture media and solutions. It was important to ensure that ingredients of these solutions would not inhibit the nuclease activity of NucB. Therefore, the nuclease activity of NucB was measured in the representative solutions such as TSB and 0.9% saline solution.

For both solutions, each assay contained the following: 12.5 μL of 5mM $MnSO_4$ (stock 100mM), 168.9 μL equivalent to 125 μg CTDNA (Stock 0.74mg/ml), 2.5 μL equivalent to 10ng NucB (Stock 1μg/ml), and 66.1 μL of either 0.9% normal saline solution or TSB. The final reaction volume was 250 μL. The mixtures were incubated at 37°C for 10 min before the reaction was started by addition and gentle mixing of NucB followed by further incubation for 15 min, 30 min, and 60 min intervals at 37°C. A negative control made up with the same ingredients with no enzyme was included. A standard NucB activity assay in the optimal buffer was performed as previously described in section 2.7.1 under the same conditions. The NucB activity was then determined using both spectroscopic measurement and gel electrophoresis as described previously in section 2.6.3.

2.7.3 Nanodrop spectrophotometry

DNA concentrations and purity were measured using a Nanodrop® ND-1000 spectrophotometer. The device was blanked using 2 μL of elution buffer loaded on the NanoDrop stage. Following this, 2 μL of the DNA sample was placed on the stage to measure DNA concentration. To estimate DNA purity, the ratio of A_{260} to A_{280} was determined and a ratio of 1.8-2.0 was deemed acceptable.

2.8 Anti-biofilm activity of NucB against bacterial biofilms

2.8.1 Quantification of biofilm formation by crystal violet staining assay

Crystal violet assay was employed to quantify both the ability of bacterial strains to form biofilms and to determine the anti-biofilm efficacy of NucB against monospecies biofilms using the method described by Shields et al. (2013) with some modifications. Biofilms were grown in 96-well microtitre plates (see section 2.4.7). At the end of the incubation, the supernatants were discarded, and the extent of biofilm formation was

determined by staining with 100 µL 0.5 % (w/v) crystal violet (per well). The plate was incubated for 15 min at room temperature (20-25°C), before the wells were rinsed 3 times with PBS. The microtiter plate was turned upside down in the oven at 60°C to dry for 5 min. The residual stain was dissolved in 100 µL 7 % acetic acid (v/v) and the A_{570} was measured using a microplate reader (Synergy HT). The A_{570} values of a negative controls (stained and washed wells with no bacterial cells) were subtracted from sample absorbance values. Each assay was performed independently at least three times.

2.8.2 Assessing the sensitivity of in vitro biofilms to DNase enzymes

To quantify the efficacy of DNase enzymes (NucB, Bovine DNase I (catalogue no. 04716728001, Roche Diagnostics, Mannheim, Germany) to disperse pre-formed bacterial biofilms, bacterial biofilms were established in 96-well microtitre plates, treated with nuclease enzyme (DNase I at 5 µg ml^{-1}, NucB at 10-1000 ng ml^{-1}) for 1 h at 37°C, and biofilm biomass was quantified using CV staining method. When assessing the efficacy of DNase enzymes to inhibit biofilm formation, the filter-sterilised enzyme was added with growth media and bacterial inoculae at the beginning of biofilm formation. Again, the biofilm biomass was then measured using CV staining assay. These were compared with control biofilms that had been treated with enzyme buffer only. Each assay was repeated at least three times independently.

2.8.3 Assessing efficacy of NucB for improving culturing recovery of bacteria from MEE fluids

The efficacy of NucB for improving the recovery of microorganisms from MEEFs of COME patients was assessed by incubating MEEF with or without NucB. Initially, two aliquots of MEEF were placed in separate sterile microcentrifuge tubes. The first was treated with 100 units of NucB and the second left without treatment as a control. Samples were incubated for 1 h at 37°C in air. Following the incubation period, 50 µl inoculum from each sample was spread evenly on blood agar, chocolate agar, and FAA in duplicate. Blood and chocolate agar plates were incubated at 37˚C with 5% CO_2 for 10-14 days and the cultures were checked for colony growth daily. Pre-reduced FAA plates were incubated anaerobically for 7-14 days. The cultures were checked for growth at >40 hr, at 5 days, and at 14 days. Plates were photographed periodically using a Canon IXUS 22HS camera and the number of colonies was counted using ImageJ (1.48v) computer software. Taking into account the dilutions, the colony forming units (CFU) per millilitre were then calculated for both NucB treated and control samples. Only plates containing 1-400 colonies were counted.

2.9 Antibiotic susceptibility of bacterial biofilms

2.9.1 Determination of minimum inhibitory and minimum bactericidal concentrations of planktonic bacterial cultures

Before assessing antibiotic susceptibility of bacterial cells within biofilms, the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of planktonic cultures were determined using the broth microdilution method as previously described (Andrews, 2001). MIC represents a standard parameter used in clinical practice to determine the sensitivity and resistance of free-living bacterial cells to antibiotics. Co-amoxiclav (Amoxicillin + Clavulanic acid 5:1, Duchefa Biochemie, Netherland) was selected for use in antibiotic susceptibility assays of *S. aureus* strains in planktonic cultures and within biofilms.

Co-amoxiclav stock solution was prepared at 40 mg ml^{-1} in sterile dH_2O, filter sterilised and stored at -80°C. The working solution of Co-amoxiclav was prepared at twice the maximum concentration to be tested in the challenge plate to compensate for the addition of an equal volume of inoculum. To prepare antibiotic challenge plates**,** twofold dilutions of Co-amoxiclav were made in CAMHB. A suitable range of antibiotic concentrations was chosen (0.0625-16 mg L^{-1}). Each well contained 100 µL of Co-amoxiclav, and triplicate wells were used for each concentration. Growth (no antibiotics) and sterility (uninoculated) controls were included in each plate. Following this, the inoculum was prepared by suspending 3-4 large or 6-8 small isolated colonies selected from an overnight culture on agar plate (a nonselective medium, such as blood agar) in 5 ml of PBS. The suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard. This results in a suspension containing approximately 1 to 2 × 10^8 CFU ml^{-1} that was validated by determination of the total viable counts on TSA plates. This solution was diluted 1 in 20 with CAMHB and it was used within 30 min. An equal volume of the adjusted bacterial inoculum (100 µL of 10^6 CFU ml^{-1}) was added to each well except the sterility control wells. The plate was covered with the lid, sealed with parafilm and incubate aerobically overnight at 37°C. The MIC endpoint was recorded as the minimum concentration of antibiotic at which there was no visible growth. Each experiment was repeated three times independently.

2.9.2 Minimal Biofilm Eradication Concentration (MBEC) assay

Bacterial biofilms can tolerate high concentrations of antibiotics in comparison to their planktonic counterparts. Thus, it was important to determine minimal biofilm eradication

concentration (MBEC) of Co-amoxiclav for *S. aureus* strains before assessing the effects of NucB on this susceptibility.

MBEC™ Biofilm Inoculator (peg lid with 96-well microtiter plate base) (Innovotech Inc., Canada) was used to measure MBECs for *S. aureus* strains according to the manufacturer's instructions with minor modifications. An overnight culture was prepared by streaking out from a -80°C cryogenic stock of desired *S. aureus* strain on a blood agar plate. The culture was checked for purity the next day. The inoculum was prepared as previously described in section 2.9.1. The subsequent steps were carried out in a biological safety cabinet. Each well of the MBEC plate was inoculated with 150 µl of the adjusted inoculum, the peg lid was placed onto the plate and sealed by Parafilm. The device was placed on an orbital shaker (IKA KS 4000i control, IKA® England LTD., Oxford, UK) at 100 rpm in a humid environment at 37°C for 18- 24 h in air. A sample of the inoculum was validated by determination of the total viable counts on TSA plates to verify the starting cell number for biofilm formation.

At the end of the incubation period, the peg lid was removed from the plate and pegs were submersed in the wells of a pre-prepared PBS rinse plate (200 µl in each well) for 1 to 2 min to remove loosely adherent cells. Using flamed sterilised pliers, three pegs were removed from different points on the lid, placed in microcentrifuge tubes containing 200 µl of TSB, and sonicated with an Aquasonic sonicating water bath (Decon FS200 frequency sweep, Decon Ultrasonics Ltd, Sussex, UK) for 30 min at energy of 44 kHz frequency, 10 kPa rms pressure, and 67 w/m^2 intensity as calculated using a calibrated Hydrophone. Viable counts were determined on TSA plates to establish the number of CFU per peg prior to exposure to Co-amoxiclav.

The antibiotic challenge plate was prepared as previously described in section 2.9.1. A suitable range of Co-amoxiclav concentration was chosen (0.25-1024 mg L^{-1}). The final volume of Co-amoxiclav solution in each well of the challenge plate was 200 µl. This was to ensure complete submersion of the biofilm in the antibiotic solution. Each plate included growth and sterility control wells in triplicate.

The peg lid of the MBEC™ device was transferred to a standard 96-well plate in which desired dilutions of Co-amoxiclav were prepared in CAMHB. Antibiotic plates were incubated for 20-24 h at 37°C in air. After 24 h, the challenge plate was removed from the incubator. In the laminar flow hood, the lid was removed, rinsed in PBS for 1-2 min, and placed in a second 96-well plate containing 200 µl of CAMHB in each well. The biofilm was removed from the peg lid of the MBEC™ device by sonication for 30 min, and a new cover

was placed over the plate. The biofilm recovery plate was incubated overnight at 37°C in air. To determine the MIC values, the wells of the challenge plate were examined for turbidity. Alternatively, a plate reader was used to measure A_{650}. The MIC is defined as the lowest concentration of antibiotic that inhibits growth of the organism. Wells with $A_{650} < 0.1$ were considered to be growth-inhibited. To determine the minimum biofilm eradication concentration (MBEC) values, the wells of the recovery plate were examined for turbidity. Alternatively, a plate reader was used to measure A_{650}. Wells with $A_{650} < 0.1$ were considered to be evidence of biofilm eradication.

2.9.3 Colorimetric tetrazolium salt XTT viability assay

Another method used to assess the susceptibility of bacterial biofilms to antibiotics treatment was the tetrazolium salt XTT viability assay. The assay is based on the ability of viable and metabolically active cells to reduce the tetrazolium salt XTT into a highly-coloured water soluble formazan salt which can be measured spectrophotometrically. Dead or damaged cells will rapidly lose the ability to produce formazan salt. There is a proportional relationship between the amount of formazan produced and the number of live cells present in the culture.

The bacterial inoculum for XTT assay was prepared and verified as described previously in section 2.9.1. Each well of a 96-well plate was inoculated with 150 µL of the adjusted bacterial inoculum. Plates were incubated statically for 18-20 h at 37◦C in humid environment to allow biofilm formation.

At the end of the incubation period, the supernatants were discarded, and biofilms were washed once with 200 µL of PBS. Biofilms were then treated in triplicate with 200 µL of Co-amoxiclav solution prepared in CAMHB for 18-20 h at 37◦C in air. Growth with no antibiotic and sterility controls were included. The applied concentrations of the Co-amoxiclav solution were 32-8192 mg L^{-1}. After incubation, the antibiotic solution was removed, and each well was rinsed three times with 200 µL of PBS. To quantify the antibiotic susceptibility of biofilms with XTT staining, the method described by Koban et al. (2012) was used with some modifications. Biofilms in each well were incubated with 200 µL of XTT solution (Cell proliferation kit II (XTT), version 17, Roche Diagnostics GmbH, Mannheim, Germany) in the plate chamber of the plate reader for 1 h at 37◦C and A_{450} was measured every 10 min. The rate of XTT reduction (MAX V (A_{450} test- A_{450} blank)) per min was calculated over the time period. The XTT solution was prepared freshly in 10% TSB as per the manufacturer's instructions. The mixture contained 0.2 mg ml^{-1} XTT, and 0.02 mg ml^{-1} phenazine methosulphate (PMS). Each experiment was repeated three times independently.

2.10 Assessing effects of NucB on the antibiotic susceptibility of bacterial cells

2.10.1 Assessing effects of NucB on antimicrobial sensitivity of planktonic bacterial cultures

The XTT viability assay was used to determine effects of NucB on MICs and cell viability of planktonic bacterial cultures. The challenge plate containing treatment groups (NucB alone, NucB+ Co-amoxiclav, Co-amoxiclav alone) was set up.

The antibiotic challenge plate was prepared as previously described in section2.9.1. A suitable range of Co-amoxiclav concentration was chosen (0.0625- 8 mg ml^{-1}). Each well contained 100 µL of either Co-amoxiclav alone, Co-amoxiclav plus NucB (0.5 µg ml^{-1}), or NucB (0.5 µg ml^{-1}) alone in triplicate. Each plate included growth and sterility (uninoculated) controls. The bacterial inoculum was prepared as previously described in section 2.9.1. An equal volume of an adjusted inoculum of the test microorganism (100 µL) was added to each well except the sterility control wells. Wells containing Co-amoxiclav only received buffer solution equivalent to the volume of NucB added to wells containing either NucB alone or NucB plus Co-amoxiclav. The plate was covered with the lid, sealed with parafilm and incubated at 37◦C for 18- 20 h in air.

The MIC endpoint was recorded as the minimum concentration of Co-amoxiclav or Co-amoxiclav plus NucB (0.5 µg ml^{-1}) at which no turbidity was observed. To determine effects of NucB on planktonic bacterial cell viability and MIC, 80 µL of each treatment group solution were transferred onto a new sterile 96-well plate in triplicate and 20 µL of XTT solution (0.2 mg ml^{-1} XTT, and 0.02 mg ml^{-1} PMS) prepared in PBS were added to each well. Absorbance at 450 nm was measured using a plate reader. Each experiment was repeated three times independently.

2.10.2 Assessing effect of NucB on antibiotic sensitivity of in vitro S. aureus biofilms

The XTT viability assay was also used to assess effect of NucB on antibiotic susceptibility of *in vitro* biofilms of *S. aureus* clinical isolates. Biofilms of the tested *S. aureus* strains were grown in a 96-well microtitre plate for 18-20 h as previously described in section 2.4.7. At the end of the incubation, the supernatants were discarded, and biofilms were washed once with 200 µL of PBS to remove loosely attached bacterial cells. Biofilms were then treated in triplicate with 200 µL of either NucB (1 µg ml^{-1}) alone, or NucB (1 µg ml^{-1}) plus Co-amoxiclav (512 µg ml^{-1} or 1024 µg ml^{-1}) or Co-amoxiclav (512 µg ml^{-1} or 1024 µg ml^{-1}) alone for 18-20 h at 37°C in air. The treatment solutions were prepared in CAMHB.

Negative (no treatment) and sterility (no cells) controls were included. Following the treatment period, all solutions were discarded and biofilms were washed three times with 200 µL of PBS. Each well then received 200 µL of freshly prepared XTT solution in 10% TSB and the plate was incubated in the microplate reader for 1 h at 37°C. Absorbance at 450 nm was measured every 10 min. The rate of XTT reduction (MAX V (A_{450} test- A_{450} blank)) per min was calculated over the time period. Each experiment was repeated at least three times independently.

2.11 Investigation of NucB toxicity for bacterial and human respiratory epithelial cells

2.11.1 Human bronchial epithelial cell culture

BEAS-2B (obtained from ATCC; LGC Standards, Teddington, UK) is a human bronchial epithelial cell line derived from normal human epithelial cells immortalised using a hybrid of adenovirus 12 and simian virus 40. Using a Costar™ 12-well flat bottom cell culture plate (Thermo Fisher Scientific, UK), BEAS-2B cells were seeded at a density of 6-7 $\times 10^5$ cells ml^{-1} and allowed to adhere and grow in 500 µL of bronchial epithelial growth medium (BEGM) (Lonza, Cambridge, MA, USA) on 5% CO_2 at 37◦C. BEGM was supplemented with 2 mL bovine pituitary extract (0.004 mL^{-1}), 0.5 mL insulin (5 µg mL^{-1}), 0.5 mL hydrocortisone (0.5 µg mL^{-1}), 0.5 mL retinoic acid (0.1 ng mL^{-1}), 0.5 mL transferrin (10 µg mL^{-1}), 0.5 mL tri-iodothyronine (6.7 ng mL^{-1}), 0.5 mL adrenaline (0.5 µg mL^{-1}), 0.5 mL recombinant epidermal growth factor human (10 ng mL^{-1}), 2 mM L-glutamine, 100 U mL^{-1} penicillin G and 100 µg mL^{-1} streptomycin (Sigma, Gillingham, UK).

2.11.2 CellTiter-Blue® cell viability assay

The effect of NucB on human bronchial cell viability and proliferation was assessed using a fluorometric CellTiter-Blue® viability assay according to the manufacturer instructions. This assay utilises the ability of cells to convert dark blue resazurin reagent into highly fluorescent and pink colour resorufin as an indicator for their viability and active metabolic status. Compromised or non-viable cells rapidly lose this ability to reduce resazurin dye, and therefore do not produce a florescent signal.

BEAS-2B cells were cultured as previously described in section 2.11.1. At the end of incubation, the culture medium was removed and cells were challenged with a range of NucB concentrations (final concentrations: 1, 10, 50, 100 µg ml^{-1}), NucB buffer (50 mM Tris pH 8.0) or Dornase alfa (100 µg ml^{-1}) (from Dornase Alfa stock concentration of 2.5 mg ml^{-1},

Roche, UK), that were prepared in 500 µl of BEGM, or left untreated with BEGM only and incubated for 24–48 h in 5% CO_2 at 37°C. Following each treatment period, 100 µl of thawed CellTiter-Blue® reagent was added into each well and shaken gently before being incubated for 2 h on 5% CO_2 at 37°C. Positive controls in triplicate were treated with 500 µl of absolute methanol (Sigma Aldrich, Dorset,UK) for 2 min at room temperature before the addition of CellTiter-Blue® reagent. The plate was shaken for 20 sec and the fluorescence at 560/590 nm was recorded using the plate reader (Infinite® 200Pro, Tecan Life Sciences, UK). Each experiment was repeated three times independently.

2.11.3 Assessing effects of NucB on bacterial cell viability within biofilms

To assess the potential toxicity of NucB against cells in pre-formed *S. aureus* biofilms, 6-well tissue culture plates containing 3 ml of BHYE were inoculated with 50 µl of stock bacterial cultures per well and incubated statically in air at 37◦C for 18-20 h. Medium was carefully removed after 24 h and wells were washed once with PBS to remove loosely attached bacterial cells. Biofilms were treated with 1 ml of NucB solution (0.5 µg ml^{-1}) for 1 hr at 37ºC, and the control treated with 1 ml of PBS. The NucB, and PBS were aspirated and collected in sterile tubes separately. Biofilms were rinsed twice with 2 ml of PBS and the rinsing PBS from each well was collected and pooled to give a final volume of 5 ml. This was labelled 'Planktonic cells'. After addition of 2 ml PBS, the residual biofilms were scraped using a tissue scraper (Greiner bio-one GmbH, Germany). The PBS containing scraped biofilm cells was collected in a separate sterile tube of 2 ml final volume. The harvested cells (planktonic & biofilm phases) were serially ten-fold diluted in PBS and spot plated on organism specific agar plates using the method of Miles *et al*, (1938). After incubation at 37°C for 24 h aerobically, colonies were enumerated and total viable counts in initial samples were calculated.

2.12 Imaging

2.12.1 Quantification of NucB effects on biofilm structure using confocal laser scanning microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was used to visualise and quantify the structural alterations within biofilms grown on glass surfaces and treated with NucB as previously described by Shields et al. (2013) with some modifications. The acquired stacks of each image were further analysed using 3D imaging software (Imaris 8.0, Bitplane) and the COMSTAT2 plugin for ImageJ (Heydorn et al., 2000).

Sterile 13 mm coverslips were placed in wells of a 12-well tissue culture plate (Greiner Bio-One GmbH, Germany). When testing for biofilm dispersal, wells containing 2 ml of organism-specific culture medium were inoculated with 40 µL of -80ºC bacterial stock cultures equivalent to 5-10 x 10^6 CFU. The plate was incubated statically at the appropriate growth conditions in humid environment for 24-48 h. Culture medium was carefully removed after 24 h and replaced with fresh medium. At the end of incubation period, medium removed, coverslips were rinsed with PBS to removed loosely attached cells. Then 0.5 ml of NucB (0.5-1 µg ml^{-1}) was added to each well for 1 h at 37°C. This was compared with control biofilms that had been treated with NucB buffer only. Coverslips were rinsed again with PBS and then submerged in 300 µl of LIVE/DEAD® *BacLight*™ stain solution (Molecular Probes) and incubated at room temperature in the dark for 15 min.

LIVE/DEAD® *BacLight*™ stain consists of a combination of two fluorescent dyes, SYTO® 9 and propidium iodide (PI), which are employed to assess microbial cell viability (Boulos et al., 1999). Both stains can selectively bind to DNA but SYTO® 9 specifically stains intracellular genomic DNA, whereas PI binds to both intracellular and extracellular DNA. In intact cells, SYTO® 9 outcompetes and excludes PI from cells. In membrane-damaged cells, PI can enter the cells more easily and outcompetes the SYTO® 9 for DNA binding. These features allow the application of LIVE/DEAD® *BacLight*™ stain for assessing the viability of bacterial cells, and for visualising eDNA within biofilms.

Excess stain was removed by careful washing in PBS twice. Coverslips were inverted onto adhesive Gene Frame® (Thermo Fisher Scientific, UK) that had been placed on a microscope slide and filled with 25 µl PBS. Biofilms were examined using a Leica confocal microscope (Leica TCS SPE) with an argon/neon laser for visualisation of SYTO 9 (excitation 485 nm, emission 519 nm), and PI (excitation 536 nm, emission 617 nm). For biofilm inhibition, NucB was included (with the culture media and inoculae) during biofilm formation and processed using same protocol described above. Each experiment was repeated three times independently.

2.12.2 Testing antibiotic susceptibility of bacterial biofilms using combined microfluidic biofilm system and Live/Dead CLSM imaging

To assess effects of antibiotics on biofilm structure and viability, a combination of BioFlux microfluidic system (BioFlux200 system, Fluxion Biosciences Inc., San Francisco, USA), with LIVE/DEAD® *BacLight*™ staining and a Nikon A1R (invert) CLSM imaging was used (Nance et al., 2013). A forty-eight-well BioFlux plate (Biosciences Inc., San

Francisco, USA) was pre-conditioned with Tryptone soya broth (TSB) to enhance cell adhesion and biofilm formation. One hundred microlitres of TSB were added to each outlet well then flowed towards the inlet well at 1.0 dyn/cm^2 for 2 min at room temperature. Flow was stopped and the plate was incubated for 20 min at room temperature. At the end of pre-conditioning incubation, the residual growth medium in the outlet wells was transferred to matching inlet wells. The outlet wells were inoculated with 100 µl of adjusted inoculum (20x diluted and filtered overnight culture). The inoculum was flowed again towards the inlet at 1.0 dyn/cm^2 for 6 sec at 37°C to insert bacterial cells into BioFlux channel for biofilm formation. The plate was then incubated at 37°C for 45 min cell attachment and early growth. When bacterial seeding was confirmed with a Nikon Eclipse TCS-100 inverted light microscope, the inoculum was removed from each outlet well and 750 µl of growth medium was added into each inlet well. The plate was then incubated at 37°C for 18-20 h at 0.2 dyn/cm^2 for biofilm formation.

At the end of incubation, the remaining growth medium and culture were removed from each inlet and outlet well. Biofilms were challenged in triplicate with a range of Co-amoxiclav concentrations diluted in CAMHB (2048, 512, 128, 32, 8, 2 µg ml^{-1}) at 37°C for 18-20 h at a flow rate of 2.0 dyn/cm^2. Growth controls with no antibiotics were included. Following overnight incubation, all wells were aspirated and rinsed with 100 µl of PBS at room temperature for 20 min at a flow rate of 0.2 dyn/cm^2 to remove residual antibiotics solution. Following this, biofilms were stained with LIVE/DEAD® *BacLight*™ stain. The stain solution was prepared according to the manufacturer instructions by mixing 3 µl of SYTO 9 and 3 µl of PI per ml of PBS and flowed at room temperature, in dark for 45 min at 0.2 dyn/cm^2.

Following biofilm staining, the stain solution that remained in each inlet well were aspirated and followed by washing with 100 µl of PBS in each inlet at room temperature for 20 min at a flow rate of 0.2 dyn/cm^2 to remove excess stain in the channel. Biofilms in the channels were imaged using Nikon A1R (invert) CLSM.

2.12.3 Assessing effects of NucB on human cell apoptosis using Dual acridine orange/ethidium bromide staining and Microscopy

Effects of NucB on epithelial cell viability and the morphology of their nuclei were assessed using dual acridine orange (AO)/ethidium bromide (EB) staining technique in conjugation with microscopic imaging as described previously (Shin et al., 2015). Both AO (Sigma-Aldrich, St Louis, USA) and EB (Sigma-Aldrich, St Louis, USA) can selectively bind

to nucleic acid, and these dyes can be used in combination to assess cellular apoptosis and functional integrity of epithelial cell membrane. Using a 12-well flat bottom cell culture plate (Thermo Fisher Scientific, UK), cells were seeded at around 1.5- 2 × 10^4 cells/cm^2 and allowed to adhere and spread over 3-4 days in 5% CO_2 at 37°C. The culture medium was carefully removed after 48 h and replaced with fresh medium. Following 3-4 days of incubation, cells were treated with a range of NucB concentrations (final concentrations: 1, 10, 50, 100 μg ml^{-1}), NucB buffer (50 mM Tris pH 8.0), Dornase alfa (100 μg ml^{-1}), that were prepared in 500 μl of BEGM, or left untreated with BEGM only and incubated for the following 24–48 h on 5% CO_2 at 37°C. After 24 or 48 h incubation, the supernatants were discarded and the cells were stained with 300 μl of AO/EB stain solution for two min under gentle shaking. Positive control wells were treated with 500 μl of absolute methanol for 2 min before staining with AO/EB dye solution. The AO/EB dye solution was prepared by mixing 100 μg ml^{-1} of EB with 100 μg ml^{-1} of AO in PBS. The dye solution was aspirated, replaced by PBS and the cells were imaged using an inverted fluorescence microscope (Eclipse Ti-E fluorescence wide field, Nikon,UK**)** at 40x magnification**.** AO and EB were excited at 488 nm and emission detected at 525/20-nm and 635/20-nm respectively.

2.13 Statistical analysis

Experimental data were analysed using statistical software IBM SPSS Statistics ver. 23. Graphs and tables were produced using either Microsoft Excel or Sigma-plot® ver.12.5. Experiments were performed independently at least three times. Data values were represented as the mean ± standard error. Sample values were analysed for normality using Shapiro-Wilk test where data with a significance value of > 0.5 were considered normally distributed, whereas those with a significance value of < 0.5 were considered not normally distributed (non-parametric data). Normally distributed data were also checked for homogeneity of the variance by measuring the Levene statistic p value, where data with a p value > 0.5 meant that there was non-significant difference in the variances and data with p value <0.5 meant that there was significant difference in the variances and the probability that the difference is due to chance is very small. Student's paired *t-test* was used to determine the statistical significance between two treatment groups with homogenic and normally distributed data (parametric data), whereas Mann-Whitney U test was used for non-parametric data. ANOVA with Tukey's *post hoc* test was used to determine the statistical significance among more than two treatment groups with parametric data, whereas Kruskal-Wallis test was used with non-parametric data. Differences with a *p*-value of < 0.05 were considered statistically significant.

Chapter 3. Anti-biofilm activity of marine nuclease, NucB, against *Staphylococcus aureus* biofilms

3.1 Introduction

Staphylococcus aureus is a major pathogen implicated in a variety of biofilm-associated human diseases. In the head and neck region, *S. aureus* infections include chronic rhinosinusitis (CRS), chronic supportive otitis media, COME, and indwelling medical device-related infections such as those on cochlear implants, ventilation tubes, and speech valves (Zheng et al., 2018). *S. aureus* is one of the most common members of the microbial flora inhabiting the human skin and anterior nasal linings. Between 9% and 37% of the population have their nasal mucosal surfaces persistently colonised by *S. aureus* and transient carriage of *S. aureus* varies between 9% and 69% in different studies (Mehraj et al., 2016) . Possible host factors that may be involved in carriage include ABO-/secretor status, Toll-like receptor (TLR) 9 polymorphisms and a range of other gene polymorphisms (Nurjadi et al., 2012, Nurjadi et al., 2018, Brown et al., 2015). Alternatively, the phenotypic properties of the strains of *S. aureus* that a person encounters may affect the outcome of colonisation (Jenkins et al., 2015).

S. aureus can form a robust biofilm encased within self-produced extracellular polymeric substances (EPS). These polymers play key roles in maintaining the structure of biofilms through cell-cell cohesion and cell-to-surface adhesion, conserving nutrients, facilitating the dissemination of genetic material among biofilm cells, and shielding biofilm cells from harsh external environmental stresses such as heavy metals, UV light, host immune action and antimicrobial agents (Teitzel and Parsek, 2003, Leid et al., 2002, Flemming et al., 2016). Furthermore, EPS plays an important role in providing a nutrient-limited environmental niche within biofilms which promotes the appearance of metabolically dormant cells and persister cells. These cells are well-known to be tolerant to high concentrations of antimicrobials and their presence may be a major factor in the reduced susceptibility of bacterial biofilms to treatment with antimicrobials that has been termed ‘antimicrobial recalcitrance’. Other mechanisms that elicit reduced susceptibility of biofilm microorganisms to treatment with antimicrobials include phenotypic alteration of the biofilm cells trigged by cellular attachment to a surface, for example by up-regulation of efflux pumps, or variations in pH, nutrients, or O_2 tension across the intracellular milieu of biofilm, which may inactivate antibiotics directly or enhance the growth of anaerobic cells toward the

core of biofilm (Hall and Mah, 2017). In addition, reduced permeability of certain antibiotics through the biofilm EPS matrix may protect cells in the inner regions of the biofilm. These mechanisms may act individually or collectively (Goltermann and Tolker-Nielsen, 2017).

The EPS of *S. aureus* can consist of different structural compounds including poly-N-acetyl-glutamate (PNAG), proteins, lipids and extracellular DNA (eDNA) (Fleming and Rumbaugh, 2017). Originally, teichoic acids and proteins derived from host and staphylococcal cells were considered to be ubiquitous and vital constituents of *S. aureus* biofilm EPS (Hussain et al., 1993). Increasingly, evidence indicates that eDNA is also a key structural component within the EPS of many microbial biofilms including *S. aureus* (Kaplan et al., 2012, Rice et al., 2007, Shields et al., 2013). The critical role of eDNA in maintaining the structural integrity of bacterial biofilms was first discovered in vitro by Whitchurch et al. (2002) when they demonstrated dramatic dispersal of *P. aeruginosa* biofilms treated with DNase I enzyme. Dornase alfa (rhDNase) is in current use as a therapeutic, and is applied to reduce the sputum viscosity in cystic fibrosis patients by disrupting neutrophil extracellular traps derived from microorganisms and host neutrophils (Manzenreiter et al., 2012). Subsequent studies performed in various bacterial species including *S. aureus* have provided evidence that eDNA plays a variety of roles in the biofilm matrix, such as facilitating the exchange of genetic material, acting as a nutrient source, maintaining structural stability in mature biofilms, enhancing initial adhesion of bacterial cells to the surface, and contributing to protection from antimicrobials and host immune actions (Kaplan et al., 2012, Mulcahy et al., 2008, Rice et al., 2007, Shields et al., 2013). Therefore, the addition of DNase enzymes could potentially inhibit biofilm formation, disperse pre-established biofilms, or increase the susceptibility of biofilms to antibiotics (Okshevsky et al., 2014).

Previous studies have largely employed crystal violet staining or qualitative microscopy methods to characterise the effects of deoxyribonuclease (DNase) enzymes on bacterial biofilms, such as those formed by *S. aureus*. To provide more detailed information on the role of eDNA in biofilm structure, this section of the study focused on optimising quantitative image analysis tools and developing an in vitro biofilm model that could be employed to investigate the sensitivity of clinically relevant bacterial isolates to DNase treatment, and to combination therapy with DNase and antibiotics. Bacteria that had previously been isolated from paranasal sinuses of patients with chronic rhinosinusitis (Shields et al., 2013) were employed since these were readily available in the laboratory and could be used while the application for ethical approval was in process. While these strains were not from otitis media, they were recent clinical isolates that had already been isolated

and identified. The microbiota of chronic rhinosinusitis is similar to that of chronic otitis media, and both are related to species found on the adenoids (Ren et al., 2013). Therefore, it was felt that strains from chronic rhinosinusitis were appropriate for developing techniques to be employed later with isolates from COME.

3.2 Aims and objectives

The main aims of this section were to optimise methods for quantification of biofilm formation and to investigate the potential of NucB to be employed as a safe (non-toxic) anti-biofilm therapy against *in vitro* biofilms of relevant *S. aureus* clinical isolates. The objectives were as follows:

1. To assess the ability of clinical isolates relevant to COME to form biofilms.
2. To develop techniques for assessing the sensitivity of *in vitro* biofilms of relevant clinical isolates to NucB treatment.
3. To investigate the ability of NucB to enhance the impact of antibiotics against *in vitro* biofilms.
4. To investigate the potential toxicity of NucB against bacteria, and the possible cytotoxicity to human epithelial cells relevant to the middle ear.

3.3 Efficacy of NucB against in vitro biofilm models of *S. aureus*

3.3.1 Test the sensitivity of S. aureus biofilms to NucB treatment using 96-well microtitre plate model

While waiting for ethical approval for isolation of COME strains, we established the key techniques for biofilm growth and analysis using clinically relevant strains that had previously been isolated from a project on chronic rhinosinusitis (CRS). A range of isolates derived from patients with CRS were assayed for biofilm formation in 96-well MTP using organism-specific media and growth conditions. The selected isolates are shown in Table 3. 1. *Staphylococcus aureus* SB14 and SB17 strains showed the greatest capability to form robust and consistent *in vitro* biofilms (Table 3. 1). Therefore, these strains were taken forward as model organisms for optimising the methodologies that would be employed later in the thesis.

Table 3. 1 Biofilm formation of selected CRS isolates quantified by CV staining assay

Bacterial species and strain	Biofilm formation (A_{570} (SE))
Haemophilus influenzae SB11BBAII	0.47 (0.08)
Moraxella catarrhalis SB11BBAIV	0.62 (0.09)
Streptococcus pneumoniae SB11BBAI	0.21 (0.04)
Streptococcus pneumoniae SB10BBAI	0.50 (0.1)
Staphylococcus aureus SB14	2.43 (0.2)
Staphylococcus aureus SB17	1.52 (0.3)

S. aureus SB14 and SB17 showed the highest capabilities to form *in vitro* biofilms among the assayed CRS clinical isolates.

In order to determine the effective biofilm inhibitory and dispersal concentration of NucB to be used in subsequent experiments, a range of NucB concentrations (see Figure 3. 1) were either included during biofilm formation for 20 h (for inhibition assays) or incubated for one hour with 24 h old pre-established biofilms of *S. aureus* SB14 grown in a 96-well MTP (for dispersal assays; see Materials and Methods section 2.8.2). For the inhibitory effect of NucB on biofilm formation (see Figure 3. 1(A)), a statistically significant concentration-dependent reduction in biofilm formation was observed over a concentration range of 25-500 ng ml^{-1} in comparison to PBS treated controls ($p<0.05$; One-Way ANOVA with Tukey's Honestly Significant Difference (HSD) *post-hoc* test after Normality and Homogeneity of variances test had indicated that the data satisfied the assumptions for ANOVA test). Biofilm biomass was reduced by approximately 50% in the presence of 500 ng ml^{-1} of NucB. When testing the efficacy of NucB to disperse pre-formed *S. aureus* SB14 biofilms (see Figure 3. 1

(B)), similarly significant dose-dependent biofilm removal was demonstrated over a concentration range of 10-500 ng ml^{-1} NucB. Interestingly, NucB at 100-500 ng ml^{-1} concentrations showed more than 50% dispersal of pre-established biofilm when compared to PBS treated control biofilms. For the inhibition assay, NucB at 10 ng ml^{-1} showed a borderline effect, and for both inhibition and dispersal assays NucB did not show significant disruption of biofilms at concentrations lower than 10 ng ml^{-1} (data not shown). A similar trend of biofilm inhibition and dispersal activities of NucB was obtained when biofilms formed by *S. aureus* SB17 strain were treated with the similar range of NucB concentrations (data not shown). Based on these findings, NucB at 500 ng ml^{-1} was selected as the most suitable concentration to be used in subsequent experiments.

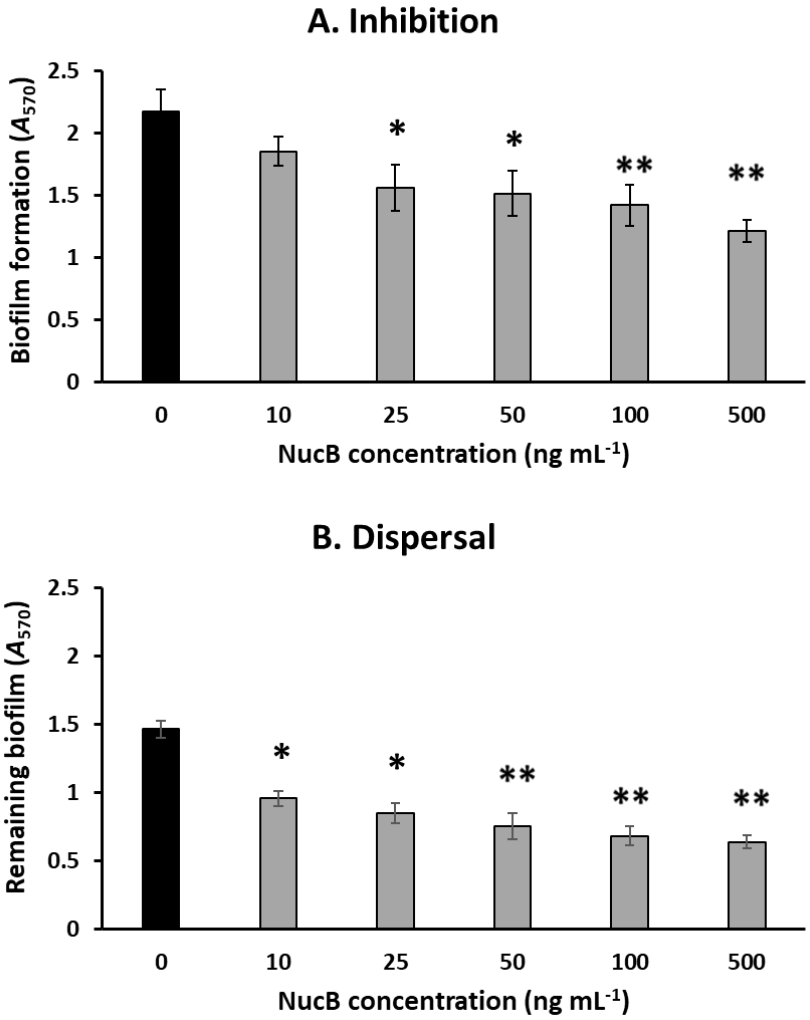


Figure 3. 1 NucB inhibits biofilm formation and disperses pre-formed biofilms of *S. aureus* SB14.

A range of concentrations of NucB were incubated with preformed (24 h) *S. aureus* SB14 biofilms at 37°C for 1 h (B), or included during biofilm formation for 20 h (A). Biofilms were stained with crystal violet, and the biofilm biomass was quantified by measuring A_{570}. Mean values from three independent assays are shown and error bars represent standard error of the mean. Statistical significance was calculated using One-Way ANOVA test with Tukey's HSD *post-Hoc* comparison (* $p<0.05$, ** $p<0.01$). NucB showed concentration-dependent inhibition and dispersal of *S. aureus* SB14 biofilms over the assayed concentration range.

To validate the DNase activity of NucB against *S. aureus* biofilms, commercial bovine DNase I at 5 µg ml^{-1} was included during biofilm formation for 20 h (inhibition assays) or was added to the 24 h-old pre-established biofilms of *S. aureus* SB14 (dispersal assays) using the standard 96-well MTP crystal violet staining assay (see Material and Methods section 2.8.2). In both biofilm inhibition and dispersal assays, DNase I showed just above 50% reduction in biofilm formation and around 50% removal of preformed biofilm of *S. aureus* SB14 (see Figure 3. 2). These reductions in biofilm biomass were similar to those observed with NucB, which is consistent with NucB acting through a DNA-degrading mechanism.

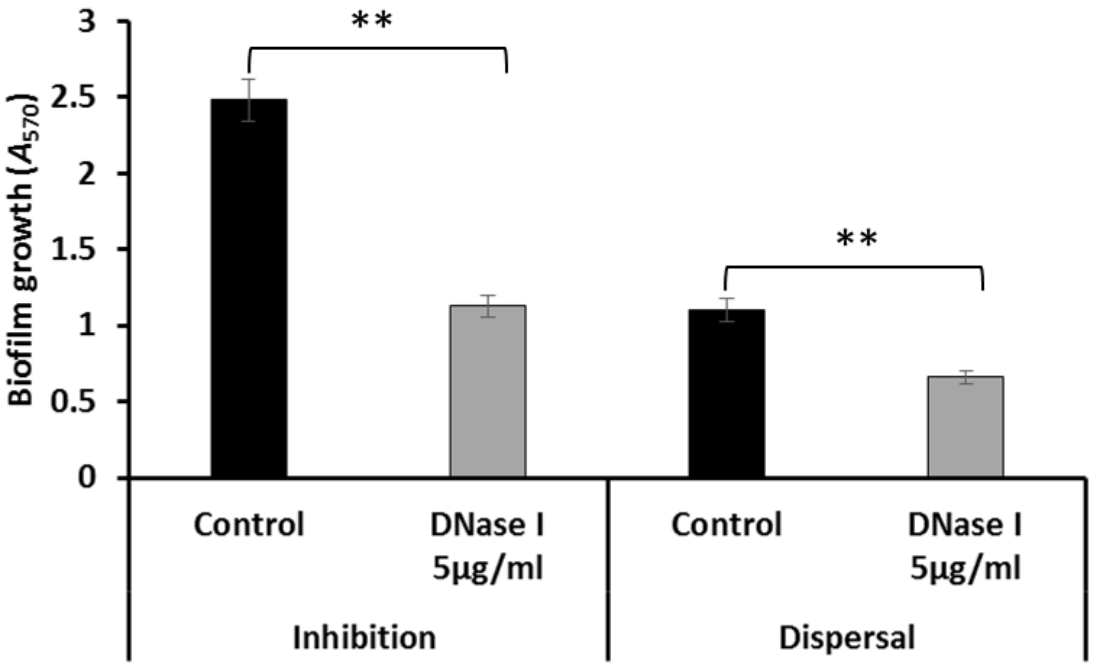


Figure 3. 2 Effect of Bovine DNase I on *S. aureus* SB14 biofilms

For biofilm dispersal, DNase I (5 µg ml^{-1}) was added to pre-formed (24 h) *S. aureus* SB14 biofilms at 37°C for 1 h or included during biofilm formation for 20 h for the inhibition assay. Biofilms were stained with crystal violet, and the biofilm biomass was quantified by measuring A_{570}. Mean values from three independent assays are shown and error bars represent standard error of the mean. DNase I significantly inhibited biofilm formation and dispersed pre-formed biofilms of *S. aureus* SB14 (**p <0.01; paired two samples *t*-test).

3.3.2 Image analysis of the structure of NucB treated S. aureus *biofilms*

Having demonstrated a potent anti-biofilm activity of NucB against biofilms of selected *S. aureus* clinical isolates, it was considered important to obtain more comprehensive information about the structural changes within the biofilms treated with NucB. A combination of CLSM and LIVE/DEAD fluorescent stains were employed to study the typical three-dimensional architecture of biofilms. Biofilms of the chosen microorganisms were grown on the surface of glass coverslips, stained with LIVE/DEAD® BacLight™ stain, and imaged by CLSM (see Materials and Methods section 2.12.1). This assay was focused on the same *S. aureus* clinical isolates (SB14 and SB17) that had been investigated in previous experiments. In the absence of NucB, biofilms of *S. aureus* SB14 were relatively thick and were mainly composed of several layers of cells that covered the majority of the surface (see Figure 3. 3 and Figure 3. 4). In most areas, heterogeneous patches or clumps of cells protruded from the surface at a thickness ranging between 7-10 μm. However, biofilms of *S. aureus* SB17 were relatively thin and consisted mostly of a single layer or a few layers of cells that were spread widely over the surface with medium size patches or clusters of cells projecting from the surfaces at 3-6 μm thickness (Figure 3. 3 and Figure 3. 4 C, G). Using LIVE/DEAD® BacLight™ stain, we were able to visualise both live cells (green) and dead or damaged cells (red). The majority of cells were alive (green) in biofilms of both *S. aureus* SB14 and SB17.

When NucB at 0.5 μg ml^{-1} was included during biofilm formation of S. *aureus* SB14 and SB17 for 20 h, biofilm formation was significantly inhibited compared with controls lacking NucB (see Figure 3. 3). Biofilms grown in the presence of 0.5 μg ml^{-1} were less extensive than the untreated controls, and were composed of irregularly distributed individual cells or very small clumps of cells (Figure 3. 3 B, F and D, H). Red (compromised) cells were difficult to be see in printed images. However, visual inspection of the images on screen showed that the proportion of dead and live cells in biofilms treated with NucB was almost equal to the control biofilms, indicating that NucB had no killing effect on biofilm cells.

Visual observation of 3D-rendered images indicated that the treatment of biofilms with NucB during growth altered the biofilm structure. In order to assess and quantify this alteration, COMSTAT 2 software was used to determine some of the key structural parameters of biofilms such as the biomass, average thickness and roughness coefficient of the biofilms (Table 3. 2).

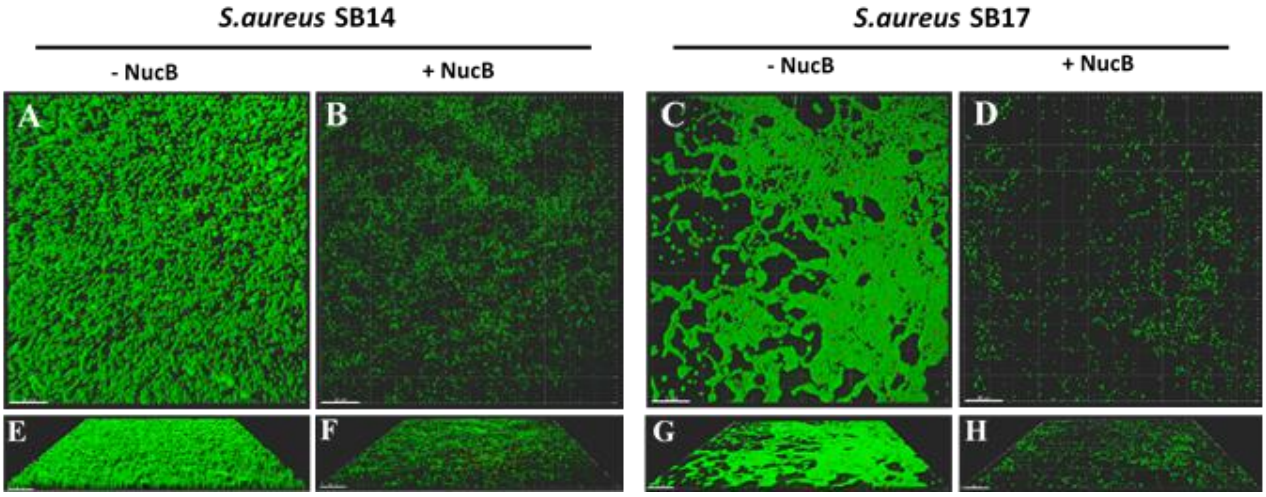


Figure 3. 3 Effect of NucB on biofilm formation by *S. aureus* SB14 and SB17, observed by CLSM

NucB (0.5 µg ml^{-1}) was included during biofilm formation on glass surfaces for 24 h and visualised with CLSM using fluorescent LIVE/DEAD® BacLight™ stain. Merged images show live cells with green signals(Syto9) and cells with red signals (propidium iodide) are dead or damaged cells. Note that it is difficult to see red signals in the printed images, however they were clearly evident when the images were inspected on the sceen, albeit in low numbers compared with green cells. (A, E and C, G) represent control biofilms with no NucB, and (B, F and D, H) represent biofilms incubated with NucB. (A-D) represent x–y plane, (E-H) represent an angled view of each plane (x–y–z) and the scale bars are 30 µm. NucB-treated biofilms are less extensive than controls, without NucB.

Table 3. 2 COMSTAT analysis of *S. aureus* SB14 and SB17 biofilm formation in the presence or absence of NucB (0.5 µg ml^{-1}).

Biofilm parameters	***S. aureus* SB14**			***S. aureus* SB17**		
	- NucB	+ NucB (0.5 µg ml-1)	P-Value	- NucB	+ NucB (0.5 µg ml-1)	P-Value
Biomass (µm3/µm2)	2.9 (0.2)	1.3 (0.3)	0.002	3.1 (0.6)	1.1 (0.4)	0.02
Average thickness: (µm)	4.9 (0.4)	2.2 (0.5)	0.001	4.0 (0.5)	1.2 (0.5)	0.001
Roughness coefficient	0.5 (0.1)	0.8 (0.2)	0.27	0.6 (0.2)	1.1 (0.3)	0.2

Data [mean (SE)] were generated from the imaging of three randomly selected areas on each coverslip from at least three independent experiments. NucB greatly altered the structure of *S. aureus* biofilms by significantly reducing biomass and average thickness of biofilms ($p<0.05$, regarded as statistically significant differences from no NucB controls; two samples *t*-test).

For *S. aureus* SB14, the average biofilm biomass and thickness of the untreated biofilms were 2.9 $\mu m^3/\mu m^2$ and 4.9 μm, respectively (Table 3. 2). The mean biofilm biomass and thickness of the NucB-treated biofilms were 1.3 $\mu m^3/\mu m^2$ and 2.2 μm, respectively (Table 3. 2). Therefore, the average biomass and average thickness of NucB treated biofilms were significantly reduced by more than 50% compared to untreated controls. In the case of S. *aureus* SB17, the inclusion of NucB during biofilm growth of S. *aureus* SB17 significantly decreased the average biofilm biomass and thickness by more than 70% in comparison with untreated controls (Table 3. 2). For both S. *aureus* SB14 and SB17, the roughness coefficient values of the biofilms incubated with NucB were increased compared with the control, although the differences were not statistically significant. Collectively, these data indicate that inclusion of NucB (0.5 μg ml^{-1}) efficiently inhibited biofilm formation by two clinically relevant *S. aureus* strains.

To determine the impact of NucB on the structure of pre-formed *S. aureus* biofilms, *S. aureus* SB14 and SB17 were cultured on glass coverslips for 48 h, and treated for 1 h (see Material and Methods section 2.12.1). Treatment with NucB resulted in significant reduction of biofilm biomass when compared to the PBS treated controls (see Figure 3. 4). From CLSM imaging, pre-established biofilms that were incubated with NucB had greatly reduced biomass than the control. No differences in the viability of biofilms cells were detected and again NucB-treated biofilms consisted of isolated cells or very small aggregates of cells. Further quantitative analysis of the images demonstrated that the mean biomass and mean thickness of *S. aureus* SB14 biofilms incubated with buffer alone were 9.5 $\mu m^3/\mu m^2$ and 12.7μm, respectively (Table 3. 3). The mean biomass and thickness of the preformed biofilms incubated with NucB at 0.5 μg ml^{-1} were 1.1 $\mu m^3/\mu m^2$ and 1.2 μm, respectively which were decreased by about 75% compared with the untreated controls. For S. *aureus* SB17, in the absence of NucB, the average biofilm biomass and thickness were 3.5 $\mu m^3/\mu m^2$ and 7.0 μm. The average biofilm biomass and thickness of NucB treated biofilms were 2.1 $\mu m^3/\mu m^2$ and 2.5 μm. When compared to biofilms incubated with PBS alone, NucB treated biofilms showed significant reduction in average biomass and thickness by about 50%. It should be noted that an approximately 3-fold reduction in the average thickness of S. *aureus* SB17 biofilms treated with NucB was associated with only a 1.7-fold reduction in biomass of the same biofilms (Table 3.3). This apparent discrepancy may have arisen because COMSTAT 2 software includes the voids (empty spaces) within the biofilm in the measurement of the average thickness but excludes them in the measurement of the biomass of biofilm (Heydorn

et al., 2000). Similar patterns were also observed in the inhibition assays of S. *aureus* SB17 & SB14 (see Table 3. 2).

Overnight (24 h-old) grown biofilms of *S. aureus* SB14 and SB17 on the glass surface, which were used in the inhibition assay, were much thinner than 48 h-old biofilms which were used in the dispersal assay (see Figure 3. 3 and Figure 3. 4). In the absence of NucB treatment, average biofilm biomass and average thickness were more than twofold less than those of untreated 48 h-old biofilms of the same strain (Table 3. 2 and Table 3. 3).

Overall, the qualitative and quantitative outputs of CSLM imaging analysis were consistent with the quantitative results obtained from 96-well plate crystal violet staining assay, and showed that eDNA is a key structural component of the extracellular matrix (EPS) of the clinically relevant *S. aureus* biofilms. Furthermore, the addition of NucB at 0.5μg ml^{-1} could efficiently inhibit biofilm formation and efficiently detach pre-established biofilms of *S. aureus* clinical isolates without negative impact on the viability of cells within these biofilms.

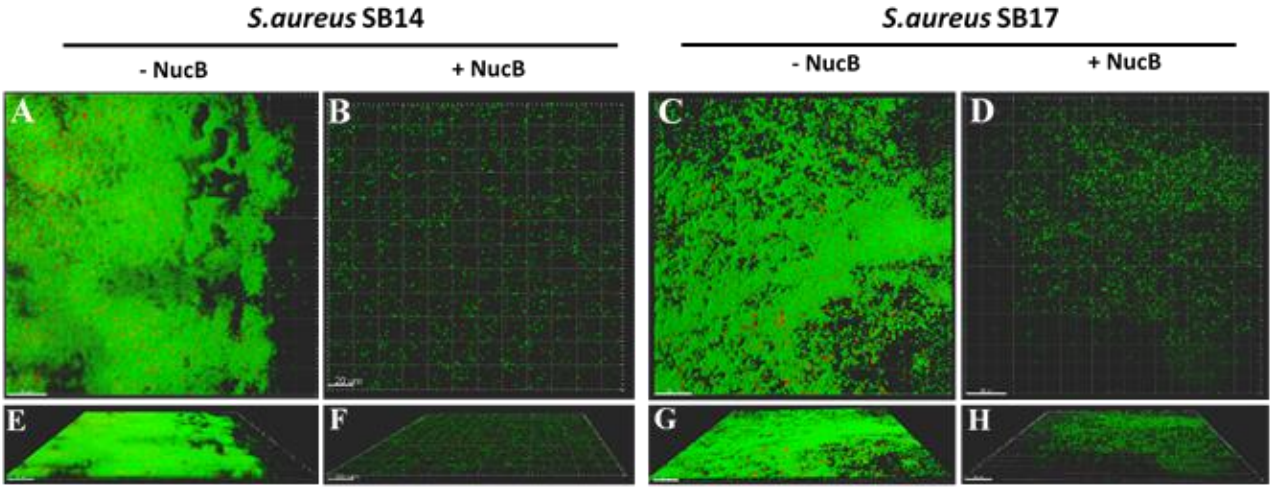


Figure 3. 4 Effect of NucB on the structure of established biofilms of *S. aureus* SB14 and SB17 observed by CLSM

Biofilms were grown on glass surfaces for 48 h, treated with 0.5 µg ml^{-1} NucB for 1 h at 37°C, stained with LIVE/DEAD® BacLight™ stain and imaged with CLSM. Merged images show live cells with green signals (Syto9) and cells with red signals (propidium iodide) are dead or damaged. NucB at 0.5 µg ml^{-1} substantially dispersed pre-established biofilms of *S. aureus* SB14 and SB17 (B, F and D, H) when compared to control (PBS) treated biofilms (A, E and C, G). (A-D) represent x–y plane, (E-H) represent an angled view of each plane (x–y–z) and the scale bars are 30 µm.

Table 3. 3 COMSTAT analysis of *S. aureus* SB14 and SB17 biofilms dispersal by NucB (0.5µg ml^{-1})

Biofilm parameters	***S. aureus* SB14**			***S. aureus* SB17**		
	- NucB	+ NucB (0.5 µg ml^{-1})	P-Value	- NucB	+ NucB (0.5 µg ml^{-1})	P-Value
Biomass ($\mu m^3/\mu m^2$)	9.5(1.1)	2(0.6)	0.0001	3.5(0.5)	2.1(0.2)	0.03
Average thickness: (µm)	12.7(1.7)	2.7(0.8)	0.0001	7.0(1.1)	2.5(0.4)	0.003
Roughness coefficient	0.26(0.1)	0.9(0.3)	0.09	0.78(0.2)	0.2(0.04)	0.006

Data [mean (SE)] were generated from the imaging of three randomly selected areas on each coverslip from at least three independent experiments. NucB substantially altered pre-established biofilms structure by significantly reducing biomass and average thickness of biofilms ($p<0.05$ regarded as statistically significant difference from no NucB controls; two samples *t*-test).

3.4 Testing the antimicrobial susceptibility of *S. aureus* planktonic cells

In clinical practice, the measurement of minimum inhibitory concentration (MIC) and minimum bactericidal concentrations (MBC) are the standards to determine the sensitivity of bacteria to antibiotics, and they provide important supportive information for clinicians to treat infections (Andrews, 2001). MIC and MBC represent the sensitivity of bacteria in a free-living state. However, in infections such as otitis media, bacteria are present in a biofilm, and may be significantly more tolerant than planktonic cells to antimicrobial agents. To compare the level of antimicrobial tolerance of *S. aureus* biofilms with planktonic cells, it was necessary to assess antimicrobial sensitivity in both biofilm and planktonic cultures. Initially the standard MICs and MBCs of *S. aureus* SB14 and SB17 clinical strains for Co-amoxiclav (clavulanic acid/amoxicillin) were determined using the standard micro-dilution method (see Material and Methods section 2.9.1). Co-amoxiclav was selected because of its well-known action against *S. aureus* which was the focus of the *in vitro* experimental investigations outlined in this chapter. Moreover, Co-amoxiclav is also effective against the three most common pathogens associated with COME (*Haemophilus influenzae, Streptococcus pneumoniae,* and *Moraxella catarrhalis*), and has been used for the treatment of CRS and otitis media. To validate the method, the MIC and MBC of *S. aureus* NCTC 6571 reference strain were also determined (see Table 3. 4). The MIC and MBC obtained for *S. aureus* NCTC 6571 was similar to the quality control (QC) value for this organism (0.12 μg ml^{-1}) (Andrews, 2001). Measurement of the Co-amoxiclav susceptibilities of the planktonic cultures of these clinical strains enabled us to estimate the concentration range to be used in measuring the antimicrobial sensitivity of biofilms.

Table 3. 4 MICs and MBCs of *S. aureus* strains for Co-amoxiclav determined by standard microdilution method.

***S. aureus* strains**	**MIC (μg ml^{-1})**	**MBC (μg ml^{-1})**
SB14	1	4
SB17	1	4
NCTC6571	0.125	0.25

3.5 Investigating the antibiotic susceptibility of *S. aureus* biofilms

Following determination of MICs and MBCs of the selected planktonic *S. aureus* cultures for Co-amoxiclav, the sensitivities of these bacterial biofilms to Co-amoxiclav treatment were investigated using a range of assays that had been described in the literature.

3.5.1 Testing the susceptibility of S. aureus *biofilms to antibiotics using MBEC assay.*

The minimum biofilm eradication concentration (MBEC) assay is a quick and reproducible high-throughput technique developed for measuring the antimicrobial sensitivity of microbial biofilms. Thus, MBEC™ (HTP) microtitre plates with peg inserts attached to the lids were used to determine MBECs of *S. aureus* SB14, SB17, and NCTC6571 for Co-amoxiclav. Initially, the time of sonication required to dislodge biofilms cells attached to pegs were optimized using a combination of total viable counts and crystal violet (CV) staining to determine release of cells. Biofilms of *S. aureus* SB14 were grown on pegs in MBEC™ (HTP) plates for 24 h according to the manufacturer's instructions (see Materials and Methods section 2.9.2). After 24 h, the peg lid was rinsed for 1-2 min and three pegs from randomly selected places were removed. The extent of biofilm formation was assessed by CV staining which represented the '0' sonication time point (see Figure 3. 5). Following this, the peg lid was placed in fresh 96-well plate containing 200 µl of TSB and sonicated for different time periods (10, 20, 30, 60 min). After each sonication time, 3 pegs were removed, and biofilm biomass was quantified by CV (see Figure 3. 5 (B)). At the same time, the bacterial suspension released from each peg was serially diluted and spot plated to calculate total viable count (see Figure 3. 5(C)). A sonication time of 30 min released the majority of cells from biofilm formed on the peg surface (see Figure 3. 5). Longer sonication times dislodged greater numbers of cells, which was evident by reduced biofilm biomass in comparison to other time points. However, this increase in bacterial release was associated with a negative impact on cell viability. Therefore, sonication for 30 min was applied in subsequent experiments.

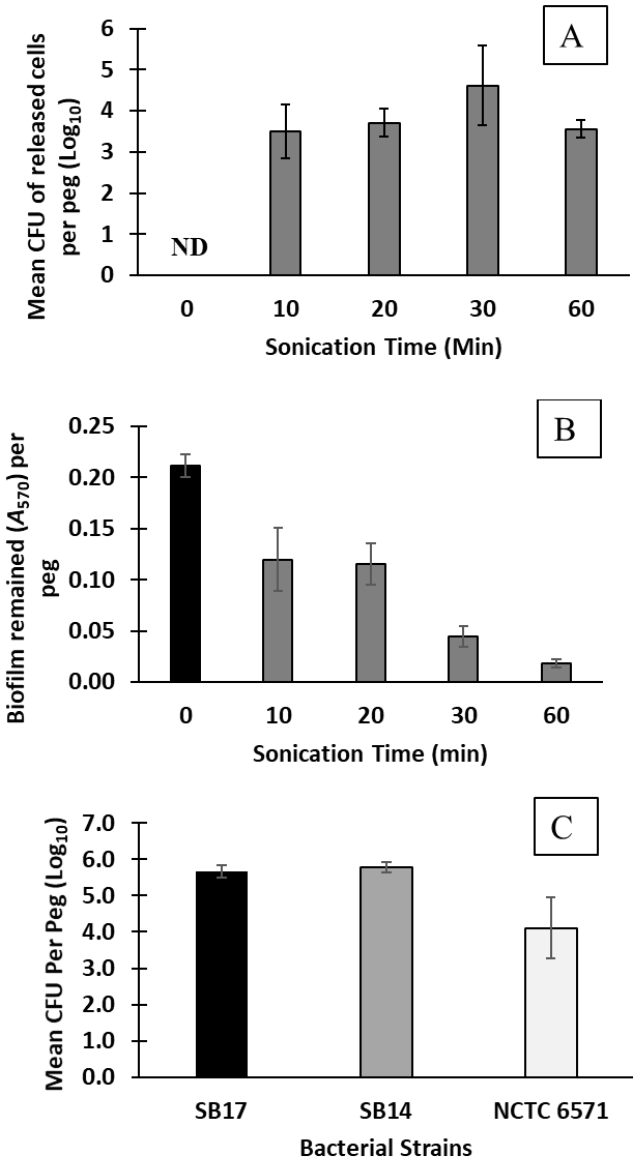


Figure 3. 5 Optimisation of biofilm removal from MBEC pegs using sonication.

S. aureus SB14, SB17, and NCTC6571 biofilms were grown on MBEC pegs for 24 h. The pegs were sonicated for 10-60 min. (A) The number of cells released from biofilm, determined by plating and calculating mean total viable counts of released bacteria (CFU per peg); 'ND' = 'not determined'. (B) Extent of biofilm remained per peg, quantified by CV staining assay. (C) Mean numbers of *S. aureus* CFU released from biofilms by sonication for 30 min. In all panels, bars represent means and standard deviations from 3 independent repeats are shown. The majority of cells were released from biofilm formed on peg by sonication for 30 min without negative impact on cell viability.

To assess the reproducibility of biofilm formation on each peg, biofilms of each *S. aureus* strain were grown for 24 h (see Materials and Methods section 2.9.2). The peg lid was sonicated for 30 min and the suspensions of at least 3 randomly selected wells were individually serially diluted and spot plated. There were no significant differences in biofilm formation among the selected pegs for each strain, and the mean biofilm formation for each *S. aureus* strain is shown in Figure 3. 5(A).

Following these optimisation steps, the determination of MBECs of *S. aureus* SB14, SB17 and NCTC6571 for Co-amoxiclav was performed. One of the well-recognized features of bacterial biofilms is a reduced susceptibility to antimicrobial treatment compared with their planktonic counterparts. Hence, a higher range of Co-amoxiclav concentrations, up to 1000 fold greater than their MICs, was used to challenge preformed biofilms of these *S. aureus* strains in order to determine the concentration of antibiotics necessary to eradicate bacterial cells within biofilms. Pre-established 24 h biofilms of *S. aureus* SB14, SB17, and NCTC6571 were formed on the peg lids of MBEC plates (see Materials and Methods section 2.9.2), and treated with a range of Co-amoxiclav concentrations (0.25-1,024 μg ml^{-1} for SB14 and SB17 and 0.25- 256 μg ml^{-1} for NCTC6571) for 24 h. Biofilms were removed from the peg lid by sonication and placed in a fresh 96-well plate containing recovery medium (CAMHB), and the MBEC values were determined following 24 h of incubation at 37°C in air by checking for turbidity in the wells and measuring OD_{650} using a microtitre plate reader. Also, it was possible to determine the MICs of these bacteria from MBEC device by reading OD_{650} in wells of the challenge plate following 24 h incubation of the isolates in Co-amoxiclav.

The MICs of Co-amoxiclav for the tested *S. aureus* strains (SB14, SB17, and NCTC6571) determined by MBEC device were similar to those obtained by standard microdilution methods (see Table 3. 4). However, despite several attempts, it was not possible to obtain consistent and reproducible readings for the MBEC values for all tested strains (see Figure 3. 6). An inability to obtain reproducible MBEC values could be due to the step of overnight incubation of biofilm cells released from pegs by sonication in the recovery medium (CAMHB) according to the manufacturer's instructions following 24 h antibiotic challenge. Low numbers of surviving cells such as persisters would potentially lead to growth in samples that had been almost eliminated by antibiotic treatment. Survival of even a single bacterial cell could eventually produce heavy bacterial growth during overnight incubation resulting in inconsistent and non-reproducible MBECs. Furthermore, minor modifications in the method employed such as using an incubator with a rotating shaker rather than tilting one during biofilm formation could also influence the reproducibility of the MBEC assay, as has

been reported in other studies (Dall, 2013). The MBECs of Co-amoxiclav for *S. aureus* SB14, SB17, and NCTC6571 were greater than the highest concentration of antibiotic in all attempts and were at least 100-1000 fold greater than the MICs for planktonic culture of these strains.

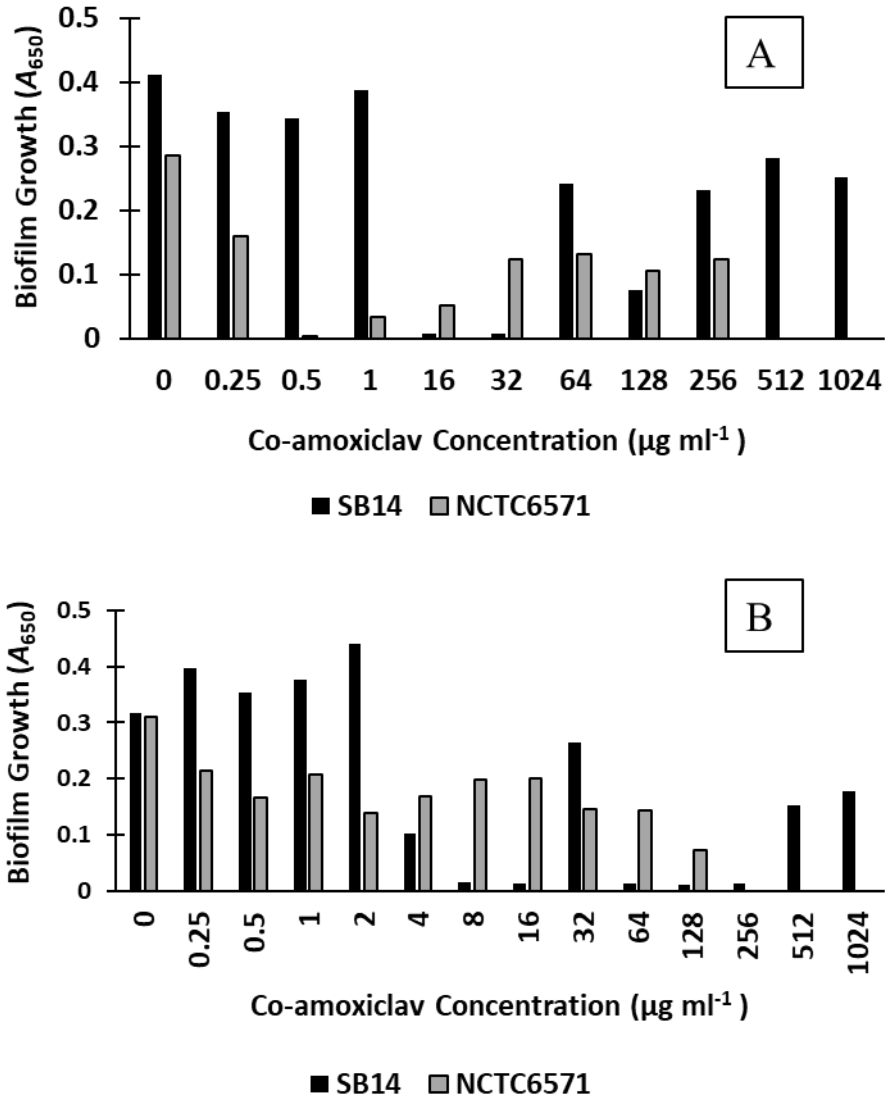


Figure 3. 6 Determination of MBECs of Co-amoxiclav for *S. aureus* biofilms.

Biofilms of *S. aureus* (SB14 and NCTC6571) were grown in MBEC plates as described in Materials and Methods, challenged with a range of Co-amoxiclav concentrations for 24 h at 37°C and biofilms were recovered from peg lids by sonication. MBEC values were determined by measuring OD_{650} following 24 h incubation at 37°C in air. (A) and (B) are examples of many experiments performed showing inconsistent MBECs of co-amoxiclav determined for *S. aureus* SB14 and NCTC6571 biofilms. MBEC values for *S. aureus* NCTC6571 were 0.5 and 128 µg ml^{-1} in the two different assays. The MBEC values of *S. aureus* SB14 were 8 and 16 µg ml^{-1}, with significant growth in cultures at concentrations higher than the estimated MBEC values. Therefore, overall the MBEC data must be treated with caution.

3.5.2 Testing antibiotic susceptibility of S. aureus *biofilms using combined microfluidic biofilm system and Live/Dead CLSM imaging*

As an alternative to the MBEC device and for illustration purposes, a medium-throughput microfluidic biofilm system was used to assess the susceptibility of *S. aureus* SB14 biofilms to a range of Co-amoxiclav concentrations using combined LIVE/DEAD BacLight stain and CLSM imaging. Biofilms were grown within the channels of 48-well plates (24 channels) for 24 h according to the protocol described in Materials and Methods section 2.12.2 and challenged with Co-amoxiclav ranging from 2- 2048 μg ml^{-1} ml for up to 24 h. Negative (no antibiotics) and positive (treated with 70% ethanol) controls were included and samples were stained using LIVE/DEAD BacLight stain and imaged by CLSM. One of the drawbacks observed using this system was the inability to obtain uniform biofilm formation in all channels despite several attempts at optimisation, which included using different dilutions of organism specific broth, bacterial inoculae, and using different flow rates for biofilm formation.

Visual inspection of the preliminary 3D-rendered images showed that the treatment of biofilms with different concentrations of Co-amoxiclav caused an increased cell death which was proportional to the increased Co-amoxiclav concentrations (see Figure 3. 7). The vast majority of biofilm cells were dead/damaged at Co-amoxiclav concentrations ranging from 128-2048 μg ml^{-1}. By contrast, the majority of cells in the negative control remained viable. In conclusion, the preliminary results obtained using the microfluidic biofilm system combined with LIVE/DEAD staining and CLSM imaging showed that the concentration of Co-amoxiclav required to kill the vast majority of cells within *S. aureus* SB14 biofilm was about 128-fold greater than the MIC for Co-amoxiclav (compare with the MIC of Co-amoxiclav for *S. aureus* SB14, which was 1 μg ml^{-1}; see Table 3. 4).

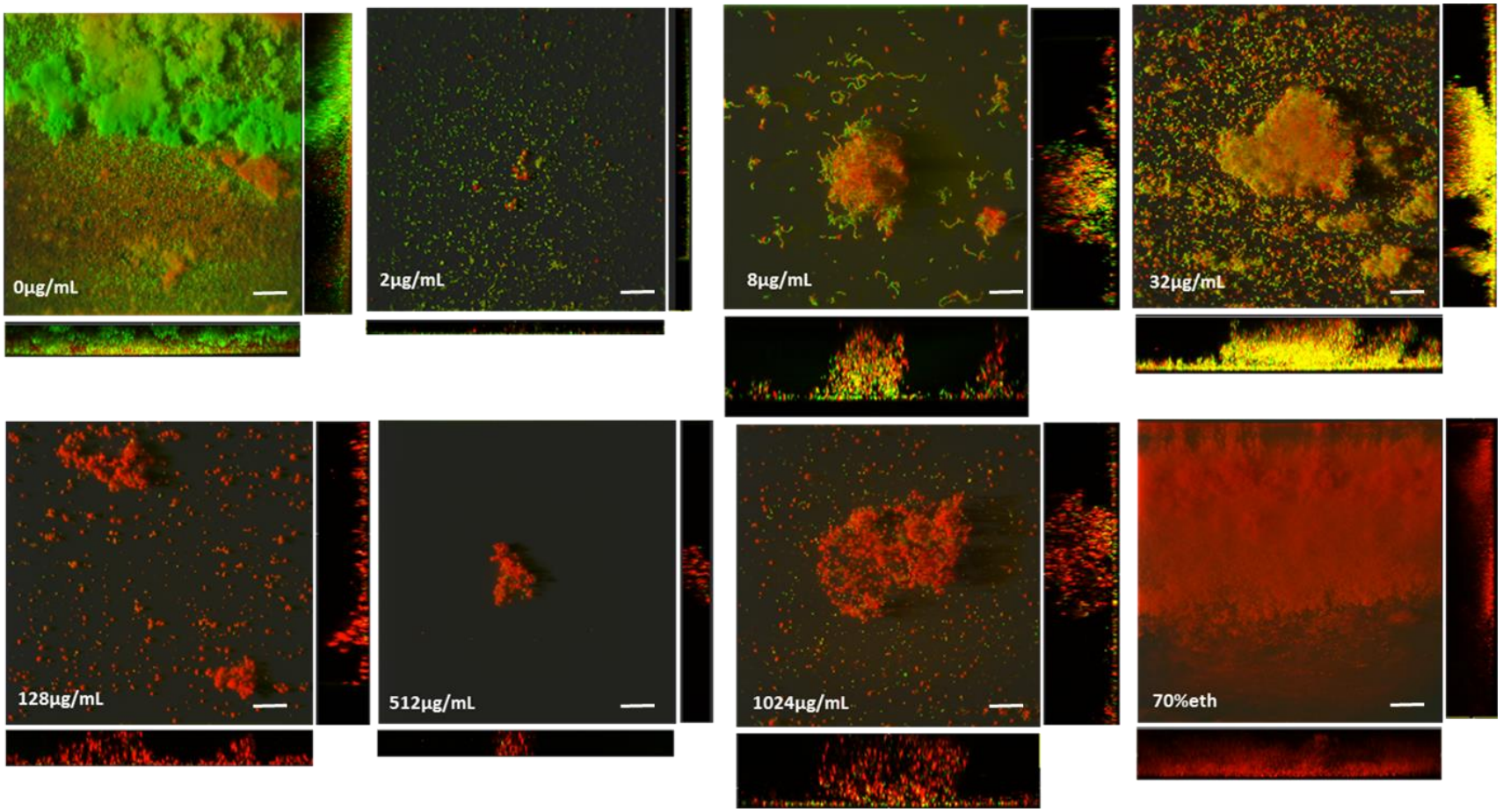


Figure 3. 7 Confocal laser scanning microscopy of *S. aureus* SB14 biofilms treated with a range of Co-amoxiclav concentrations

A Bioflux microfluidic plate was inoculated with *S. aureus* SB14 and fed with 25% TSB for 20 h at 37°C. Biofilms were treated with Co-amoxiclav (2-2048 µg ml^{-1}) for 24 h at 37°C, stained with LIVE/DEAD® BacLight™ stain and examined using CLSM and 3D imaging software (Imaris, Bitplane). Merged images are shown; green signal indicates viable live cells (Syto 9), red signal indicates damaged/dead cells (propidium iodide). Negative (no Co-amoxiclav) and positive (Ethanol 70% - '70%eth') controls were also shown. The majority of *S.aureus* SB14 cells were compromised (red) at Co-amoxiclav concentrations of 128 µg ml^{-1} and above. Scale bars are 30 µm.

3.5.3 Antibiotic susceptibility of S. aureus *biofilms using colorimetric tetrazolium salt (XTT) assay*

Due to the limitations of the previous assays in providing consistent and reproducible data about the antibiotic susceptibility of *S. aureus* biofilms, a colorimetric tetrazolium salt (XTT) assay was used. The XTT assay is a quick method which measures the vitality and the metabolic activity of eukaryotic and prokaryotic cells based on lactate dehydrogenase enzyme activity of the viable and metabolically active cells, which causes a reduction of a yellow color XTT salt to a soluble orange coloured formazan derivative. The degree and the rate of color changes are proportional to the number of live and metabolically active cells present in biofilms.

Biofilms of *S. aureus* SB14 and NCTC6571 were formed in 96-well MTPs for 24 h (see Material and Methods section 2.9.3), and challenged with a range of Co-amoxiclav concentrations (32-4096 μg ml^{-1}) for 24 h. The viability of biofilms was quantified by the addition of XTT solution and measuring A_{450} after incubation for 2 h at 37°C aerobically. Despite several attempts, it was not possible to obtain end-point readings for the biofilm eradication concentrations for *S. aureus* SB14 and NCTC6571 (see Figure 3. 8), even though extremely high concentrations of Co-amoxiclav (up to 4,000 fold greater than the MICs for planktonic cells) were used.

Therefore, as an alternative to a simple endpoint measurement, the rate of XTT reduction over one hour of incubation was measured. Over this time, there was a linear increase in XTT reduction (data not shown). Biofilms of *S. aureus* SB14 and NCTC6571 were grown in 96-well MTPs for 24 h (see Method and Materials section 2.9.2) and processed as previously described. The range of Co-amoxiclav concentrations used in this assay was 250-16,384 μg ml^{-1} for *S. aureus* SB14 and 128-8,192 μg ml^{-1} for *S. aureus* NCTC6571. Biofilm cell viability was quantified by obtaining A_{450} every 10 min for 1 h at 37ºC following the addition of XTT solution, and calculating the increase in Relative Light Units per min ($RLU.min^{-1}$) (see Figure 3. 9).

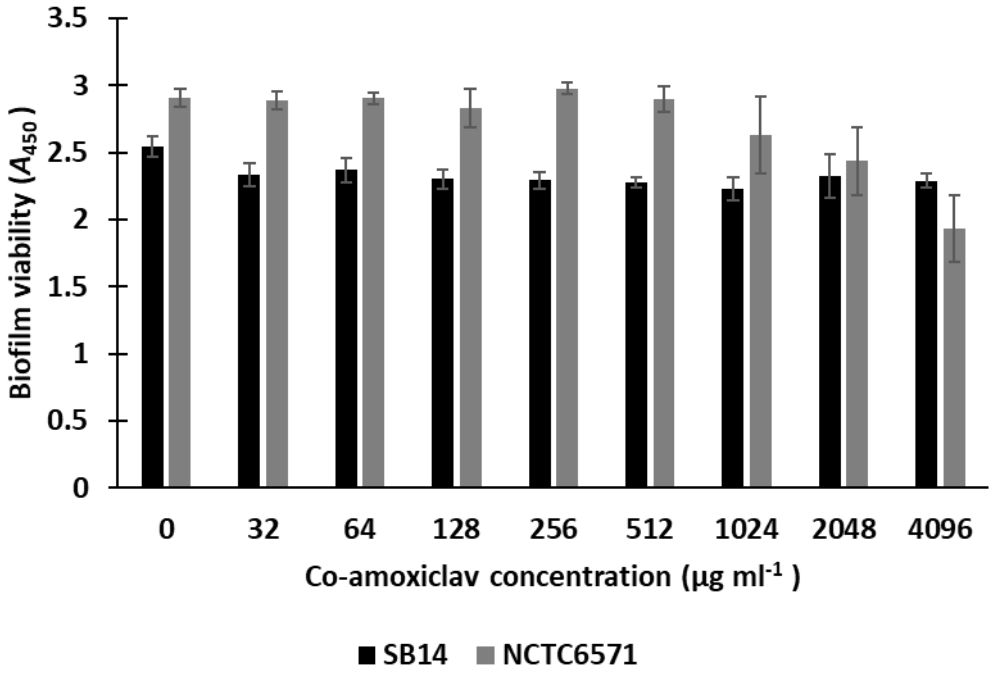


Figure 3. 8 Testing antibiotic susceptibility of *S. aureus* biofilms by XTT assay.

Biofilms of *S. aureus* (SB14 and NCTC6571) were grown in 96-well MTPs for 24 h, challenged with a range of Co-amoxiclav concentrations for 24 h at 37ºC, and bacterial cell viability within biofilm was determined by XTT staining assay through measuring endpoint A_{450}. Even at high Co-amoxiclav concentrations, colour was observed in *S. aureus* SB14 and NCTC6571 cultures, indicating that XTT had been reduced.

Using the kinetic assay, there was statistically significant concentration-dependent reduction in the rate of XTT conversion over the whole concentration range assayed for both *S. aureus* SB14 & NCTC6571 biofilms in comparison with controls without Co-amoxiclav (One Way ANOVA and Dunnett Multiple Comparison with control) (see Figure 3. 9). For *S. aureus* SB14, there was more than 50% reduction in the rate of XTT conversion at the concentration range 4,096-16,384 μg ml^{-1} when compared with no antibiotic controls (see Figure 3. 9 (B)). Co-amoxiclav at 2,048 μg ml^{-1} produced approximately a 50% decrease in XTT reduction rate. There was approximately 30-40% decrease at Co-amoxiclav concentrations between 512-1,024 μg ml^{-1}. For *S. aureus* NCTC6571, again there was statistically significant reduction (>50%) in the rate of XTT conversion at concentrations 4,096-8,192 μg ml^{-1} in comparison with controls lacking Co-amoxiclav (see Figure 3. 9 (A)). There was an approximately 45% decrease in XTT reduction rate at 2,048 μg ml^{-1} of Co-amoxiclav, whereas, XTT reduction was decreased by approximately 27-35% at concentrations ranging from 512-1,024 μg ml^{-1}. Co-amoxiclav at concentrations less than 512 μg ml^{-1} showed minimal effects on XTT reduction rate within biofilms of both *S. aureus* SB14 & NCTC6571 (decreased by ≤ 25%). Co-amoxiclav concentrations of 512 and 1,024 μg ml^{-1} demonstrated statistically significant moderate impacts on cell viability (represented by XTT conversion rate) within the biofilms of both *S. aureus* SB14 & NCTC6571 which were between the highly effective concentrations (4,096-16,384 μg ml^{-1}) and minimally effective concentrations (128-256 μg ml^{-1}). On the basis of these observations, it was decided to use concentrations of 512 and 1,024 μg ml^{-1} for the next series of experiments, investigating the potential for a DNase enzyme (NucB) to enhance antibiotic killing.

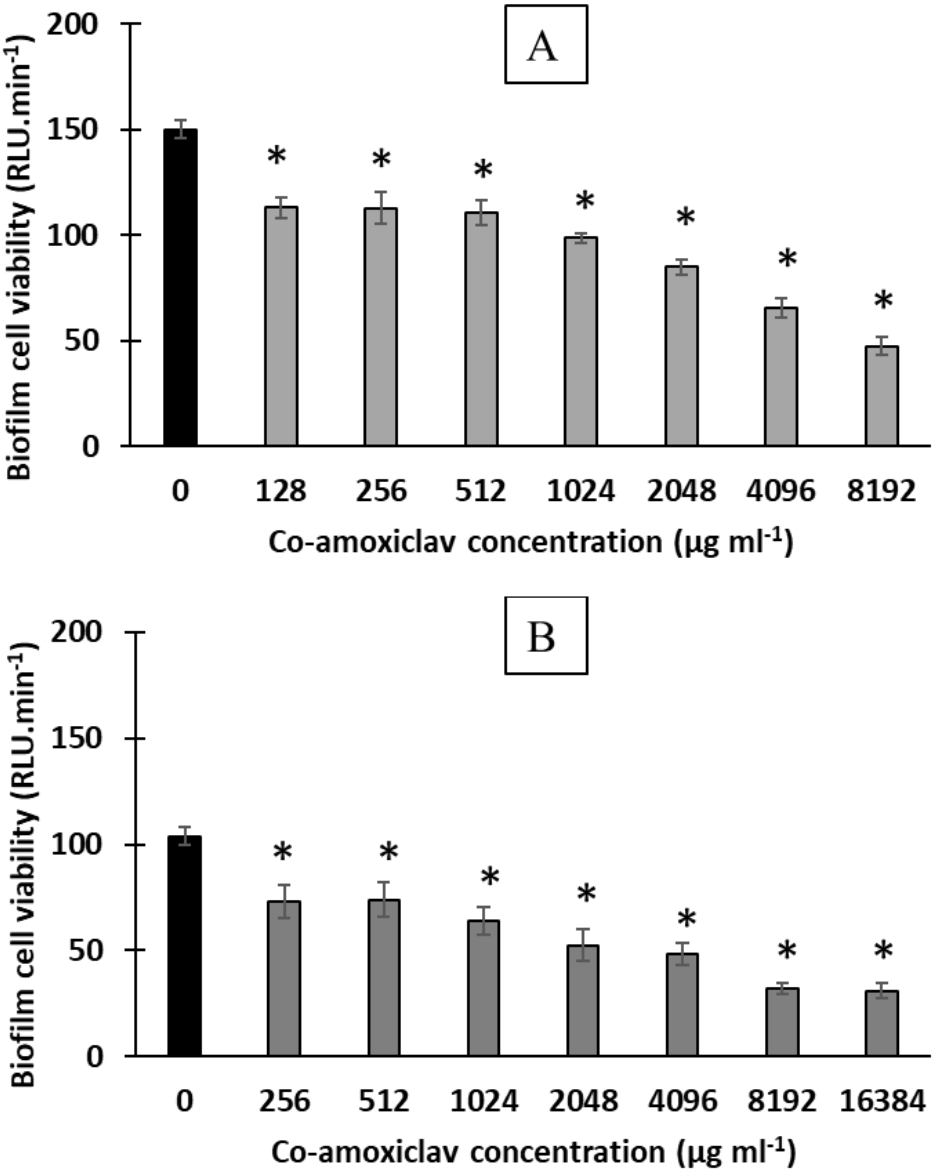


Figure 3. 9 Antibiotic susceptibility of *S. aureus* biofilms determined by XTT assay

Biofilms of *S. aureus* (NCTC6571 (A) and SB14 (B)) were grown on 96-well MTP plates for 24 h as described in Materials and Methods, challenged with a range of Co-amoxiclav concentrations for 24 h at 37ºC, and bacterial cell viability within biofilms was determined by measuring the rate of XTT reduction at 450 nm for 1 h ($RLU.min^{-1}$). Asterisks indicate a significant difference from the control (ANOVA, post-hoc Dunnett's test). There was a significant dose-dependent decrease in cell viability ($RLU.min^{-1}$) within biofilms of both *S. aureus* SB14 and NCTC6571 by Co-amoxiclav.

3.6 Investigating effects of NucB on antibiotic susceptibility of *in vitro* biofilms of *S. aureus*

3.6.1 Effects of NucB on the antimicrobial sensitivity of planktonic bacterial cultures of S. aureus

To investigate the effects of NucB on MICs of planktonic cultures of the same *S. aureus* clinical isolates (SB14 & SB17), an adjusted planktonic bacterial suspension of *S. aureus* SB14 & SB17 in 96-well plates was challenged with a range of Co-amoxiclav concentrations with and without NucB (0.5 µg ml^{-1}) for 24 h (see Materials and Methods section 2.10.1), and quantified by colorimetric XTT viability assay.

The MICs of Co-amoxiclav determined for *S. aureus* SB14 & SB17 in the absence of NucB were similar to the MICs determined in the presence of NucB (see Figure 3. 10 A&B). Interestingly, MICs of Co-amoxiclav measured by the standard microdilution method and XTT viability assay were also similar for *S. aureus* SB14 & SB17 (1 µg ml^{-1}). Similarly, there was no significant difference in the cellular viability of both *S. aureus* strains over the assayed Co-amoxiclav concentration range with or without NucB (0.5 µg ml^{-1}). Finally, the cell viability in planktonic cultures of both *S. aureus* clinical isolates treated with NucB alone were almost equivalent to those of negative controls (no NucB and no antibiotics), which indicates that NucB has no bacteriostatic or bactericidal effect on *S. aureus* cells (see Figure 3. 10 A&B). This finding confirms the previous result obtained from visual analysis of LIVE/DEAD stained CLSM images, which showed no killing effects of NucB on both *S. aureus* SB14 & SB17 biofilm cells. In conclusion, NucB at 0.5 µg ml^{-1} did not affect the antimicrobial sensitivity and viability of both *S. aureus* SB14 and SB17 planktonic cultures.

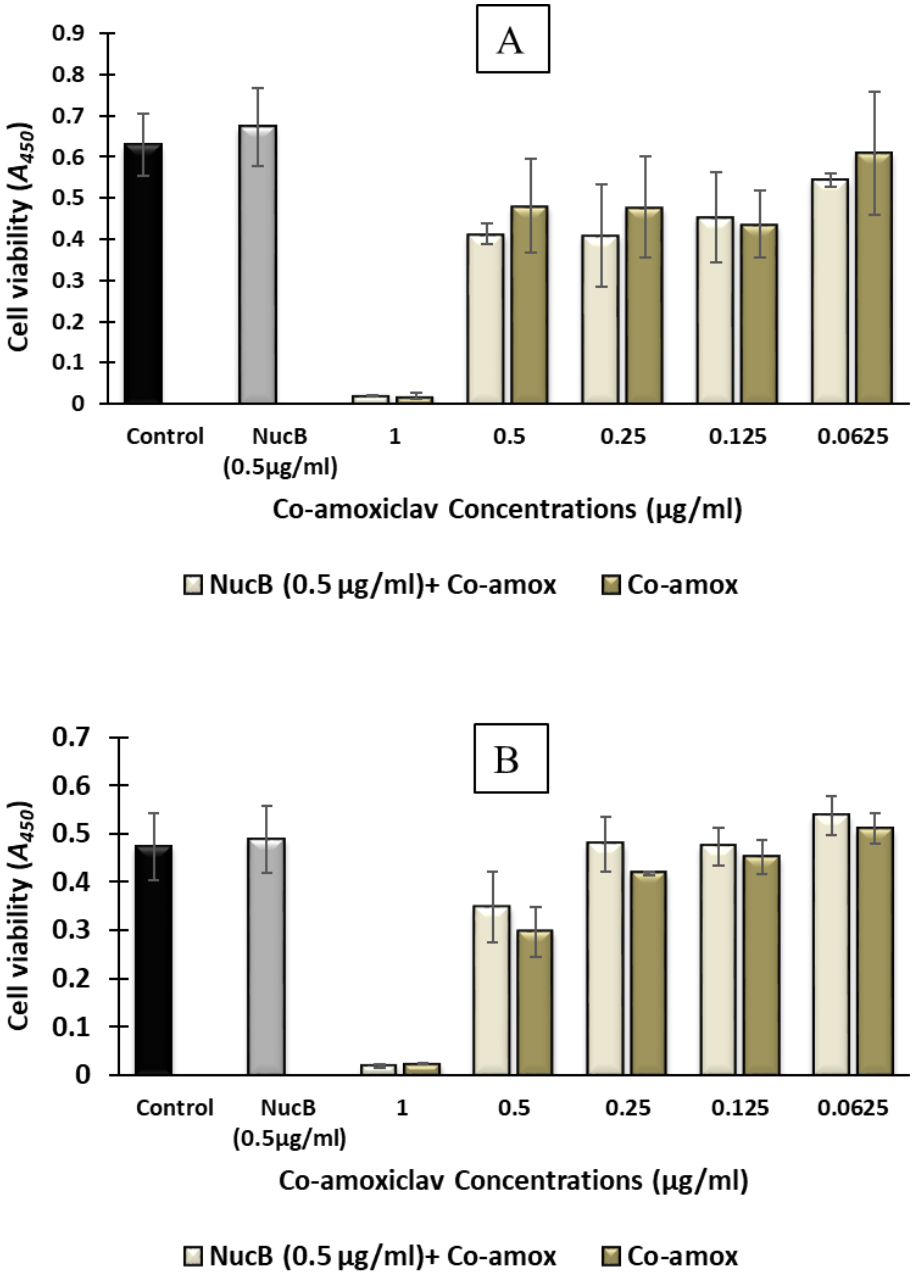


Figure 3. 10 Effect of NucB on antibiotic susceptibility of planktonic *S. aureus* cells.

Standardised bacterial cultures of *S. aureus* SB17 (A) and SB14 (B) were challenged with a range of Co-amoxiclav concentrations with or without NucB (0.5 µg ml^{-1}) in 96-well MTPs for 24 h at 37ºC. NucB alone and untreated controls were included. Bacterial cell viability was determined by measuring endpoint A_{450} using XTT assay. NucB had no effect on cell viability and antibiotic susceptibility of both *S. aureus* SB17 and SB14 planktonic cells.

3.6.2 Effect of NucB on antimicrobial susceptibility of in vitro S. aureus biofilms

After assessing the effects of NucB on antimicrobial sensitivity of planktonic cultures of *S. aureus*, the next step was to investigate the ability of NucB to enhance the susceptibility of *S. aureus* biofilms to antibiotic treatment. Initially, the sensitivity of 24 h old pre-formed *S. aureus* SB14 biofilms to a range of NucB concentrations (0.156-1 µg ml^{-1}) with or without Co-amoxiclav (512 µg ml^{-1}) was studied using XTT viability (see Material and Methods section 2.10.2) to identify an appropriate concentration range of NucB for more detailed investigations. There was concentration-dependent decrease in the rate of XTT reduction observed in biofilms treated with NucB alone in comparison with growth controls (Figure 3. 11), which was likely due to the dispersal action of NucB on these biofilms. At the highest concentrations tested (0.5-1 µg ml^{-1}) the reduction was generally more than 50% compared with controls lacking NucB. The pattern and the extent of XTT reduction within pre-formed biofilms of *S. aureus* SB14 mediated by NucB were consistent with the pattern of reduction in biofilm biomass obtained using the crystal violet staining assay (see Figure 3. 1(B)). Interestingly, combinations of NucB at different concentrations (0.156-1 µg ml^{-1}) with 512 µg ml^{-1} Co-amoxiclav appeared to show additional dose-dependent reduction in cell viability of *S. aureus* SB14 biofilms when compared with biofilms treated with Co-amoxiclav only (512 µg ml^{-1}). However, this experiment was only performed once, and it was not possible to determine statistical significance. Therefore, to investigate the effects of NucB on antibiotic susceptibility in more detail, experiments were repeated using Co-amoxiclav at 512 µg ml^{-1} or 1,024 µg ml^{-1} and NucB at 1 µg ml^{-1}. These experiments were performed on *S. aureus* SB14 & SB17 clinical isolates as well as the reference strain *S. aureus* NCTC6571.

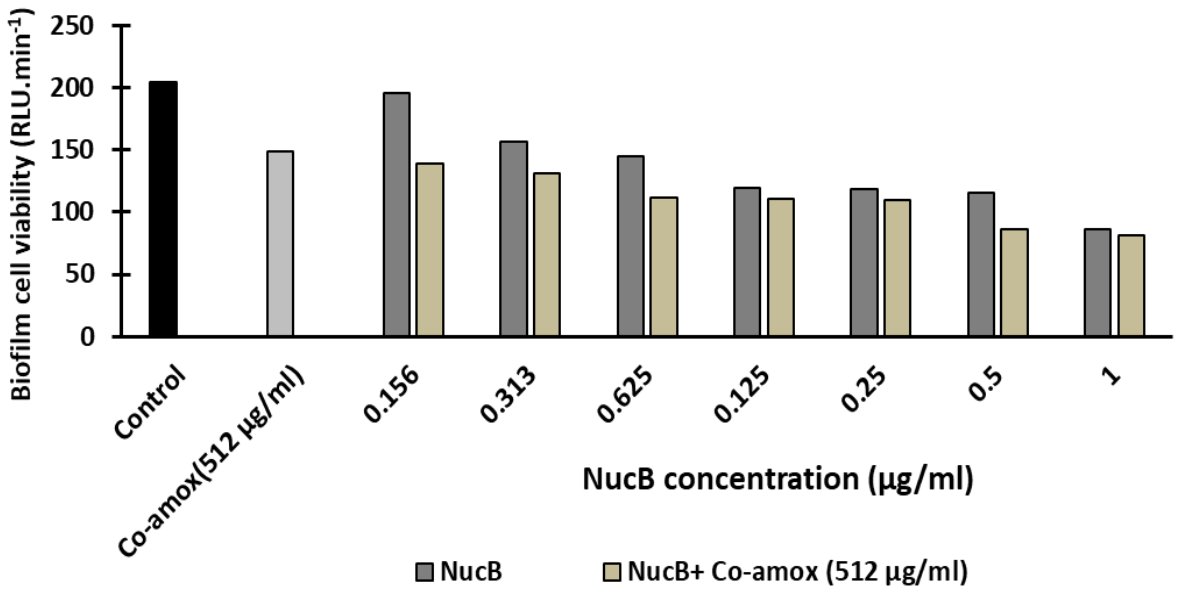


Figure 3. 11 Effects of different NucB concentrations on antibiotic susceptibility of *S. aureus* biofilms.

Biofilms of *S. aureus* SB14 were grown on 96-well MTPs for 24 h as described in Materials and Methods, and challenged with a range of NucB concentrations with or without Co-amoxiclav (512 µg ml^{-1}) for 24 h at 37ºC. Co-amoxiclav only and untreated controls were included. Bacterial metabolic activity within biofilms was determined by measuring the rate of XTT reduction at 450 nm for 1 h ($RLU.min^{-1}$). NucB with or without Co-amoxiclav showed dose-dependent decreases in cell viability within biofilms compared with biofilms treated with Co-amoxiclav alone or with no treatment.

NucB alone at 1 µg ml^{-1} or in combination with Co-amoxiclav at either concentration significantly reduced XTT reduction rate within pre-established biofilms of all tested *S. aureus* strains compared with controls that were not treated with NucB (see Figure 3. 12). For *S. aureus* SB14, there was a significant reduction in metabolic activity of cells within biofilms amongst all treatment groups compared with no treatment controls (one way ANOVA, p <0.001). The metabolic activity in biofilms treated with NucB alone was approximately 58% reduced compared with the negative control and this difference was significant (Tukey's post hoc test, p <0.001, see Figure 3.12A). Under similar conditions, the combination of NucB with Co-amoxiclav at 512 or 1,024 µg ml^{-1} resulted in further dose-dependent reductions in activity compared with biofilms treated by Co-amoxiclav only (p <0.001 in both cases). Similarly, addition of NucB alone at 1 µg ml^{-1} to pre-formed *S. aureus* SB17 biofilms significantly reduced (p <0.001) viable cells remained in biofilms by approximately 55% compared with no treatment controls (see Figure 3. 12 (B)). Again, NucB together with Co-amoxiclav at 512 or 1,024 µg ml^{-1} produced an additional significant dose-dependent decrease in cell viability within biofilms by approximately >35% compared to biofilms treated with Co-amoxiclav alone. Finally, pre-formed biofilms of *S. aureus* NCTC6571 were more sensitive than those of the clinical isolates to NucB treatment alone. Treatment with NucB substantially decreased the metabolic activity within biofilms by approximately 80% compared with the control biofilms (see Figure 3. 12 (C)). Once again, NucB in combination with Co-amoxiclav at 512 or 1,024 µg ml^{-1} showed an additional significant reduction (p <0.001) in metabolic activity of *S. aureus* NCTC6571 biofilms by >50% compared with Co-amoxiclav only treated biofilms.

In conclusion, NucB at 1 µg ml^{-1} significantly reduced the metabolic activity of cells within biofilms formed by all assayed *S. aureus* strains by approximately 55-80%. This reduction in activity was likely due to the dispersal of attached cells from the biofilm. Importantly, when NucB was combined with Co-amoxiclav, there was an additional reduction in the metabolic activity of biofilms over and above Co-amoxiclav treatment alone. These data demonstrate that NucB has the potential to enhance the efficacy of Co-amoxiclav against *S. aureus* biofilms.

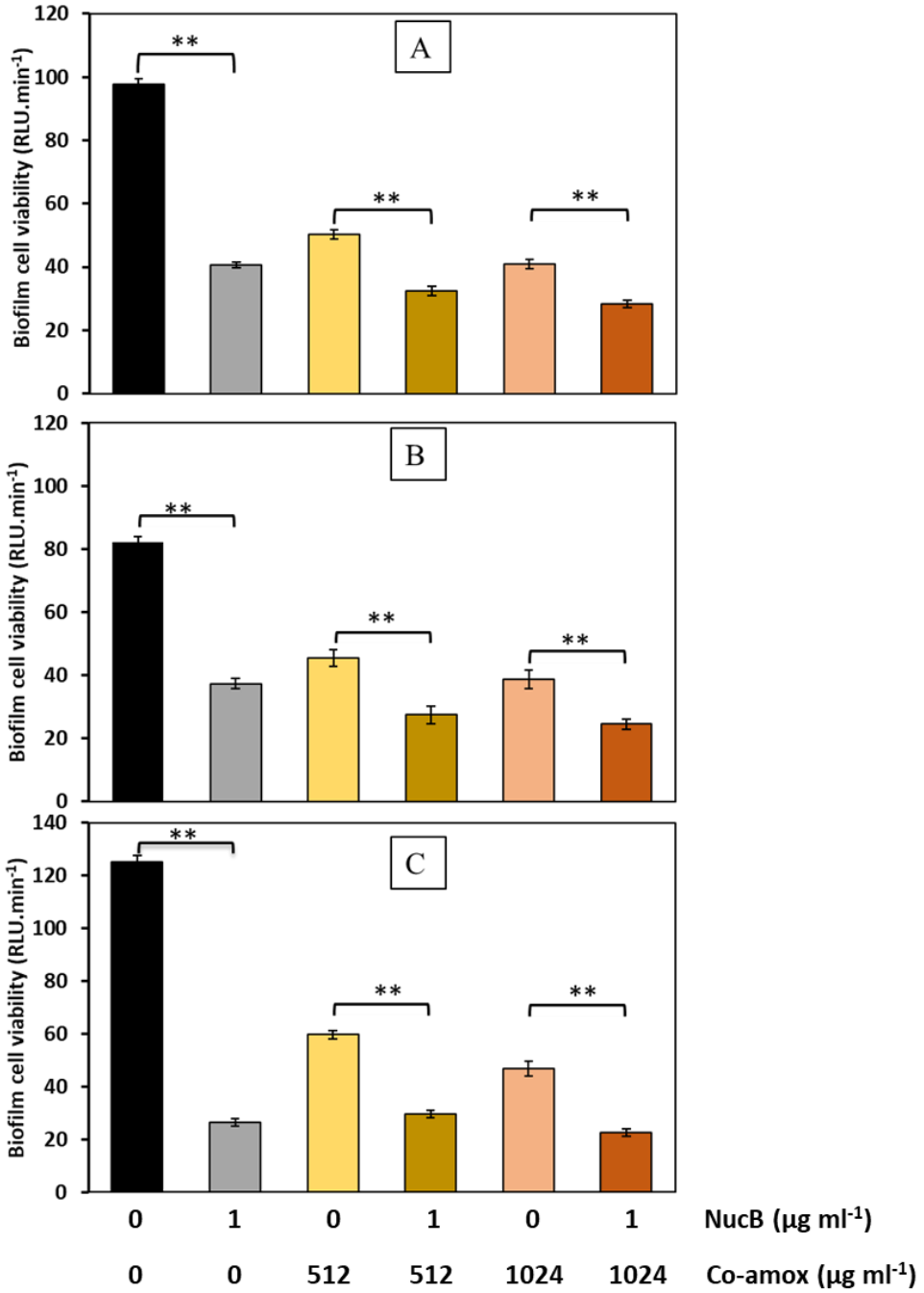


Figure 3. 12 Effects of NucB on the antibiotic susceptibility of *S. aureus* biofilms.

Biofilms of *S. aureus* SB14 (A), SB17 (B), and NCTC6571 (C) were grown on 96-well MTPs for 24 h as described in Materials and Methods. Biofilms were challenged for 24 h at 37 ºC with Co-amoxiclav (0, 512 µg ml^{-1} or 1,024 µg ml^{-1}) in the presence or absence of 1µg ml^{-1} NucB. Bacterial metabolic activity within biofilms was determined by measuring the rate of XTT reduction at 450 nm for 1 h ($RLU.min^{-1}$). NucB treatment resulted in significant reductions in metabolic activity at all Co-amoxiclav concentrations compared with equivalent samples without NucB treatment (**p <0.001; ANOVA with Tukey's HSD post-hoc comparison test).

3.7 Investigating the toxicity of NucB against microbial and human cells

3.7.1 Testing the effects of NucB on bacterial cell viability

Previous data from LIVE/DEAD microscopic analysis and colorimetric XTT assays indicated that incubation of NucB at up to 1 µg ml^{-1} with *S. aureus* biofilms and planktonic cells for 1-24 h at 37ºC did not have a negative impact on the cell integrity or metabolic activity of these cells. To assess the impact of NucB on cell viability, *S. aureus* SB14 biofilms were grown in a 6-well tissue culture plate for 24 h and treated with 1 ml NucB (0.5 µg ml^{-1}) for 1 h at 37ºC (see Materials and Methods 2.11.3). The concentration of NucB at 0.5 µg ml^{-1} is similar to that which was used in the LIVE/DEAD microscopic analysis. Non-bound or loosely bound planktonic cells were recovered directly from the supernatant, whereas biofilm cells were recovered using a cell scraper. Viability was assessed by viable cell counting. Treatment with NucB did not lead to an overall reduction in the total CFU/ml present in planktonic or biofilm cells compared to the control group (see Table 3. 5). In fact, the total number of cells was slightly, though not significantly, increased with NucB, as noted below. Treating *S. aureus* biofilms with NucB resulted in approximately two-fold more viable cells in the planktonic phase (increased from 2.0 x 10^8 to 4.2 x 10^8 CFU/ml) in comparison to buffer-treated controls. By contrast, the number of viable cells in the biofilm phase was reduced by approximately 15%. Biofilms in other wells on the same plates, which were also treated with NucB under same conditions and quantified simultaneously by CV staining, showed approximately two-fold decrease in biofilm biomass compared to control biofilms, confirming that NucB was effectively dispersing biofilms (Figure 3. 13).

It is not clear why the total CFU appeared to be approximately two-fold higher following NucB treatment than in controls treated with PBS. It is possible that bacterial cells aggregates were present in control samples, and that these led to an artificially low viable count (see Table 3. 5). To address this hypothesis, the harvested cells from both NucB- and PBS-treated biofilms (planktonic and biofilm phases) were sonicated with a high power sonicator to break down cell aggregates. Samples were compared with the matched non-sonicated cell suspensions. However, in both treatment conditions, sonication for 1 or 3 min significantly reduced the total viable counts present in planktonic and biofilm phases from 9.25 x 10^8 and 5.13 x 10^7 to 2.98 x 10^8 and 2.54 x 10^7, respectively. Due to this significant negative impact on cell viability, sonication of the harvested cells suspension was avoided. Nevertheless, the results overall indicate that NucB acts specifically through disruption of pre-established biofilms without influencing cell viability.

Table 3. 5 Total viable counts (CFU ml-1) of in vitro S. aureus biofilms following treatment with NucB.

Bacterial phase	Control CFU ml^{-1} ± SE	NucB (0.5 µg ml^{-1}) CFU ml^{-1} ± SE
Planktonic (P)	$2.0x10^8$ ($1.9x10^8$)	$4.2\ x10^8$ ($3.8x10^8$)
Biofilm (B)	$2.8\ x10^7$ ($7.5x10^6$)	$2.4\ x10^7$ ($1.3x10^7$)
Total (P+B)	$2.3\ x10^8$ ($2.0x10^8$)	$4.4\ x10^8$ ($3.8x10^8$)

NucB did not reduce viable cell counts within *S. aureus* biofilms

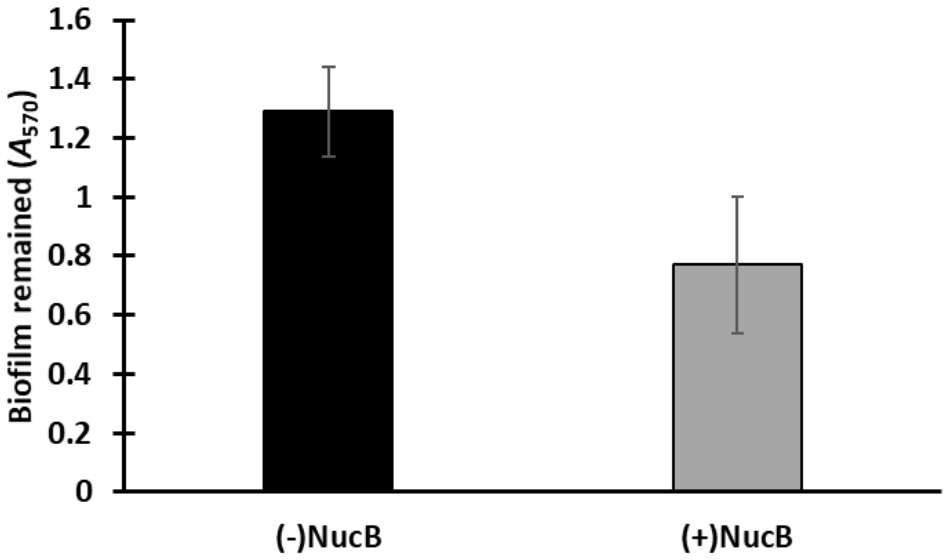


Figure 3. 13 Crystal violet quantitative analysis of the effect of NucB on pre-formed S. *aureus* SB14 biofilms

S. aureus SB14 biofilms were grown on the same 6-well tissue culture plates as those in table 3.5 for 24 h and treated with 0.5 µg ml^{-1} NucB for 1 hr at 37ºC. Biofilms were stained with crystal violet, and the biofilm biomass was quantified by measuring A_{570}. PBS treated control biofilms were included. Mean values from three independent assays are shown and error bars represent standard error of the mean. NucB treated biofilms had approximately two-fold less biomass compared to control biofilms.

3.8 Investigation of NucB toxicity for human respiratory epithelial cells

Following the *in vitro* evaluation of the anti-biofilm activity of NucB against clinically relevant *S. aureus* biofilms, it was important to assess the safety of NucB on human cells relevant to the epithelial lining of the middle ear as a mandatory preliminary stage in the process of developing new drug for medicinal use. Human bronchial epithelial cells (BEAS-2B) were treated with different concentrations of NucB (1-100 µg ml^{-1}) and cell viability and morphology were quantified using fluorometric CellTiter-Blue® cell viability assay and Dual acridine orange(AO)/ethidium bromide (ED) staining apoptosis assay respectively.

3.8.1 Assessing NucB safety on human cells using CellTiter-Blue® cell viability assay

To assess the effects of NucB on human cell viability, BEAS-2B cells were seeded in 12-well tissue culture plate (see Materials and Methods section 2.11.2) and allowed to grow in a 5% CO_2 atmosphere at 37ºC. Cells were challenged with different concentrations of NucB (1-100 µg ml^{-1}) and Dornase alfa (rhDNase I) at 100 µg ml^{-1} for 24-48 h. Positive (absolute methanol treated) and negative (media treated) controls for cell death were included. Subsequently, cell viability was quantified using the CellTiter-Blue® cell viability assay. The CellTiter-Blue® cell viability assay is a quick high-throughput fluorometric method that utilises resazurin dye as an indicator to monitor the metabolic activity of viable cells. Live and metabolically active cells can reduce dark blue resazurin into fluorescent pink resorufin. However, damaged or non-viable cells lose their capability to convert the indicator dye and therefore fluorescent signals will not be generated by these cells.

At the end of both incubation time points (24-48 h), the epithelial cells exhibited normal cell viability with the addition of NucB at concentration up to 100 µg ml^{-1} and Dornase alfa at 100 µg ml^{-1} compared with cells incubated with growth medium only (see Figure 3. 14). There was essentially no metabolic activity (<5% reduction of dye) in positive controls that were treated with absolute methanol for 2 min. Cells treated with Dornase alfa at 100 µg ml^{-1} for 24-48 h, displayed similar proportions of viability and metabolic activity as those treated with medium alone (>99%). Treatment of cells with up to 50 µg ml^{-1} NucB had no significant effect on their viability (approximately 95% activity remained). Only a slight reduction in the activity of cells (to 86.3% of levels in untreated controls) was observed after 24 h treatment with NucB at 100 µg ml^{-1}. However, the activity of cells was slightly higher at 93% after 48 h treatment with 100 µg ml^{-1} NucB. Again, no significant differences in metabolic activity were observed following 48 h incubation of bronchial cells with NucB at concentrations up to 100 µg ml^{-1} compared with negative controls. After 24-48 h, the viability of cells treated with NucB at an anti-biofilm concentration (1 µg ml^{-1}) was generally remained

unaffected (93-98%) in comparison to control cells. Overall, NucB at concentrations up to 100-fold greater than those required for anti-biofilm activity was not toxic to human bronchial epithelial cells.

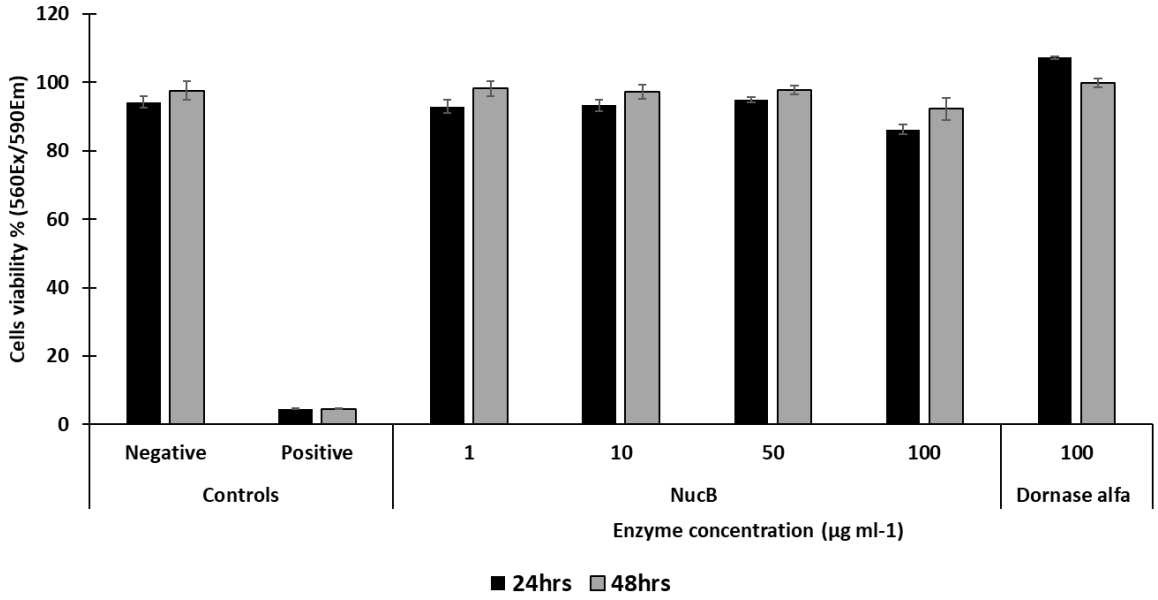


Figure 3. 14 Effects of NucB on viability of human bronchial epithelial cells.

Using a 12-well plate, BEAS-2B cells were grown at a density of 6-7 × 10^5 cells ml^{-1} and incubated for 24–48 h in the presence of NucB (1–100 µg ml^{-1}), and Dornase alfa (100 µg ml^{-1}). Positive (100% methanol) and negative (no treatment) controls were included. At 24 and 48 h time points, cell viability were assessed using CellTiter-Blue® Cell Viability assay by measuring florescence (560Ex/590Em). Mean values from three independent assays are shown and error bars represent standard error of the mean. Addition of NucB and Dornase alfa at concentrations up to 100 µg ml^{-1} for 24-48 h had no significant effects on the viability of human bronchial cells (One-Way ANOVA test with Tukey's HSD *post-Hoc* comparison).

3.8.2 Effects of NucB on the apoptosis of human cells using Dual acridine orange (AO)/ethidium bromide (EB) apoptosis assay

To obtain more detailed information about the effects of NucB on the viability and nuclear morphology of human cells, AO/EB DNA binding fluorescent stains coupled with microscopic imaging were employed as described in Materials and Methods section 2.12.3. Again, human BEAS-2B cells were treated with a range of NucB concentrations (1-100 µg ml^{-1}) and Dornase alfa (100 µg ml^{-1}) for 24-48 h. Cells were then stained with AO/EB and imaged using fluorescent microscopy. A combination of AO/EB and microscopic imaging can quickly and accurately assess nuclear apoptotic alterations and cytoplasmic membrane integrity simultaneously. At both time points (24-48 h), in the absence of NucB, almost all cells were alive with intact cell membranes and appeared green by AO. In these cultures, a negligible number of cells were orange-stained apoptotic or red-stained necrotic cells stained by EB. It was possible to recognise bright green stained spindle, oval or kidney shape shaped cellular nuclei with smooth outline in the majority of live cells (see Figure 3. 15). Under cell death-inducing conditions (positive controls), the majority of the epithelial cells were either apoptotic or necrotic. Apoptotic cells showed abnormal shape nuclei contained condensed and fragmented chromatin (green or orange colour), whereas necrotic cells had red or orange-stained nuclei which were similar in shape to those of live cells with the absence of condensed chromatin.

At both time points, qualitative observation of images showed that cells exhibited tolerance to high concentrations of NucB and Dornase alfa. After 24 h treatment with Dornase alfa at 100 µg ml^{-1} and NucB of up to 100 µg ml^{-1}, the majority of cells were viable with a normal nuclear morphology. Minimal apoptotic changes were observed in a small proportion of cells at 100 µg ml^{-1} NucB (see Figure 3. 15 white circles). However, under similar conditions these nuclear apoptotic changes were absent in cells treated with the same concentration of NucB for 48 h. Similarly, the vast majority of cells were alive with normal morphological features after 48 h of treatment with NucB at concentrations 1-100 µg ml^{-1} and Dornase alfa at 100 µg ml^{-1}. Once more, cells at anti-biofilm concentrations of NucB (1 µg ml^{-1}) exhibited normal viability and nuclear morphology in comparison to negative controls. In conclusion, the results obtained from the morphological examination of human bronchial epithelial cells treated with NucB of up to100 µg ml^{-1} were consistent with the results derived using CellTiter-Blue® cell viability analysis which indicate that NucB has no toxic effect on human bronchial epithelial cells.

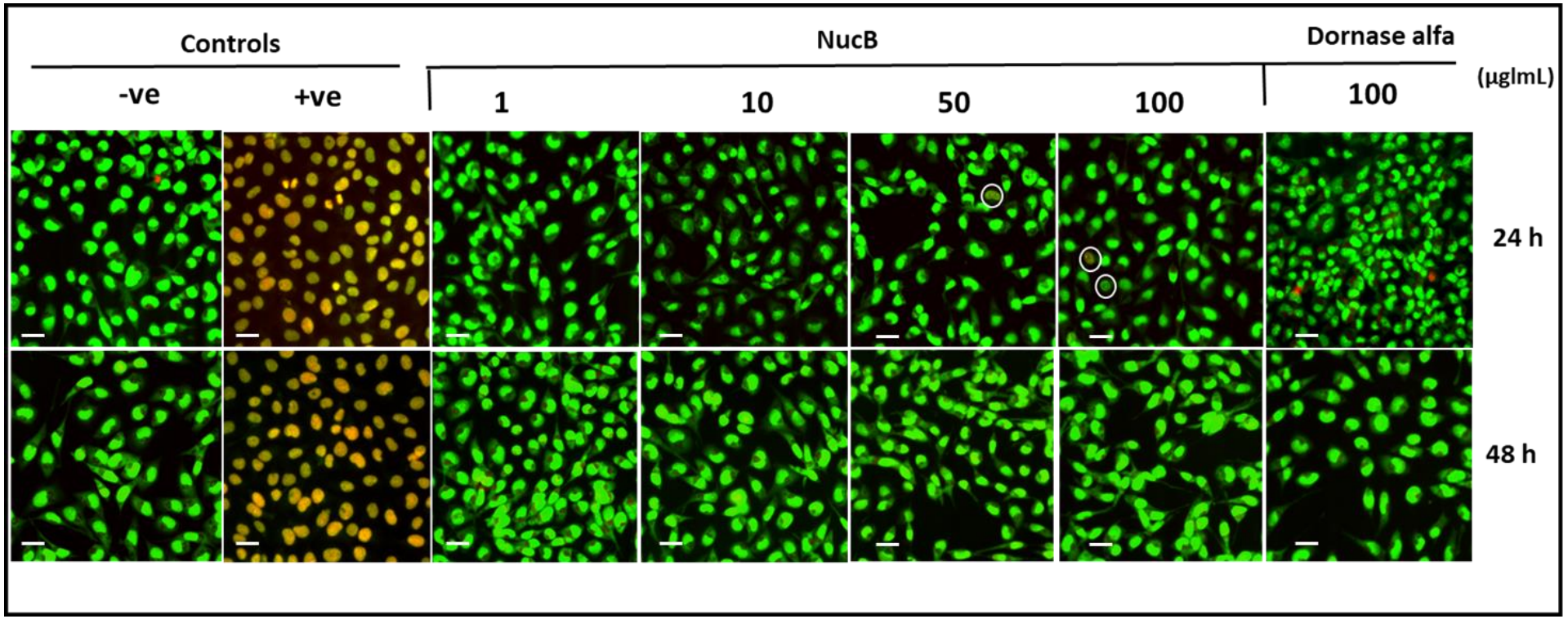


Figure 3. 15 Effects of NucB on human bronchial epithelial cells morphology and apoptosis observed by fluorescence microscope.

BEAS-2B cells were grown as described in Material and Methods and challenged with NucB (1–100 µg ml^{-1}), and Dornase alfa (100 µg ml^{-1}) for 24–48 h in 5% CO_2 at 37ºC. Positive (100% methanol treated) and negative (no treatment) controls for cell death were included. Cells were stained with dual AO/EB stains and visualised by fluorescence microscopy. Merged images show that the nuclei of cells with normal membrane integrity stained green by AO. Early apoptotic cells stained green but contained bright green dots in the nuclei due to chromatin condensation and nuclear fragmentation. Late apoptotic cells stained with EB and appeared orange, and characteristic apoptotic phenotypes were observed in some samples (white circles). Necrotic cells stained orange or red but the nuclear morphology resembled the viable cells with absence of chromatin condensation. Representative images from three independent experiments are shown. Scale bars are 20 µm. After 24-48 h incubation, NucB and Dornase alfa at concentrations up to 100 µg ml^{-1} had no obvious effect on cell viability and nuclear morphology of human bronchial cells.

3.9 Discussion

Up to now, over 80% of all bacterial infections affecting humans are estimated to be associated with biofilm formation (Romling and Balsalobre, 2012, Davies, 2003). *Staphylococcus aureus* is among the most common pathogens isolated from human head and neck biofilm-associated infections including chronic rhinosinusitis and chronic otitis media with effusion (Daniel et al., 2012b, Shields et al., 2013). Biofilm formation by *S. aureus* is thought to be critical for infection since it provides bacterial cells with a plethora of advantages including, but not limited to, protection against host immunity and up to 1000-fold increase in resistance to antibiotics and antimicrobials (Elgharably et al., 2013, Rogers et al., 2010). Increasing evidence indicates that extracellular DNA (eDNA) is a key structural component within the matrix of many microbial biofilms including *Staphylococcus aureus* (Tetz et al., 2009, Fleming and Rumbaugh, 2017). In this section of the study, the main aim was to assess the efficacy of NucB, a DNase from *Bacillus licheniformis*, as a potential anti-biofilm treatment against clinically relevant *in vitro* biofilms of *Staphylococcus aureus*.

To date, many researchers have employed a variety of DNases such as bovine DNase I, recombinant human DNase I (rhDNase I), also known as Dornase alfa, restriction endonucleases, and micrococcal nuclease to control *in vitro* and *in vivo* biofilms formed by different Gram positive and Gram-negative bacterial species by targeting eDNA with the extracellular matrix of these bacterial biofilms (Fleming and Rumbaugh, 2017, Kaplan et al., 2012, Tetz et al., 2009). In this study, among the assayed microorganisms relevant clinically to COME, *S. aureus* exhibit greatest capability to form robust and reproducible *in vitro* biofilms. Daniel et al. (2012a) also chose *S. aureus* as a suitable model microorganism to investigate eradication of biofilms with biodegradable modified-release antibiotic pellets due to its superior biofilm formation capability compared to other bacterial species implicated in COME. It follows that the species that produces the strongest biofilms will present the greatest challenge for approaches to eradicate biofilm-associated infections. Therefore *S. aureus* was felt to be a suitable species for developing methods to investigate biofilms and to assess the efficacy of biofilm control agents. It should be noted that *S. aureus* was among the species that were subsequently identified in COME patients and strains isolated from COME were analysed in Chapters 4 and 5.

The next objective was to determine the best therapeutic concentration, which would efficiently inhibit biofilm formation and disperses pre-established biofilm of *S. aureus* clinical isolates. Results of this study showed that incubation of NucB at 25-500 ng ml^{-1} during

biofilm formation resulted in efficient dose–dependent inhibition (25-50%) of *S. aureus* biofilm formation as quantified by CV staining (Figure 3. 1 (A)). A maximum reduction of around 50% in the amount of attached biofilm biomass was observed in the presence of 500 ng ml^{-1} of NucB. This finding is consistent with that of Kaplan et al. (2012) who found that the inclusion of rhDNase I (Dornase alfa) at 0.125-4 µg ml^{-1} under conditions similar to those employed in the current study caused efficient inhibition of biofilm formation by five different strains of *S. aureus*. In dispersal assays, the concentration of rhDNase I required to induce about 90% reduction in biofilm biomass of these *S. aureus* strains was approximately 1 µg ml^{-1}. Bovine DNase I at 100 mg ml^{-1} also significantly inhibited biofilm formation by *S. aureus* strains SH1000, MRSA252 (Izano et al., 2008). Furthermore, Mann et al. (2009) found that inclusion of 28 units of bovine DNase I at different time-points of biofilm formation up to 24 h resulted in more than 50% decrease in cell attachment of *S. aureus* UAMS-1 in a static model. This study also revealed that addition of 500 mg ml^{-1} of polyanethole sulfonate (PAS), a chemical agent that inhibits cell lysis without affecting bacterial growth, at up to 2 h post-inoculation caused great reduction in biofilm biomass. Conversely, addition of PAS after 4 h of inoculation had negligible effect on cellular adhesion, highlighting the importance eDNA generated by cell lysis in mediating the attachment of bacterial cells during the early stage of biofilm formation by *S. aureus* which is consistent with the present study. There is evidence that eDNA is particularly important during infections. For example, a recent study found the amount of eDNA in 6 h biofilms of *S. aureus* and *S. epidermidis* was higher in strains isolated from infected knee and hip prostheses that in control isolates from the skin of healthy volunteers (Zatorska et al., 2017). Therefore, targeting eDNA is a promising approach to prevent the accumulation of staphylococcal biofilms in a variety of clinical situations.

Data in this study also revealed that the exogenous addition of NucB at 10-500 ng ml^{-1} to 24 h pre-formed *S. aureus* static biofilms resulted in efficient detachment of approximately 30-60% of biofilm biomass in comparison to the buffer treated control biofilms (Figure 3. 1 (B)). The addition of various DNases such as NucB, rhDNase, and bovine DNase I to staphylococcal biofilms including *S. aureus* clinical isolates also exhibited significant biomass dispersal effect, indicating that eDNA is important for promoting the stability of mature biofilms (Izano et al., 2008, Kaplan et al., 2012, Shields et al., 2013, Sugimoto et al., 2018). Pre-established biofilms produced by many bacterial species such as *E. coli, Micrococcus luteus, Bacillus subtilis, Haemophilus influenzae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Streptococcus pyogenes*, and *Acinetobacter baumannii* have also

been found to be sensitive to NucB or DNase I treatment (Nijland et al., 2010, Tetz et al., 2009). Overall, the data presented here are consistent with a model in which eDNA is important both at the early stages of attachment and biofilm formation by *S. aureus* and later, for the stability of mature biofilms.

It was noted in this study that NucB at 500 ng ml^{-1} was the best therapeutic concentration that dramatically inhibited and detached biofilms produced by the assayed *S. aureus* clinical isolates. Consequently, this concentration was employed in subsequent experiments. To validate this DNase activity, a commercial bovine DNase I at 5µg ml^{-1} was used under conditions identical to those applied for NucB biofilms inhibition and dispersal assays. In both assays, DNase I resulted in significant inhibition of biofilm formation and disruption of pre-formed biofilms of S. *aureus* by about 50% (Figure 3. 2). Therefore, bacterial DNase, NucB at 0.5 µg ml^{-1}, has approximately 10-fold greater anti-biofilm (inhibitory and dispersal) activity against *S. aureus* biofilms than bovine DNase I (5 µg ml^{-1}). The bovine DNase I used here was not highly purified, and therefore it is impossible to know how much DNase I was in the preparation. Although it was not possible to compare specific activities of NucB versus DNase I, the findings here supported the hypothesis that the antibiofilm action of NucB against biofilms is due to its DNase activity rather than any other property of the protein. One of the limitations with this study is that we did not included heat inactivated NucB enzyme in our control. However, a previous study found no anti-biofilm activity associated with the addition of heat inactivated NucB (Shields et al., 2013).This finding is consistent with other studies that compared the dispersal activity of different concentrations of two different NucB preparations with the similar range of commercial bovine DNase I concentrations against *B. licheniformis* biofilms (Nijland et al., 2010). Nijland et al. (2010) have found that the concentration of NucB required for efficient dispersal of *B. licheniformis* was approximately 5-fold lower than that of bovine DNase I. However, no previous study has compared the inhibitory activities of NucB and DNase I as reported in this study.

Another objective of the present study was to assess effects of NucB on the structure of biofilms using CLSM imaging and LIVE/DEAD® BacLight™ staining. CLSM images of 48 h old *S. aureus* biofilms exposed to NucB were visibly reduced in comparison to control biofilms (Figure 3. 3 and Figure 3. 4). Shields et al. (2013) also have found that CLSM images of *S. aureus* FH7 and *S. constellatus* FH20 biofilms treated with 3 µg/ml NucB were clearly more dispersed compared to PBS treated control biofilms. To provide quantitative data on biofilm structure, we employed a well-developed computer software (COMSTAT 2) to

analyse 9 stacks of CLSM images for each treatment condition and calculated a range of parameters representing the spatial and three-dimensional structure of biofilms (Heydorn et al., 2000).

Consistent with observational analysis, in both inhibition and dispersal studies, quantification of CLSM images by COMSTAT 2 software generally showed that biofilms treated with NucB had significantly reduced average thickness and biomass attached to the surface than those treated with the buffer only (see Table 3. 2 and Table 3. 3). Interestingly, in accordance with a previous study (Shields et al., 2013), CLSM images of biofilms grown on glass coverslip surface treated with NucB showed a slightly greater reduction in biomass than that observed in 96-well MTP model. In the MTP system, the reduction in biomass was approximately 50% for both assays (Figure 3. 1) compared to approximately 50-75% on glass coverslips (Table 3. 2 and Table 3. 3). This difference may arise from variation in the interactions of eDNA with the substrata, either glass coverslips or the polystyrene of MTPs. Alternatively, differences may have been due to the analysis techniques. It was noted that CLSM imaging shows microbial cells only in contrast to CV staining technique, which stains bacterial cells and matrix together. Consistent with our findings, CLSM imaging of 48 h old *S. aureus* UAMS-1 biofilms established in a flow-cell system and treated with 0.5 U ml^{-1} DNase I for 24 h, showed significant reductions in biomass compared to control biofilms imaged before the addition of DNase I (Mann et al., 2009). Furthermore, *S. aureus* UAMS-1 biofilms cultured for three days using a higher inoculum ($1x10^8$ CFU ml^{-1}) were more sensitive to DNase I treatment. In contrast, 72-h old biofilms initiated with lower inoculum ($5x10^5$ CFU ml^{-1}) were more resistant to treatment with DNase I, indicating that biofilm dispersal by DNases could be influenced by different growth conditions such as biofilm age and the amount of inoculum. In both techniques (CV staining and CLSM imaging) higher numbers of bacterial cells (2-5 $x10^8$ CFU ml^{-1}) were used to grow biofilms in our study which may explain the observed sensitivity of these assayed biofilms to NucB treatment. However, further work is required to assess the sensitivity of *S. aureus* biofilms older than 48 h to NucB treatment.

Having shown that NucB efficiently controls biofilms of clinically isolated *S. aureus* strains, the next stage aimed to assess the effects of NucB on antibiotic susceptibility of biofilms. To compare the level of antimicrobial tolerance of *S. aureus* biofilms with planktonic cells, it was necessary to assess antimicrobial sensitivity in both biofilm and planktonic cultures. Initially, MICs and MBCs of *S. aureus* clinical strains for Co-amoxiclav were determined. The rationale behind selecting Co-amoxiclav in this study was described in

section 3.4. Both strains were susceptible to Co-amoxiclav because their MICs were within MIC breakpoints according to the NCCLS criteria and methodology that separates susceptible organisms from intermediately susceptible or resistant organisms. A range of methods was attempted to measure antimicrobial sensitivity of these bacterial biofilms. Although the minimum biofilm eradication concentration (MBEC) assay is a well-established approach for determining the sensitivity of in vitro biofilms to antibiotics (Ceri et al., 1999), it did not perform well here. Difficulties in getting reproducible MBEC values with this device in addition to other disadvantages such as high cost of the device, risk of contamination when pegs are removed from the device's lid for additional analysis and the possibility of incomplete dislodgement of biofilms cells from pegs by sonication have been reported previously (Dall, 2013). These limitations in the MBEC assay indicate that more standardization of the methods are required in order to be confidently introduced in clinical practice to guide clinicians' decisions about treatment of biofilm infections (Coenye et al., 2018).

As an alternative, a colorimetric tetrazolium salt (XTT) assay was used to assess effects of antibiotics on metabolic activity of *S. aureus* biofilms. The principle of this assay was described in section 3.5.3. There are many advantages to the XTT assay. Perhaps the most important is the ability to be automated with a microplate reader for a quick processing of multiple biofilm samples without sample manipulation as biofilm removal from the surface is not required. However, there are also some limitations which should be considered in this assay, such as the difference in the metabolic rate of bacteria between biofilm and planktonic states and the susceptibility of the assay to bacterial growth rate and biofilm thickness (Bueno, 2014). The relationship between the time required by XTT reagents to reach the core of biofilms and biofilm thickness requires further investigation. Here, the MICs of Co-amoxiclav measured for planktonic *S. aureus* SB14 & SB17 cells using colorimetric XTT assay were similar to those determined using the standard microdilution method. Assessment of bacterial viability in this study showed that cells within biofilms of *S. aureus* SB14 & NCTC6571 were not susceptible to killing even when the concentrations of Co-amoxiclav reached as high as 4000 times greater than their MICs (Figure 3. 8). Since the metabolic activity of bacterial cells has been shown to be in linear relationship with the production of orange water-soluble formazan (Alonso et al., 2017, Peeters et al., 2008), we assumed that measuring the rate of tetrazolium salt conversion over the incubation time of 1 h (at 10 minute intervals) would provide greater sensitivity to determine differences in cell viability among the treatment groups of the assay. This approach revealed a significant dose-dependent

reduction in bacterial viability (represented by the rate of XTT salt reduction during the incubation period) in biofilms associated with *S. aureus* SB14 & NCTC6571 (Figure 3. 9). The MBEC values obtained in this assay were extremely elevated and they were more than 8000- fold greater than the established MIC breakpoints. This finding broadly supports previous studies demonstrating that bacterial cells within biofilms are highly tolerant to very high concentrations of antibiotics in comparison to their free-living counterparts (Fleming and Rumbaugh, 2017). A recent study has also shown that biofilms of 11 staphylococcal clinical isolates including three *S. aureus* strains were resistant to wide range of antibiotics including Penicillin and Oxacillin at the highest concentration assayed (256 µg ml^{-1}) when compared with their sensitive planktonic bacteria (Brady et al., 2017). Assessment of bacterial viability also showed that Co-amoxiclav at 512 and 1,024 µg ml^{-1} demonstrated borderline killing efficacy against biofilms of both *S. aureus* SB14 and NCTC6571, and these concentrations were used for investigating the effect of NucB on the antibiotic sensitivity of these *S. aureus* biofilms.

An important goal of this study was to assess the effects of NucB on susceptibility of *S. aureus* planktonic cells and biofilms to antibiotic treatment. Incubation of NucB at 0.5 µg ml^{-1} with *S. aureus* planktonic cultures in the presence of a range of Co-amoxiclav concentrations did not enhance their susceptibility to killing by Co-amoxiclav (Figure 3. 10) , indicating that eDNA possibly do not have a role in protecting *S. aureus* cells from Co-amoxiclav action at the planktonic state phase. This observation agrees with that of Tetz and Tetz (2010) who showed that cultivation of *E.coli* and *S. aureus* planktonic cells in nutrient media in the presence and absence of DNase I (5 µg ml^{-1}) had no effect on the susceptibilities of these bacteria to Ampicillin and Levofloxacin. Moreover, NucB showed no effect on the cell viability of these planktonic *S. aureus* cultures when compared with the growth controls without NucB. This finding was also observed in the bacterial cells within biofilms quantified by total viable counts (CFU ml^{-1}) in parallel with CV staining where NucB was shown to be acting specifically through degradation of the pre-formed biofilms rather than by affecting cell viability itself (Table 3. 5 & Figure 3. 13). These findings are in agreement with Shields et al. (2013), who showed that incubation of NucB at 5 µg ml^{-1} for an hour had no effect on the cell viability of *S. aureus* FH7, *S. constellatus* FH20, *S. salivarius* FH29 and *M. catarrhalis* FH4 planktonic cultures in comparison to controls lacking the enzyme. Furthermore, previous studies carried out on *in vitro S. aureus* biofilms also have shown that addition of DNase I and rhDNase I for up to 24 h did not exhibit significant effects on bacterial growth and viability (Kaplan et al., 2012, Mann et al., 2009, Rice et al., 2007).

The next stage was to use a combination of 1 µg ml^{-1} of NucB with Co-amoxiclav at 512 and 1,024 µg ml^{-1} to assess the effect of NucB in enhancing antibiotic susceptibility of the selected *S. aureus* biofilms. Our results showed that addition of NucB alone at 1 µg ml^{-1} to pre-formed *S. aureus* biofilms significantly reduced the viability of bacterial cells within these biofilms by approximately 55-80% (Figure 3. 12). This reduction most likely owing to its dispersal action on these pre-formed biofilms without having a killing effect on bacterial cells within these biofilms. Importantly, when NucB was combined with Co-amoxiclav at either concentration (512 or 1,024 µg ml^{-1}), there was an additional reduction in the metabolic activity of biofilms by approximately >35% compared to Co-amoxiclav treatment alone. These data demonstrate that NucB has the potential to enhance the susceptibility of *S. aureus* biofilms to Co-amoxiclav treatment. Furthermore, these data also show that anti-biofilm activity of NucB was not compromised when it was used in combination with Co-amoxiclav. Additional investigation of the compatibility of NucB with other antibiotics is required. In accordance with present findings, previous studies showed that bovine DNase I effectively disrupted *in vitro* biofilms formed by many bacterial species and enhanced their susceptibility to various antimicrobial agents (Kaplan, 2009, Tetz et al., 2009, Waryah et al., 2017). Similarly, rhDNase I has been demonstrated to inhibit biofilms produced by *S. aureus* and *S. epidermidis* and increase the sensitivity of bacterial cells within biofilms to biocide treatment (Kaplan et al., 2012).

Finally, another important aim of this section was to investigate the safety of NucB on human cells relevant to the middle ear and nasal cavity. Data in this study demonstrated that human bronchial epithelial cells were highly tolerant to high concentrations of NucB up to 100 µg ml^{-1} when compared with negative controls. Incubation of human bronchial epithelial cells for up to 48 h with NucB at concentrations >200-fold greater than the minimum concentration for anti-biofilm activity (0.5 µg ml^{-1}) did not demonstrate significant effect on the viability of these cells (Figure 3. 14) . Consistent with the cell viability assay, when these cells were incubated in direct contact with NucB at 1-100 µg ml^{-1} for 24-48 h, apoptosis was almost absent (Figure 3. 15). These data, while preliminary, show that *in vivo* application of NucB, even at high concentrations possibly would be safe. Kaplan et al. (2012) have shown that treatment of the *in vivo* model of *S. aureus* infection with a combination of tobramycin and rhDNase I (2.5mg/l) successfully extended the survival of *C. elegans* in comparison to the control treated with antibiotic only. Moreover, in many clinical trials, daily administration of rhDNase I (Dornase alfa) inhalational solution, which also had no effect on cell viability and apoptosis in this study, has proven to be effective in slowing down the deterioration of

pulmonary function and reducing the frequency of lung infections in young and elderly patients with mild to advanced cystic fibrosis by reducing the viscosity of bronchial secretions (Parsiegla et al., 2012, Pressler, 2008). In conclusion, the key techniques to quantify *in vitro* biofilm formation were successfully developed and have shown that NucB effectively disrupts biofilms both alone and in combination with antibiotics. It was also shown that NucB is potentially non-toxic for *in vivo* use. Next, it will be important to determine whether NucB is effective against isolates from COME. In order to do this, representative fresh clinical isolates from the MEEFs of COME patients were required. Isolation of strains was a key goal of the next section.

Chapter 4. Characterisation of microbial populations associated with COME patients

4.1 Introduction

Chronic otitis media with effusion (COME) is a chronic inflammatory condition of multifactorial cause, characterised by the persistence of middle ear fluid behind an intact ear drum for more than 12 weeks, typically without clinical manifestations of acute inflammation. It is the most common cause of acquired hearing loss and elective surgery in childhood. It is estimated that 80% of children have had at least one episode of COME by the age of 10 years (Kubba et al., 2000, NICE, 2016). Most cases of COME are self-limiting and may undergo spontaneous resolution. However, unfavourable consequences are not uncommon. Long-standing hearing loss due to COME at this critical stage of young children development may significantly deteriorate speech and language development in addition to negative impacts on older children's social skills and progress at school (Rosenfeld et al., 2016, NICE, 2016). The aetiopathogenesis of COME is still not well understood. In the past, middle ear effusion fluids from COME patients were considered sterile because a high percentage of them were culture negative. However, subsequent studies conducted using culture-independent approaches such as targeted PCR have demonstrated the presence of pathogenic bacterial DNA co-existing with bacterial-specific mRNA in more than 80% of culture-negative cases, indicating the presence of metabolically active microorganisms in these effusions (Rayner et al., 1998, Kubba et al., 2000). This discrepancy between high culture-negative rate and high PCR-positive rate in middle ear effusions of COME patients was later attributed to the presence of surface associated bacterial communities known as biofilms (Fergie et al., 2004). Biofilms of otopathogens were frequently observed on middle ear mucosa and effusions of patients with COME (Daniel et al., 2012b, Hall-Stoodley et al., 2006, Thornton et al., 2011, Van Hoecke et al., 2016, Kania et al., 2019, Novotny et al., 2019), strongly supporting the important role of biofilm infection in the aetiology of COME.

The most commonly cultured bacterial pathogens from middle ear effusions of children with COME are *Haemophilus influenzae*, *Streptococcus pneumoniae, Moraxella catarrhalis, Staphylococcus aureus,* and coagulase-negative staphylococci. All of these have the capability to develop biofilms *in vitro* and *in vivo* (Hall-Stoodley and Stoodley, 2009, Starner et al., 2006, Van Hoecke et al., 2016, Daniel et al., 2012b, Silva and Sillankorva, 2019). Studies suggest that biofilms on adenoids may be essential for the initiation and development of biofilms in the

middle ear, and that adenoids potentially act as a reservoir for biofilm-forming otopathogens (Hoa et al., 2009, Hoa et al., 2010, Van Hoecke et al., 2016, Kania et al., 2019, Nistico et al., 2011).

Traditional culture-based surveys generally underestimate the total population of fastidious, uncultivable microbes and bacteria residing in biofilms because they tend to detect only dominant bacterial species that can grow efficiently and are present in high numbers in the samples (Swidsinski et al. 2007a; Stol et al. 2013). Therefore, these studies potentially delivered partial or biased characterisation of the microbial community composition (Ren et al. 2013). The development of advanced culture-independent molecular-based techniques such as 16S rRNA gene sequencing now enables characterisation of microbial communities present in different anatomical sites of human body, especially those that reside in biofilms, at a level of detail that far exceeds previous culture-dependent or other targeted-PCR based molecular methods (Ari et al., 2019, Boers et al., 2018, Chan et al., 2017b). Culture-independent methods are also biased to some extent. There are biases in DNA extraction, PCR amplification, DNA sequencing, and bioinformatics analysis (Fouhy et al., 2016, Eisenhofer et al., 2019). Detection of microbial DNA also does not necessarily indicate the presence of viable bacteria. Even so, the culture independent analysis still provide a new and detailed understanding of microbial community composition. Recent microbiome studies utilising 16S rRNA gene sequencing have shown that *Alloiococcus otitis* is the most predominant bacteria in the MEEFs of children with COME, followed by three common otopathogens genera (*Haemophilus influenzae*, *Streptococcus pneumoniae*, *Moraxella catarrhalis*) (Ari et al., 2019, Boers et al., 2018, Chan et al., 2016, Chan et al., 2017b, Jervis-Bardy et al., 2015). Furthermore, *Turicella otitidis* has also been reported as a dominant bacteria within middle ear microbiota of children with COME (Ari et al., 2019, Boers et al., 2018, Krueger et al., 2017). To our best knowledge, there are no published studies on the identification of bacterial population present in middle ear effusion fluids (MEEFs) of patients with COME using a combination of conventional culture and 16S rRNA gene sequencing techniques.

4.2 Aims and objectives

The aims of this section were to characterise microbial populations present in MEEFs and adenoids of patients with COME using combined culturing and culture-independent 16S rRNA gene sequencing methods, and to investigate the efficacy of NucB enzyme in improving the recovery of bacteria from middle ear effusions. The objectives were as follow:

1. To culture and identify microorganisms present in MEEFs collected from patients undergoing myringotomy and grommet tube insertion to obtain representative clinical isolates that can be used in the subsequent experiments.
2. To measure the potential efficacy of NucB to disperse putative bacterial biofilms present in MEEFs.
3. To analyse the microbiome of selected MEEFs and adenoids of COME patients including uncultivable species and estimate the efficacy of traditional culture methods in identifying microbial community members of COME.

4.3 Patients and samples

In total, 39 patients were recruited in this study. Thirty-four of these were listed for myringotomy and grommet tube insertion for the treatment of COME, seven with adenoidectomy, and 27 without adenoidectomy. Control adenoid samples were also collected from 5 patients who were listed for adenotonsillectomy for the treatment of obstructive sleep apnoea (OSA) without clinical features of COME. From 34 patients, a total of 59 MEEF samples were collected. Twenty-five (73.5%) of 34 patients had bilateral COME, whereas 9 had unilateral COME. There was significant variation in MEEFs consistency, colour, and volume.

4.4 Isolation and identification of microbial populations associated with COME

To characterise microbial populations associated with COME, a portion of MEEF was inoculated on a range of growth media such as blood, chocolate, and fastidious anaerobe agars using methods adopted from the UK Standards for Microbiology Investigations for ear infections and associated specimens (UKSMI)(PHE, 2014)(see Materials and Methods 2.4.2).

Initially, a pilot isolation and identification of microorganisms from 12 MEEFs of 6 COME patients was performed using the standard operating procedure for the investigation of ear infections. The plates were inoculated in duplicates with 20 µl of thawed MEEF that had been stored overnight at -80˚C. Blood and chocolate agars were incubated at 37˚C with 5% CO_2 for 40-48 h, whereas FAA was incubated anaerobically for 7-14 days. To monitor the development of different colony types, plates were inspected for growth daily. Microbial colonies with different morphologies, colours, or sizes were sub-cultured three times on agar to establish axenic cultures. This approach was also employed for samples that produced abundant microbial colonies (> 400 colonies) (Figure 4. 6). All samples were plated in duplicate on each type of agar, and the total number and type of colonies were similar on each duplicate. In total, 8 strains of bacteria were isolated from 6 patients, consisting of 2 different genera and 6 separate species (Table 4. 1). In total, 7 (58.3%) out of 12 MEEFs were culture positive for at least one bacterium, whereas 5 (41.7%) were culture negative.

Table 4. 1 Bacteria isolated from MEEFs of 6 patients with COME in the pilot sample set

Species	Number of MEEFs	Number of patients
Staphylococcus auricularis	4	2
Staphylococcus epidermidis	1	1
Staphylococcus pastueri	2	2
Staphylococcus spp.	2	1
Staphylococcus caprae	2	1
Micrococcus luteus	1	1

Staphylococcus spp. (CoNS) were most frequently isolated bacteria from pilot set of MEEFs

Table 4. 2 Bacteria isolated from MEEFs of patients with COME in a follow-up set of 16 patients, using an improved culture method

Species	Number of MEEFs	Number of patients
Turicella otitidis	5	4
Alloiococcus otitis	2	2
Staphylococcus auricularis	4	3
Staphylococcus epidermidis	3	2
Staphylococcus aureus	2	2
Staphylococcus simulans	2	1
Staphylococcus schleiferi	1	1
Staphylococcus pastueri	1	1
Staphylococcus sp.	1	1
Streptococcus oralis	2	2
Streptococcus parasanguinis	1	1
Streptococcus pneumoniae	1	1
Streptococcus pyogenes	1	1
Streptococcus mitis	1	1
Haemophilus influenzae	1	1
Moraxella catarrhalis	1	1
Neisseria sp.	1	1
Actinomyces odontolyticus	1	1
Pseudomonas nitroreductans	2	1

Wide range of bacterial species were isolated using the “improved” culture method.

Coagulase-negative staphylococci (CoNS) including *Staphylococcus auricularis* and *Staphylococcus pastueri* were the most commonly isolated bacteria that were present in almost all culture positive MEEFs. These data in combination with the early culture independent 16S rRNA gene sequencing data obtained from these MEEF samples (see section 4.8) clearly showed that the culturing methods used needed improvement as they failed to isolate most of the potential middle ear colonisers identified by 16S rRNA gene sequencing. Therefore, the methods used to culture and isolate microorganisms from the subsequent 26 MEEFs collected from 16 patients (10 with bilateral COME, 6 with unilateral COME) were optimised. Modifications included (i) transferring MEEF samples quickly (within 30-60 min) following collection for immediate processing, (ii) inoculating the plates with 50 µl of MEEF instead of 20 µl, and (iii) incubating blood and chocolate agars at 37˚C with 5% CO_2 for a longer duration (10-14 days instead of 40-48 h; see Materials and Methods section 2.4.2).

Following these optimisation steps, 32 strains of bacteria were isolated from the 16 patients, comprising 9 different genera and 19 different bacterial species (Figure 4. 2). Fourteen (53.8%) of the 26 MEEFs were culture positive, and 12 (46.2%) MEEFs were culture negative. The most prevalent bacterial species present in MEEFs of COME was *Turicella otitidis*, which was isolated from 19.2% of the samples. Colonies of *T. otitidis* started to appear on culture media (blood, chocolate, FA agars) after 72 h. Coagulase-negative staphylococci (CoNS) were still the most frequent microorganisms associated with COME and were isolated from 12 of 26 MEEFs (46.2%). *S. auricularis* (15.4%), *Staphylococcus epidermidis* (11.5%), and *Staphylococcus simulans* (7.7%) were the most frequently isolated species among CoNS. *Alloiococcus otitis* was isolated from 7.7% of samples. Colonies started to appear on growth media after around 5-7 days of incubation. *S. aureus, Streptococcus oralis, and Pseudomonas nitroreductans* were each isolated from 2 MEEFs (7.7%). Other bacterial species that were less frequently isolated from MEEFs included *H. influenzae, M. catarrhalis, S. pneumoniae, Streptococcus pyogenes, Streptococcus mitis, and Actinomyces odontolyticus.* The modifications adopted in culture methods significantly improved the isolation of many bacterial species associated with COME, particularly fastidious microorganisms such as *T. otitidis, A. otitis, H. influenzae, M. catarrhalis, S. pneumoniae, S. mitis, Streptococcus parasanguinis,* and *A. odontolyticus*.

However, an extended incubation of a significant number of samples was associated with an increase in the rate of contamination, which started to appear on blood and chocolate agars as patches of fungal growth with an appearance resembling cotton wool following 72-96 h incubation at 37˚C with 5% CO_2. These patches occasionally covered most of the surface of

the agar, which made the visualisation of bacterial growth impossible and interfered with attempts to obtain axenic cultures (Figure 4. 1). Therefore, further optimisation was required to prevent contamination during long incubation of the growth media. Initially, this work focussed on determining the source of contamination. Blood agar plates were prepared as described in Materials and Methods section 2.4.1. Plates were poured on the bench and left uncovered to solidify, or were poured in the Class 1 biological safety cabinet and either left uncovered to solidify or covered immediately. Empty (uninoculated) control blood agar plates were incubated at 37˚C with 5% CO_2 for up to 10 days and checked for growth daily. At the end of incubation, plates that were left uncovered to solidify on either the bench or in the biological safety cabinet showed patches of growth similar to those that had appeared on culture plates during the extended incubation. By contrast, no growth was observed on plates that were covered immediately after being poured in the biological safety cabinet (see Figure 4. 2). These results were consistent with contamination being introduced to the plates from the air during growth media preparation. To minimise the risk of airborne contamination by fungal spores or bacteria, the biological safety cabinet was cleaned and thoroughly decontaminated using both antibacterial and antifungal disinfectants. The benchtop incubator and the candle jars were also disinfected, and plates were always covered immediately after pouring from this point forward. Overall, these steps dramatically reduced the levels of contamination during the longer (10-14 day) incubation period.

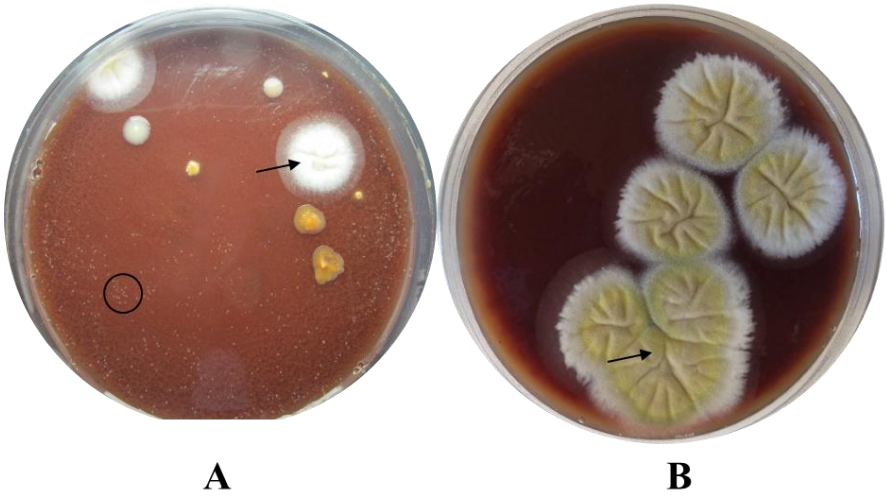


Figure 4. 1 Appearance of contamination on growth media.

(A) Chocolate agar from MEEF 20L, (B) blood agar from MEEF 17L. The growth of white patches of fungal contamination (black arrows) are visible on both blood and chocolate agars. Occasionally, these patches covered a large area of the plate's surface. *Alloiococcus otitis* small colonies are also visible (black circle), covering the agar surface following 5-7 days of incubation at 37˚C with 5% CO_2.

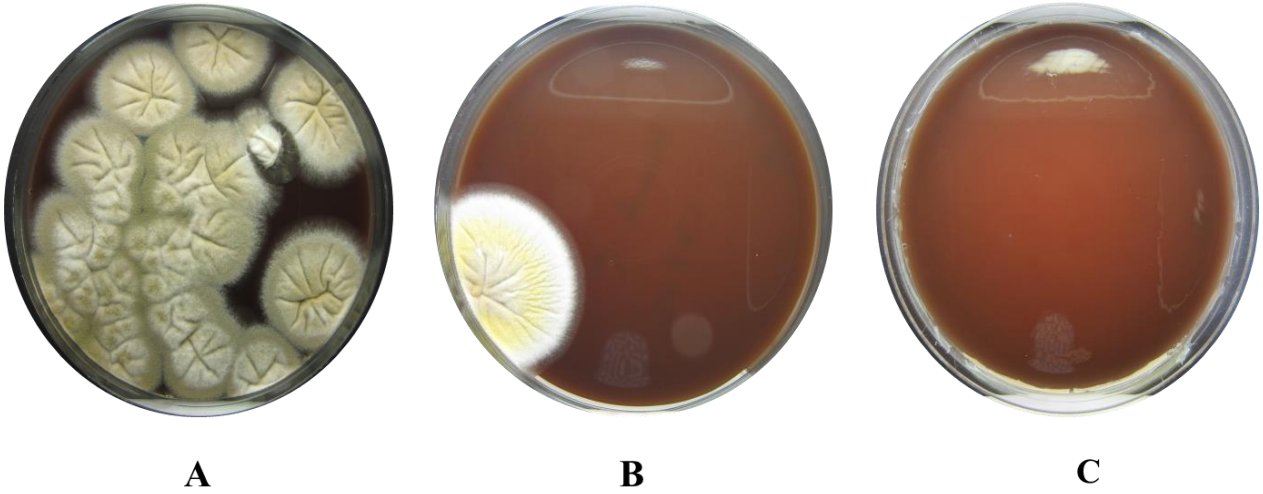


Figure 4. 2 Outcomes of an extended incubation of empty (control) blood agar plates

Blood agar was prepared as described in Materials and Methods before being poured into petri dishes and either left uncovered to solidify on the bench or in the biological safety cabinet or covered immediately in the biological safety cabinet. Empty (uninoculated) plates were incubated for up to 10 days at 37˚C with 5% CO_2. Patches of white growth were observed on plates that were left uncovered on the bench (A) or in the biological safety cabinet (B) at the end of the incubation time. No growth was observed on plates that were covered immediately after the growth medium was poured in the biological safety cabinet (C). These results show that contamination was likely introduced from the air during growth media preparation.

Following these additional optimisation steps, a total of 21 MEEF samples from 12 patients (9 with bilateral COME, and 3 with unilateral COME) were analysed. Nineteen (90.5%) MEEFs were culture positive for at least one microorganism, whereas only 2 (9.5%) MEEFs were culture negative. Forty-five microbial strains were identified in total from 12 patients, including 10 different genera and 16 separate species (Table 4. 3). Each MEEF contained between 1 to 6 microbial species, and an average of 2.3 (SE 0.5) bacterial species were identified per MEEF sample. There was no significant difference (p= 0.47; unpaired two samples *t*-test) in the number of bacterial species isolated from both ears in patients with bilateral COME. *T. otitidis* again was the most common microorganism detected in MEEFs and was isolated from 11 of 21 samples (52.4%). *A. otitis* was the second most common bacterial species and was identified in 38.1% of samples. CoNS were isolated from 76.2% of samples including *S. epidermidis* (28.6%), *S. auricularis* (19%), *Staphylococcus capitis* (14.3%), and in addition to other CoNS, which were isolated from 14.3% of samples. *H. influenzae, M. catarrhalis,* and *S. pneumoniae* have previously been shown to be the most frequently isolated pathogens. These microorganisms were isolated from 4 of 21 MEEFs (19%), and they frequently co-existed with other bacterial species. Other microbial species such as *Propionibacterium acnes, Streptococcus salivarius, Neisseria flavescens, Kocuria rhizophila,* and *Candida parapsilosis* were also identified, although less frequently. The vast majority of microbial isolates were facultative anaerobes. However, obligate aerobes (such as *N. flavescens* and *M. catarrhalis*) and obligate anaerobes (*P. acnes*) were also identified. In conclusion, adoption of the optimised culture techniques significantly improved culturing and isolation of microorganisms associated with COME. Moreover, a significantly higher number of culture positive MEEF samples were identified using the optimised culture methods in comparison to the traditional culture methods used in this study.

Table 4. 3 Bacteria isolated from middle ear fluids of 12 patients with COME

Species	Number of MEEFs	Number of patients
Turicella otitidis	11	6
Alloiococcus otitis	8	5
Staphylococcus epidermidis	6	4
Staphylococcus auricularis	4	3
Staphylococcus capitis	3	2
Haemophilus influenzae	2	1
Propionibacterium acnes	2	2
Staphylococcus simulans	1	1
Staphylococcus hominis	1	1
Staphylococcus pettenkofen	1	1
Streptococcus pneumoniae	1	1
Streptococcus salivarius	1	1
Moraxella catarrhalis	1	1
Neisseria flavescens	1	1
Kocuria rhizophila	1	1
Candida parapsilosis	1	1

Most of MEEFs were culture positive when the modified “improved” culture method was used. High proportion of fastidious species such as *Turicella otitidis* and *Alloiococcus otitis* in addition to other microorganisms were isolated.

4.5 Efficacy of NucB for improving culturing recovery of microorganisms from a pilot set of MEEF samples

Bacteria residing in biofilms are often difficult to culture. In Chapter 3, it was shown that NucB from *Bacillus licheniformis* can disperse biofilms formed by clinically relevant *S. aureus* strains. I hypothesised that NucB can disperse biofilms present in MEEFs and boost recovery of microorganisms from these biofilms. Initially, 12 MEEFs collected from 6 patients with COME were used to optimise methods. These samples were divided into two portions. The first portion was stored at -80˚C until microbial DNA extraction for 16S rRNA gene sequencing could be performed. The second portion of MEEF was used for culturing cells and was either processed immediately or stored at -80˚C for processing the next day. Twenty microliters of MEEF were inoculated on blood agar, chocolate agar, and FA agar in duplicate (see section 4.4) before being treated with NucB (1 µg ml^{-1}) for 1 h at 37°C aerobically. Following the incubation, 20 µl from NucB-treated MEEF were plated on each growth medium and incubated under the same conditions as described in section 4.4. The colony forming units (CFU) per millilitre were then calculated for NucB-treated and control samples. Of the 12 MEEFs, 6 were excluded from analysis because 5 were culture-negative, and one produced heavy growth (>400 colonies) that was impossible to count accurately.

Overall, MEEFs treated with NucB a showed 2-fold or more increase in the number of CFU ml^{-1} of microorganisms in 3 out of 6 MEEFs cultured on blood agar plate in comparison to negative controls (Table 4. 4), whereas only 1 out of 6 MEEFs showed greater counts of bacteria in the absence of NucB, which could be due to uneven distribution of microbial cells across the MEEF samples. By contrast, in 2 out of 6 MEEFs there were at least two-fold greater CFU ml^{-1} on FA agar in untreated controls compared with NucB-treated cultures (Table 4. 5). Only one NucB-treated MEEF had more microorganisms cultured on FA agar than the control.

Table 4. 4 Colony forming unit numbers (CFU ml^{-1}) of microorganisms cultured from middle ear fluids, in the presence and absence of NucB (1 µg ml^{-1}), on chocolate agars.

Patient No.[a]	Control	(+) NucB (1 µg ml^{-1})*
1L	2400	150
2R	0	**12550**
3R	50	**200**
3L	100	**200**
4R	100	100
6L	500	400

Table 4. 5 Colony forming unit numbers (CFU ml^{-1}) of microorganisms cultured from middle ear fluids, in the presence and absence of NucB (1 µg ml^{-1}), on FA agar.

Patient No. [a]	**Control**	**(+) NucB (1µg ml^{-1})***
1L	450	150
2R	0	0
3R	1600	100
3L	0	**300**
4R	0	0
6L	250	150

[a] MEEF 1R,2L,4L,5R,5L, and 6R were excluded from analysis.
* Values in bold and underlined indicate NucB increased CFU ml^{-1} by 2-fold or more, whereas values that are underlined only indicate 2-fold or more CFU ml^{-1} were cultured in the absence of NucB. The CFU numbers represent the averages of CFU ml^{-1} calculated from two plates. In general, NucB did not consistently increased the recovery of bacteria from MEEFs that were cultured on both blood and FA agars.

The results obtained from these initial experiments showed an inconsistent efficacy of NucB at improving the recovery of bacteria from MEEF samples. It is likely that microbial cells were unevenly distributed across the MEEF samples, resulting in a great deal of inherent variability in the assay. These problems may have been compounded by inconsistent levels of NucB activity, since NucB was standardised based on mass rather than activity, and different preparations were used for different assays. To eliminate issues arising from inconsistent levels of NucB activity, it was important to develop methods to measure NucB nuclease activity. This would then enable consistent amounts of active NucB enzyme to be added to each experiment.

4.6 Measurement of NucB enzymatic activity

4.6.1 Measurement of NucB specific activity in the optimal buffer solution

In order to be able to add a consistent amount of active NucB to each experiment, the specific activity of NucB in the optimal buffer (50 mM Tris pH 8.0, 5 mM $MnSO_4$) was measured using both spectroscopic and gel electrophoresis techniques (see Materials and Methods section 2.7.1). It should be noted that in Chapter 3 the inhibition and dispersal of *S. aureus* biofilms by NucB was demonstrated to be dose-dependent over a concentration range of 10-500 ng ml^{-1} (see Figure 3. 1). To ensure that differences in the enzyme activity of different NucB preparations would not interfere with the assay, the enzyme was used in excess (0.5 µg ml^{-1}) during subsequent experiments throughout Chapter 3. However, the continued use of high concentrations of enzyme was problematic due to the high cost of producing the purified enzyme. Therefore, it was important to develop an assay for measuring and standardising enzyme activity.

The principle of the assay was to digest high molecular weight calf thymus DNA (CTDNA) with NucB for a certain time period and then quantitatively recover and measure the amount of perchloric acid-soluble low molecular weight DNA produced. Spectroscopic measurement does not provide accurate quantification of DNA degradation during early stage of the reaction because only DNA digestion products of less than approximately 500 bp in length are quantitatively solubilised by the addition of perchloric acid, whereas high molecular weight DNA fragments are not. Thus, the early part of the reaction was monitored by agarose gel electrophoresis (Figure 4. 3), which showed that maximum degradation of high molecular weight CTDNA into low molecular weight DNA was reached by 60 minutes in comparison with the negative controls. The spectroscopic analysis of the digestion of CTDNA (125 µg) by NucB revealed that on average 10 ng of NucB generated an A_{260} of 5.6 absorbance units (+/- 0.3, n=3), equivalent to 70 µg of DNA, in 1 h at 37ºC in 250 µl of Tris buffer containing 5 mM Mn^{+2} after taking into account the various dilutions made. A unit of NucB activity was defined as a production of perchloric acid soluble low molecular weight CTDNA that generated an absorbance of 1.0 at 260 nm, per hour at 37ºC in 50 mM Tris pH 8.0, 5 mM $MnSO_4$ buffer. Thus, the mean specific activity of NucB calculated was 1.4 x 10^5 units mg^{-1}.

To determine the best concentration of NucB to be used in the measurement of specific activity, the activity of a range of NucB concentrations (0.25, 1, 5, 10, 25, 50, 100 ng) was measured (see Materials and Methods section 2.7.1). The extent of DNA digestion increased

up to 50 ng NucB, which generate an A_{260}= 10.7 (+/- 0.3, n=3). Therefore, NucB concentration of 25 ng was considered as an ideal concentration for the assay work because it produced a large signal (A_{260}= 8.1 (+/- 0.25, n=3), 101 µg of DNA), and it lay approximately in the middle of the range (see Figure 4. 4 (B)). Visualisation of the DNA digestion products using gel electrophoresis confirmed that NucB at 25 ng fully digested high molecular weight CTDNA into low molecular weight DNA products and there were no intermediate molecular weight DNA fragments as seen with other concentrations below 25 ng (see Figure 4. 4 (A)). Furthermore, 25 ng of NucB was the lowest concentration of NucB that produced an A_{260} value that was approximately 80% of the overnight value when 125 µg CTDNA was digested to completion, using 100 ng NucB incubated overnight (A_{260}= 10 (+/- 0.6, n=3), 125 µg). On the other hand, NucB concentrations above 50 ng will saturate the reaction. Moreover, addition of NucB at concentrations lower than 25 ng produced partial digestion products that would be precipitated by perchloric acid and therefore not measured as assay products. In conclusion, the correct incubation time and NucB concentration of the assay were determined and the assay was used to monitor NucB specific activity subsequently to ensure the addition of a consistent amount of enzyme activity in the experiments.

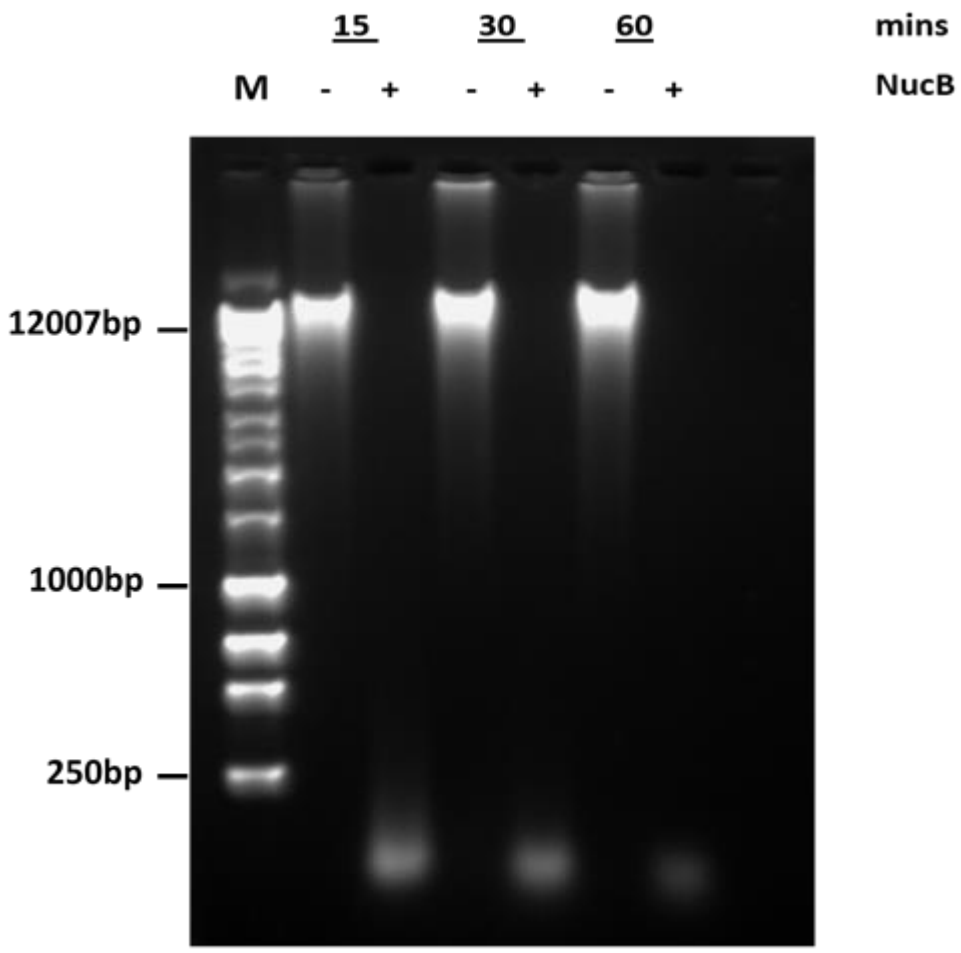


Figure 4. 3 NucB activity against high molecular weight calf thymus DNA.

Calf thymus DNA (125 µg) was incubated with (+) or without (-) 10 ng of NucB in Tris buffer containing 5 mM Mn^{+2} ions for 15, 30 and 60 min at 37ºC. Agarose (1% w/v) gel electrophoresis was used to separate the digestion products of samples and DNA visualised by staining with GelRed™. (M) represents a molecular weight marker (HyperLadder 1 kb Plus (250-12,007 bp). The CTDNA was completely digested by 60 min. The image shows total degradation of high molecular weight CTDNA into low molecular weight DNA in the presence of NucB at each time point when compared to negative controls.

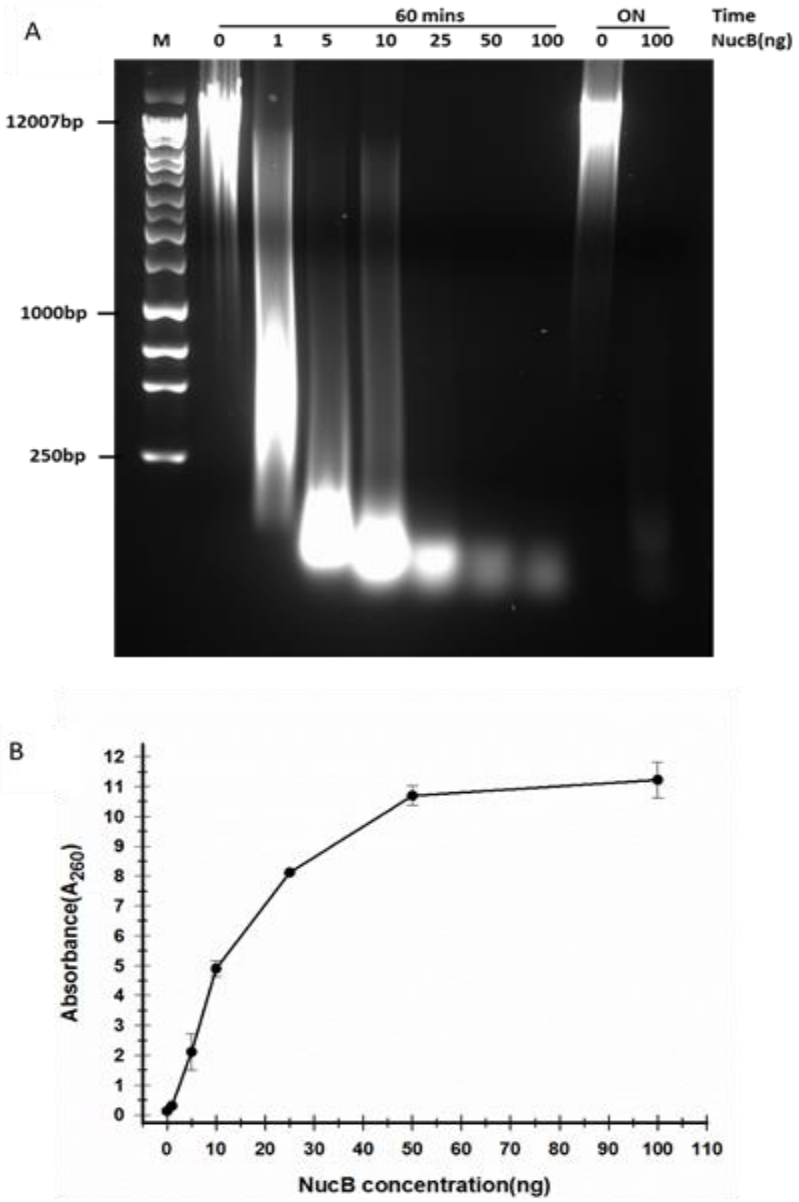


Figure 4. 4 The nuclease activity of NucB against calf thymus DNA.

(A) Calf thymus DNA (125 µg) was incubated with a range of NucB concentrations in Tris buffer containing 5 mM Mn^{+2} ions for 60 min at 37ºC. CTDNA was also incubated with or without 100 ng of NucB overnight (ON). Agarose (1% w/v) gel electrophoresis was used to separate the digestion products of samples, and DNA visualised by staining with GelRed™. (M) represents a molecular weight marker (HyperLadder 1 kb Plus (250-12,007 bp). (B) Absorbance values (A_{260}) of perchloric acid soluble DNA products generated by the digestion of 125 µg of CTDNA with a range of NucB concentrations in 250 µl of Tris buffer containing 5 mM Mn^{2+} ions for 60 min at 37ºC were measured by Nanodrop spectrophotometry. NucB showed dose-dependent degradation of High molecular weight CTDNA into low molecular weight DNA over 60 min with the full digestion of CTDNA was obtained at 25ng compared to the control (A). There was also proportional correlation between the increase in the A_{260} of perchloric acid soluble DNA products and the increase in the concentration of NucB (B).

4.6.2 Measurement of NucB nuclease activity in growth media and normal saline

The nuclease activity of NucB was measured in different solutions such as TSB and 0.9% saline solution used in the experimental methods assessing the anti-biofilm activity of NucB against various bacterial biofilms (see Materials and methods section2.7.2). These were chosen because both media were used for experiments involving NucB digestion. For example, 0.9% saline solution was used during the collection of MEEF samples (see Material and Methods section 2.3.1) that were treated by NucB to assess the efficacy in improving the recovery of bacteria. TSB was used as a culture medium to grow *S. aureus* biofilms that were treated with NucB to investigate the capacity to inhibit biofilm formation by different *S. aureus* strains as previously described in chapter 3. Generally, the nuclease activity of NucB was lower in both solutions compared with the NucB buffer. The calculated specific activity of NucB in TSB was 6.8×10^4 units mg^{-1}, which was approximately 2-fold less than that calculated in the optimal buffer (1.4×10^5 units mg^{-1}) containing 50 mM Tris-HCl pH 8.0, 5 mM $MnSO_4$. In 0.9% saline solution, the findings were similar. The calculated specific activity of NucB was 8.0×10^4 units mg^{-1}, which was again just 50% less than that calculated in NucB buffer (1.3×10^5 units mg^{-1}). The reduced DNase activity was also observed by agarose gel electrophoresis (see Figure 4. 5). In conclusion, NucB was still able to efficiently digest high molecular weight CTDNA in both TSB and 0.9% saline solution. However, the specific activity was significantly lower in TSB than in the optimal NucB buffer.

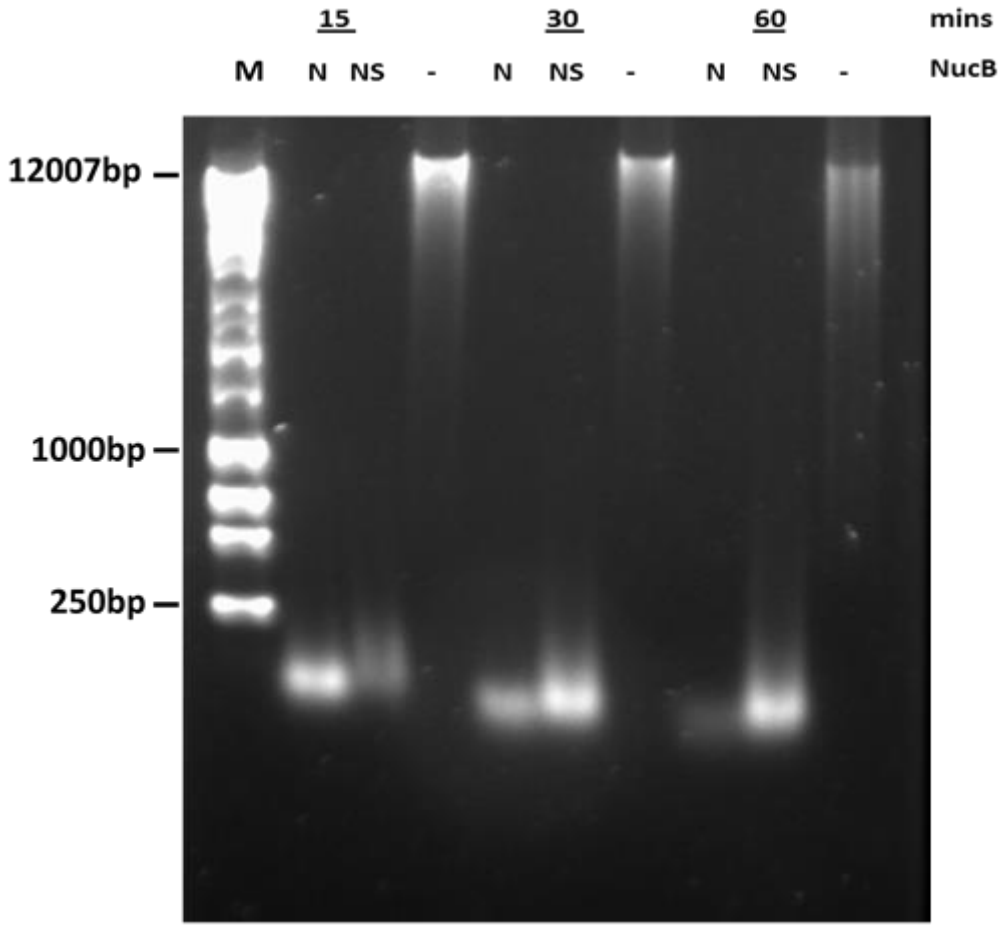


Figure 4. 5 The nuclease activity of NucB against CTDNA in 0.9% saline solution

Calf thymus DNA (125 µg) was incubated with 10 ng NucB in Tris buffer containing 5 mM Mn^{2+} (N) or in 0.9% saline solution (NS) for 15, 30 and 60 minutes at 37ºC. Control samples with no NucB (-) were included. Agarose (1% w/v) gel electrophoresis was used to separate the digestion products and DNA visualised by staining with GelRed™. (M) represents a molecular weight marker (HyperLadder 1 kb Plus (250-12,007 bp)). NucB was still able to degrade high molecular weight CTDNA into low molecular weight DNA over 15-60 min incubation in 0.9% NS but at slightly slower rate compared to that in Tris buffer.

4.7 Efficacy of NucB in improving culturing recovery of microorganisms from MEEFs of COME patients

Having established methods for measuring NucB specific activity, subsequent samples of MEEFs were treated with 100 units of NucB to assess its efficacy at improving culture recovery of bacteria from these samples. Furthermore, it was important to include additional controls that consisted of samples treated exactly the same way as the NucB samples, but without the inclusion of NucB. This 'incubation control' was included in the analysis of the subsequent MEEF samples (see Materials and Methods section 2.8.3).

A total of 44 MEEFs from 26 patients with COME were assessed. After plating 50 µl of the MEEF sample on each growth medium (blood, chocolate and FA agar) in duplicate, the remaining sample was divided into 3 portions and processed according to the optimised methods described previously in Materials and Methods section 2.8.3. Of the 44 MEEFs treated, 19 were excluded from statistical analysis because 14 were culture–negative in all conditions, 3 were lacking incubation controls, and two samples produced abundant microbial colonies in all conditions that could not be counted accurately (Figure 4. 6). Generally, addition of 100 units of NucB to MEEF did not cause a statistically significant increase (p = 0.38; paired two samples t-test) in the total numbers of microorganisms cultured on blood and chocolate agar plates when compared with controls incubated without NucB for 1 h at 37°C in ambient air (Table 4. 6). In the presence of NucB, 5 out of 25 samples (20%) showed 2-fold or more CFU ml^{-1} on blood and chocolate agar in comparison to incubation controls with no NucB (Figure 4. 7). Furthermore, 5 out of 25 MEEFs (20%) treated with NucB also showed just below 2-fold increase in the total bacterial CFU ml^{-1} cultured on blood and chocolate agar. However, there was a statistically significant reduction (p = 0.007; paired two samples t-test) in the total numbers of microbes cultured from MEEFs incubated without NucB for 1 h at 37°C aerobically on blood and chocolate agars when compared with the control MEEFs that were cultured immediately on these plates without incubation (see Figure 4. 8). Surprisingly, this reduction affected the majority of the bacterial species cultured in this study and it was not species-specific, though fastidious and slow growing bacteria were affected more than other bacteria. However, under the same conditions the total numbers of microbial colonies cultured from MEEFs treated with NucB was not significantly different (p = 0.08; paired two samples t-test) from those cultured from negative controls without incubation.

Under anaerobic conditions using FAA, microbial cells were released in greater numbers when treated with NucB than the incubation controls in only 3 out of 25 MEEFs

(12%) (Table 4. 7), which was not statistically significant (p = 0.8; paired two samples t-test). Again, 11 out of 25 MEEFs (44%) incubated for 1h at 37°C aerobically in the absence of NucB had 2-fold or more decrease in the total numbers of microorganisms when compared with the negative controls cultured on FAA without incubation (Table 4. 7). In conclusion, addition of 100 units of NucB to the MEEFs collected from patients with COME did not efficiently improved the recovery of the bacteria from these samples onto culture media, and the incubation of these samples for 1 h at 37°C on air without NucB had a significant negative impact on the viability of the microbial cells present in these samples.

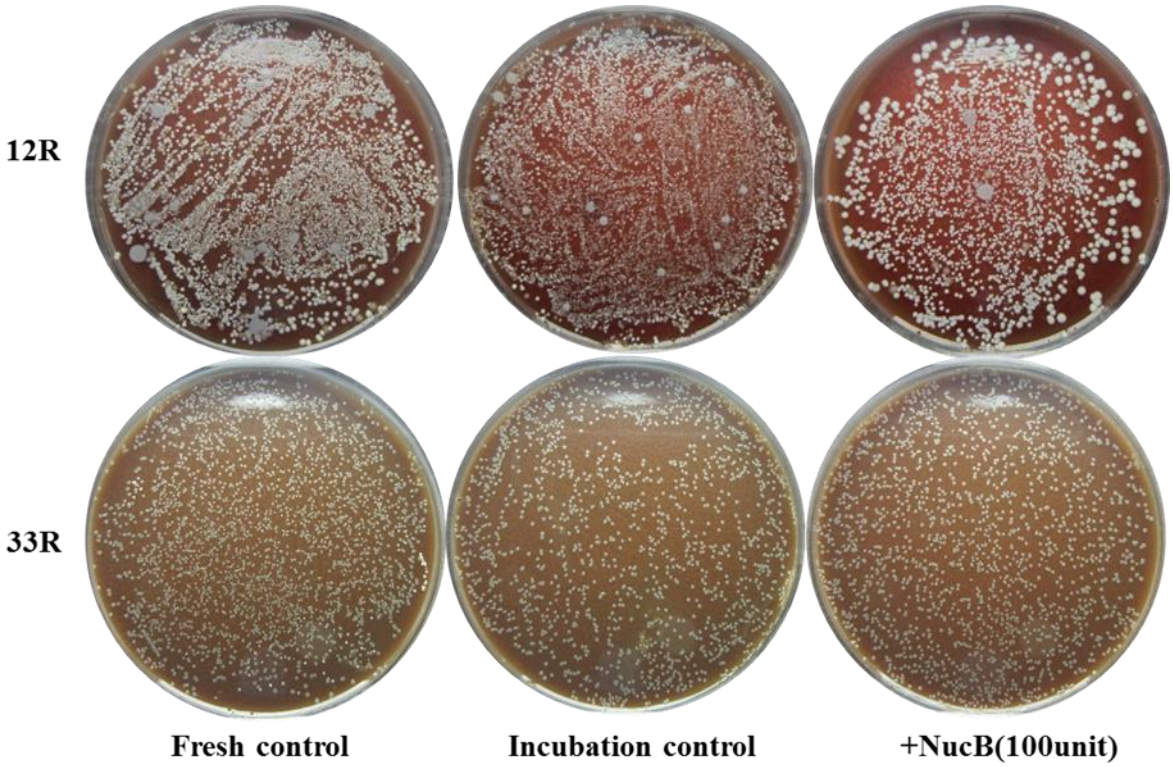


Figure 4. 6 Abundant colonies isolated from COME samples on two different growth media.

(12R) Sample from the right ear of patient 12 cultured on blood agar. (33R) Sample from the right ear of patient 33 cultured on chocolate agar. Many colonies of different morphology are visible covering both agar plate surfaces in all treatment conditions.

Table 4. 6 Colony forming units (CFU ml^{-1}) of microorganisms cultured from MEEFs, in the presence and absence of NucB (100 unit), on blood and chocolate agars.

MEEF No.[a]	Fresh control CFU	(+)100 unit NucB CFU ml^{-1} [b]	Incubation control CFU ml^{-1} [b]
9L	1845	(2385)	**540**
11L	3060	2112	2340
11R	1600	3476	3460
12L	7880	**3740**	**3800**
14L	580	**(242)**	**40**
16L	8000	**0**	**0**
19R	120	(182.4)	80
20L	16000	14700	23870
21L	3760	2275	**1440**
22L	400	**110**	**120**
23R	7720	5865	**3000**
23L	4700	3105	3600
24L	180	92	100
25R	160	138	**80**
25L	980	**0**	**0**
26R	1700	1311	**780**
26L	140	184	160
27R	700	**207**	**120**
27L	1820	**(230)**	**80**
29R	100	**0**	**0**
30L	12000	**184**	**160**
32R	3180	(3837)	**1560**
32L	4560	**0**	**0**
33L	160	430	360
34L	7640	5221	5160

[a] MEEF 7R, 7L,18L were excluded from analysis because of lack of incubation controls, 12R and 33R were excluded because their CFU count were >400, and the rest for being culture negative.[b] * Values in bold and underlined indicate MEEFs incubation for 1h at 37°C aerobically reduced total CFU ml^{-1} by 2-fold or more in comparison to fresh controls, whereas values between parentheses indicate NucB increased CFU ml^{-1} by 2-fold or more compared to incubation controls. The CFU numbers represent the averages of CFU ml^{-1} calculated from

two plates. NucB did not significantly improved culture recovery of bacteria from MEEF samples.

Table 4. 7 Colony forming unit numbers (CFU ml^{-1}) of microorganisms cultured from MEEFs, in the presence and absence of NucB (100 unit), on FAA agar anaerobically

MEEF No.[a]	Fresh control CFU	(+)100 unit NucB CFU ml^{-1} [b]	Incubation control CFU ml^{-1} [b]
9L	3330	(2790)	**1040**
11L	2100	**748**	1360
11R	1780	4532	3760
12L	7160	4114	**2460**
14L	0	0	0
16L	10400	**0**	**0**
19R	140	**0**	**0**
20L	880	**210**	**372**
21L	2860	2100	3380
22L	0	0	0
23R	3980	3588	3140
23L	2120	1610	2020
24L	0	0	0
25R	140	92	**60**
25L	660	184	**220**
26R	260	(621)	280
26L	40	**0**	**0**
27R	260	(414)	140
27L	1480	**138**	**140**
29R	80	0	**0**
30L	220	115	**100**
32R	3300	3750	2280
32L	0	0	0
33L	220	344	320
34L	3400	3312	3980

a MEEF 7R, 7L,18L were excluded from analysis because of lack of incubation controls, 12R and 33R were excluded because their CFU count were >400, and the rest for being culture negative.

b * Values in bold and underlined indicate MEEFs incubation for 1h at 37°C aerobically reduced total CFU ml^{-1} by 2-fold or more in comparison to fresh controls, whereas values between parentheses indicate NucB increased CFU ml-1 by 2-fold or more compared to

incubation controls. The CFU numbers represent the averages of CFU ml^{-1} calculated from two plates. NucB also did not significantly increased CFU ml^{-1} of bacteria cultured on FAA.

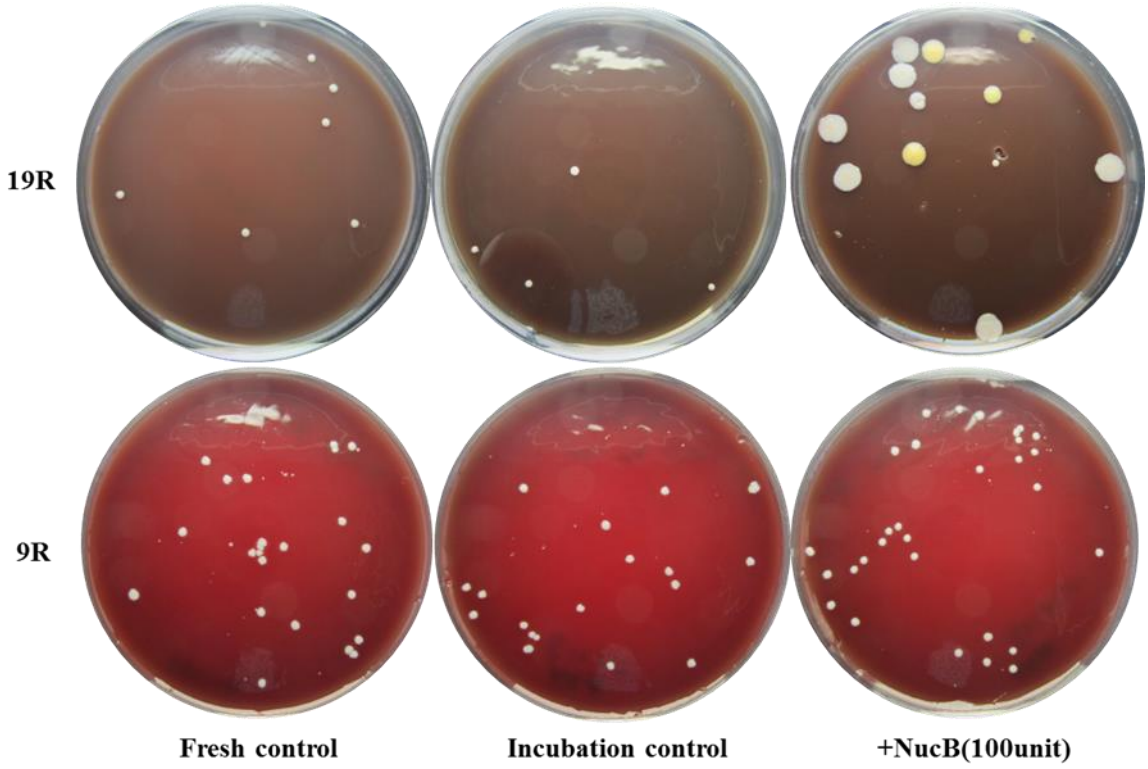


Figure 4. 7 Recovery of COME microorganisms from MEEF samples using NucB enzyme.

MEEFs were incubated for 1 h at 37°C in ambient air with or without 100 units of NucB, then cultured on agar plates and compared with negative controls that were not subjected to incubation (Fresh control). This method enabled the counting of the total number of microbial colonies released from MEEFs. An illustration of this, on chocolate agar (19R) and blood agar (9R), show greater numbers of microbes recovered with the addition of NucB, in comparison to the incubation control.

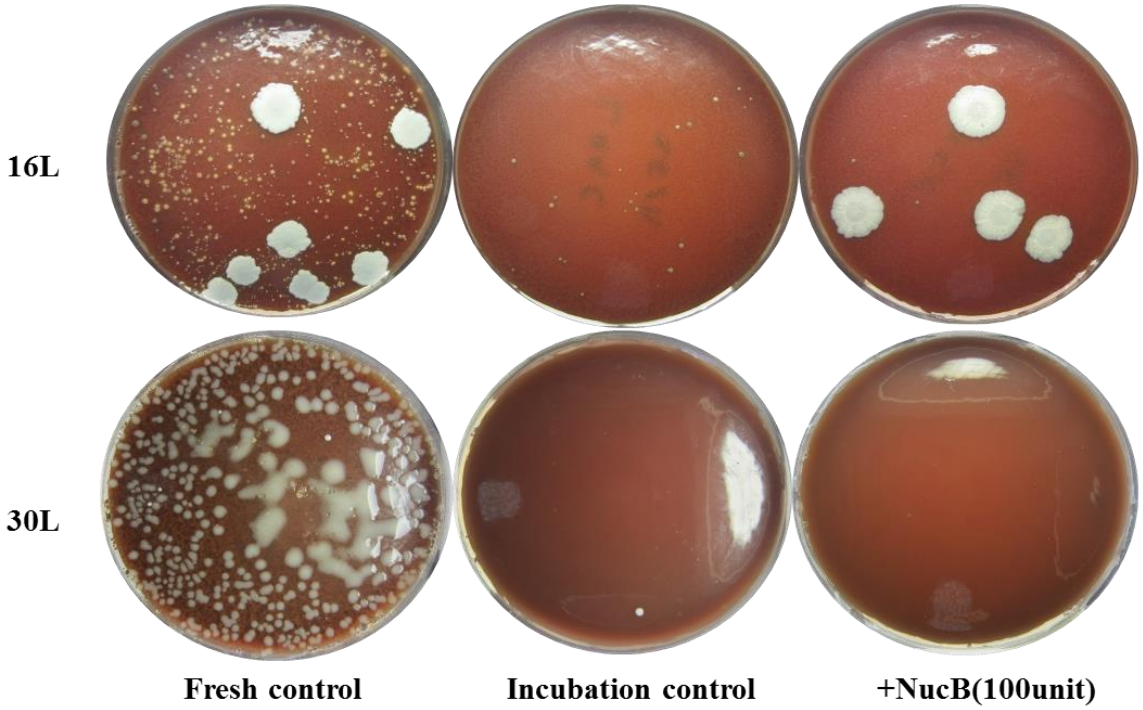


Figure 4. 8 Effect of aerobic incubation on COME microorganisms.

Examples of chocolate agars from MEEF No. 16L and 30L shows significantly less microorganisms were cultured from MEEFs incubated for 1h at 37°C aerobically with or without NucB in comparison to fresh controls that were not subjected to incubation. The microorganisms affected were *Alloiococcus otitis, Propionibacterium acnes, and Staphylococcus epidermidis* in case of 16L, and *Staphylococcus auricularis* in case of 30L.

4.8 Characterisation of microbial communities associated with COME and adenoids by 16S rRNA gene sequencing

To verify the efficacy of the traditional culture methods for identifying the bacterial population present in the MEEFs of patients with COME, and to study co-occurrence of otopathogens in the middle ear and adenoids, the composition of the microbiota present in the selected MEEFs and adenoids was also characterised using culture-independent 16S rRNA gene sequencing. MEEF and adenoid samples underwent microbial DNA extraction as previously described (see Materials and Methods, section 2.6.1 and 2.6.2) and the concentrations of DNA extracted from the samples are listed in Table 4. 8. The sequencing of PCR- amplified V1-V3 (27F-519R) fragments of 16S rRNA gene using Illumina®MiSeq™ platform alongside with bioinformatics analysis of these DNA sequences were performed by Dr Scott Dowd (see Materials and Methods, section 2.5.3). The results of 16S rRNA gene sequencing were received in the form of text files and the processed data in the form of FASTA files. The numbers and percentages of OTUs that were taxonomically assigned at kingdom, phylum, class, order, family, genus, and species level for each sample set were received in a separate text file. These data were transferred into an Excel spreadsheet by me for further analysis including sorting OTUs according to their percentages in each sample, removing OTUs with relative abundance of less than 1%, removing common contaminants that were identified based on negative control samples and literature screening, and finally presenting the processed data in bar charts and graph for further observational analysis. Initially, a pilot sequencing of 8 MEEFs from 4 patients with bilateral COME and 5 adenoids (two from patients with COME, and 3 without COME (controls)) was performed. These 8 MEEF samples in addition to 4 MEEFs from 2 other patients were also analysed using the traditional culture methods (see Table 4. 1). The bacterial profile identified in 8 MEEFs with 16S rRNA gene sequencing was greatly different from that identified using standard culture methods recommended by UKSMI (see Table 4. 1 and Figure 4. 9). Culture-negative samples (2R, 2L, 6R, and 6L) appeared to have a more diverse microbial profile when compared with culture – positive samples. It is likely that this was due to the high proportion of the contaminant DNA that was detected because of the low bacterial loads in these samples (see Table 4. 9). Therefore, common contaminant DNA were manually identified and removed based on the literature (Salter et al., 2014, Eisenhofer et al., 2019). In total, 14 different bacterial genera consisting of 18 separate species with a relative abundance of >1% were identified (see Figure 4. 9). *A. otitis* and *Pseudomonas tolaasii* were the most dominant

bacterial species, which were present in 4 out of 8 (50%) MEEF samples. *S. epidermidis* and *P. acnes* were the second most common species identified in 3 out of 8 (37%) of MEEFs. *H. influenza*e and *Mycoplasma pneumoniae* were present in 2/8 MEEF samples. Other bacterial species including *Prevotella* spp., *S. mitis*, and *S. petrasii* were less frequently present in MEEF specimens. The traditional culture methods for the initial pilot set of MEEF samples failed to identify the majority of the microorganisms present in these samples including fastidious or slow-growing bacteria such as *A. otitis, H. influenzae*, *P. acnes, M. pneumoniae,* and others. Furthermore, when 16S rRNA gene sequencing was used, the bacterial profiles in the culture positive bilateral MEEFs collected from the same patients were similar. For example, in patient 1, 3 out of 4 bacterial species identified were detected in both samples and they were dominated by *H. influenzae*, whereas in patient 6, *A. otitis* was the only dominant species in both MEEFs. Although *A. otitis, H. influenzae,* and *P. acnes* did not appear in the initial "pilot culturing" for the first set of MEEFs sample, these organisms did appear in the other sets of MEEF samples when 'improved' culturing methods were used (see Table 4. 3).

The microbiota of the adenoids consisted mainly of *Haemophilus* spp., *Fusobacterium* spp., *Prevotella* spp., *Neisseria* spp., *Streptococcus* spp., and *Gemella* spp. (see Figure 4. 10). In general, the microbial profiles of all adenoids were similar, but proportions of the microbial genera were different. The microbiota of adenoids was less diverse in patients with COME when compared with control adenoids from patients without COME, and *Haemophilus*, the classical otopathogen, was the most dominant genus with a relative abundance of about 70%. Interestingly, *H. influenzae* was detected in MEEFs of a patient (patient 1) whose adenoids were also concurrently colonised with an unidentified *Haemophilus* sp. (See Figure 4. 9 and Figure 4. 10). In conclusion, the pilot culture-independent analysis of MEEF samples showed that the traditional culture methods used here required improvement in order to improve the isolation of the species present. Additionally, the data showed that it is important to include negative controls during the process of DNA extraction from samples in order to confidently filter the background contaminant signals. Moreover, bilateral effusions from a single patient had similar microbiota. Therefore, in future the focus will be on analysing single MEEF samples from more patients. Finally, adenoids of patients with COME were dominated by otopathogens and to further investigate this, it was decided to analyse more matched adenoids and MEEF samples in future.

Following this pilot analysis, 12 unpaired MEEFs from 12 patients with COME and 5 adenoids (3 with COME, and 2 without COME (controls)) were analysed using 16S rRNA gene sequencing. A negative control of 1 ml of a sterile 0.9% saline solution that was

processed exactly the same way as MEEF samples was included. Again, culture-negative MEEFs and those with low microbial biomass showed high levels of contaminant sequences (data not shown). The negative control and common contaminant sequences were removed and only microbial genera or species with a relative abundance of >1% were included.

The most commonly identified genera in the MEEF samples were *Staphylococcus* (10/12 MEEFs), *Alloiococcus* (8/12 MEEFs), *Moraxella* (6/12 MEEFs), *Turicella* (5/12 MEEFs), and *Haemophilus* (3/12 MEEFs). Identification to the species level revealed that *S. epidermidis* was the most frequently detected *Staphylococcus* species, which was present in 6/12 (50%) samples, whereas *S. simulans*, *S. pasteuri*, *Staphylococcus pseudolugdunensis*, and *Staphylococcus* sp. were present in 4/12 (33.3%) of samples (see Figure 4. 11). *A. otitis*, *M. catarrhalis*, *Turicella* spp. were the only species identified in their corresponding bacterial genera. *H. influenzae* was the most dominant *Haemophilus* species present in 3/12 (25%) of samples. *Pseudomonas tolaasii* was not identified in this set of MEEF samples though it was one of the dominant species identified in the pilot set of MEEFs. It was identified in MEEF samples that showed higher rate of contaminant DNA (60-85%) see Table 4. 9.

Pseudomonas tolaasii is a gram-negative soil bacterium that is the most common causative agent of brown bacterial blotch on several species of edible cultivated mushroom and it is not regarded as a pathogen of humans(Lo Cantore et al., 2015). Therefore, it is likely that DNA from this species was a contaminant from environmental sources. In agreement with this hypothesis, *Pseudomonas tolaasii* was never cultured from clinical samples. Further investigations are required to determine whether it is genuinely present in human middle ear infections. *Burkholderia pyrrocinia* was also detected in 3/12 (25%) of samples with a relative abundance of 3-5%. The majority of MEEFs were dominated by a single species, with *A. otitis*, *M. catarrhalis*, *T. otitidis*, or *H. influenzae* at a relative abundance ranging between 58-97%. Comparison of the bacterial species identified using the improved culture methods and 16S rRNA gene sequencing showed that the improved culture methods were successful in isolating the majority of the representative microorganisms present in MEEF samples (see Figure 4. 11). *S. epidermidis*, *S. simulans*, *Staphylococcus* sp. (very likely to be *S. auricularis* basing on comparison with culture results), and *T. otitidis* were identified using both methods. *A. otitis* was successfully cultured in 5 out of 8 MEEF samples in which *A. otitis* was identified at relative abundance of >1%. However, the improved culture failed to isolate *A. otitis* in two MEEFs (28L and 31R) where *A. otitis* was detected at relative abundance of less than 3%, possibly due to its low relative abundance in these samples. Other bacterial species

that were also identified with both techniques were *N. flavescens* (1/1 MEEF), *S. pneumoniae* (1/1 MEEF), and *P. acnes* (1/2 MEEFs).

M. catarrhalis was the most common bacterial species that the improved culture methods failed to isolate, and it was cultured in 2 out of 6 MEEF samples, which may be due to low oxygen growth conditions. Other microorganisms that were not cultured with the improved culture methods were *B. pyrrocinia, Mycobacterium brisbanense, Corynebacterium kroppenstedtii, S. pseudolugdunensis, Haemophilus haemolyticus, S. pasteuri,* and *Peptoniphilus asaccharolyticus*. Most of these difficult-to-culture bacterial species were identified by 16S rRNA gene sequencing in culture-negative MEEF samples (see Figure 4. 11).

To study the hypothesis that adenoids act as a reservoir for otopathogens in patients with COME, the microbiota identified in 3 matched MEEFs and adenoid samples of patients with COME were compared (Figure 4. 12). In general, the microbiota of adenoids showed more diverse microbial profiles that included many species present in MEEFs. Again, the core microbiota of adenoids commonly consisted of *Haemophilus* spp., *Prevotella* spp., *Streptococcus* spp., *Veillonella* spp., and *Porphyromonas* spp. A total of 11 OTUs (operational taxonomic units) were identified in both sample types, of which 9 OTUs including *H. influenzae, S. pneumoniae, P. acnes, and M. catarrhalis* were detected in both matched sample types from 3 patients. There were 6 unique OTUs identified in these 3 MEEFs samples. These were *Staphylococcus* sp., *Turicella* sp., *S. pasteuri, S. pseudolugdunensis, Peptoniphilus asaccharolyticus,* and *Flavobacterium* sp. To assess whether the bacterial species concurrently colonising both middle ear and adenoid of COME are genetically identical, matched adenoid and MEEF samples from one patient (patient number 20) were analysed using traditional culture methods. Bacterial species isolated from matched samples are listed in Table 4. 10.

Interestingly, strains of *S. oralis* and *S. parasanguinis* were isolated from both samples (adenoid and MEEF) of the same patient. Unfortunately, both adenoid and MEEF samples of this patient were not analysed using 16S rRNA gene sequencing with the other matched samples mentioned in Figure 4. 12. Therefore, none of 9 OTUs, that were identified in both matched adenoids and MEEF samples, appeared in Table 4. 10 Bacteria isolated from matched MEEF and adenoid of one patient with COME.. To assess the genetic relatedness between these isolates, full genome sequencing was performed. Genomic comparison using Mauve multiple genome alignment software showed that they were not genetically identical: there were ~150,000 single nucleotide polymorphisms (SNPs) between the two *S. oralis*

strains and ~300,000 SNPs between the strains that were labelled '*S. parasanguinis*'. In fact, one of the *S. parasanguinis* was re-classified as *Streptococcus australis* on the basis of the genome sequence indicating that MALDI-TOF MS is good for identification of the bacterial species, but it is not 100% accurate for closely related species such as oral streptococci.

Table 4. 8 Concentrations of DNA extracted from MEEF and adenoids samples.

Sample code	Concentration of DNA (ng μl^{-1})
1R	45.4 (1.8)
1L	22.7 (1.8)
2R	3.2 (2.6)
2L	2.1 (2.6)
3R	8.6 (1.7)
3L	37.7 (1.8)
6R	1.4 (2.1)
6L	6.8 (1.6)
22R	103 (1.83)
23L	100 (1.81)
25R	12.2 (1.8)
26L	17 (1.6)
27R	354 (1.87)
28L	9.2 (1.57)
29R	120.9 (1.83)
30L	113 (1.85)
31R	49 (1.76)
32R	9.2 (1.42)
33R	83 (1.73)
34L	75 (1.86)
Control (NS)	10 (1.35)
1*	115.2 (1.8)
2	165.5 (1.8)
3*	282.3 (1.8)
4	149.7 (1.8)
5	257.3 (1.8)
6	115.2 (1.8)
7	60.9 (1.88)
30*	19 (1.74)
31*	110 (1.89)
33*	53 (1.9)

(*) Adenoid samples from patients with COME. (NS) 0.9% normal saline solution.

Table 4. 9 List of contaminant microbial species identified in the sequenced set of MEEF samples.

Contaminant Species	1R	1L	2R	2L	3R	3L	4R	4L
Acidovorax temperans	4.5	11.8	31.4	13.9			33.0	3.3
Mesorhizobium sp.			29.0					
Sphingomonas paucimobilis	2.5	5.5	18.4				14.9	
Methylobacterium sp.			2.3	3.8				10.3
Chitinophaga spp.			1.8					
Delftia spp.			1.6	14.0				3.7
Gordonia polyisoprenivorans			1.1					
Riemerella columbipharyngis								11.4
Curvibacter spp.								5.8
Acetobacter senegalensis								5.0
Agrobacterium rhizobium rhizogenes							2.5	
Ochrobactrum intermedium							1.8	
Sphingomonas koreensis							1.8	
Nocardioides aquaticus				7.2				
Acetobacter aceti								1.7
Paenibacillus provencensis								1.0
Reyranella spp.							2.8	
Stenotrophomonas sp.				6.8				
Massilia timonae								3.3
Paenibacillus sp.				4.2				1.7
Ralstonia pickettii				9.8				
Geobacillus spp.							5.2	
Chryseobacterium hominis							3.0	
Pseudomonas syringae								2.3
Halomonas sp.				2.2				
Bacillus pumilus								1.7
Bacillus simplex								2.1
Prevotella ruminicola	1.4							
Corynebacterium vitaeruminis								9.2
Total (%)	8.3	17.3	85.6	61.9	0.0	0.0	65.1	62.6

Table 4. 10 Bacteria isolated from matched MEEF and adenoid of one patient with COME.

Species	MEEF	Adenoid
Streptococcus intermedius	-	+
Streptococcus parasanguinis	+	+
Streptococcus salivarius	-	+
Streptococcus oralis	+	+
Rothia mucilaginosa	-	+
Staphylococcus aureus	-	+
Actinomyces odontolyticus	-	+
Streptococcus anginosus group	-	+
Streptococcus pneumoniae	-	+
Streptococcus mitis	+	-
Alloiococcus otitis	+	-
Staphylococcus pastueri	+	-

Various bacterial species were isolated from adenoid than that from MEEF. Similar strains of *Streptococcus parasanguinis* and *Streptococcus oralis* were isolated from the adenoid and MEEF of the same patient.

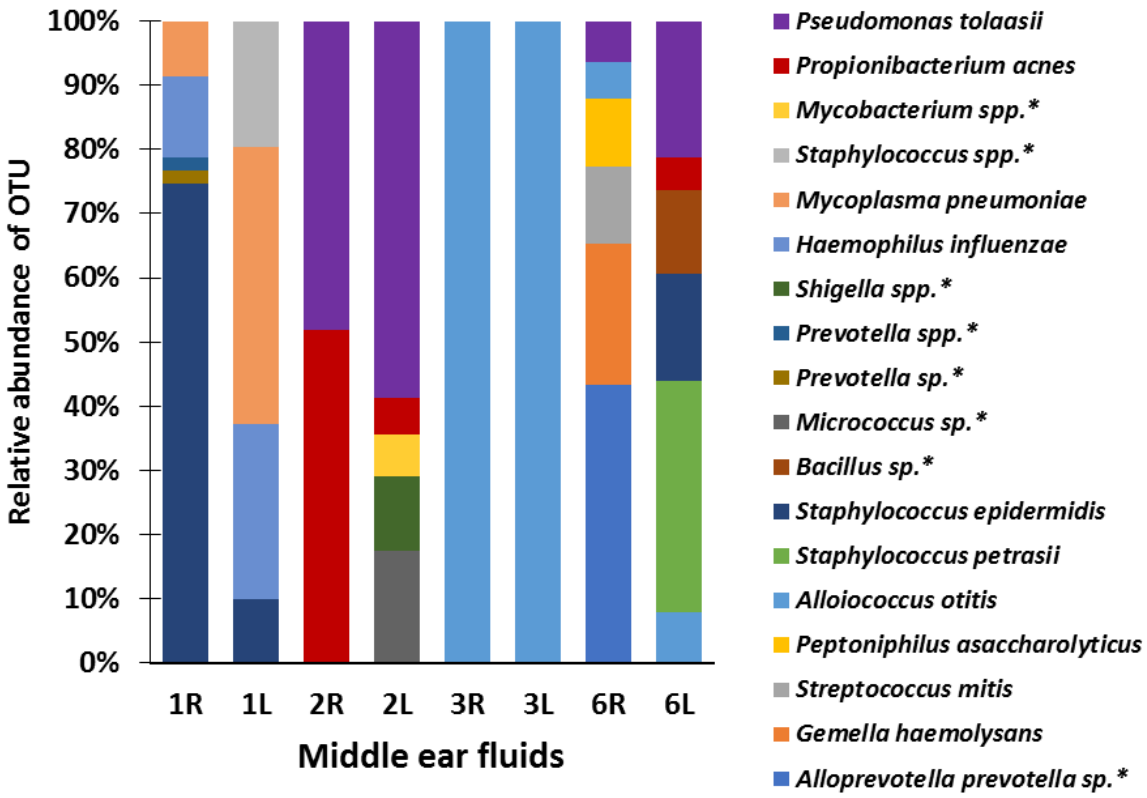


Figure 4. 9 The microbiota present in the pilot set of MEEF samples of patients with COME.

Data represent OTUs that were present at a relative abundance (%) of ≥ 1% in at least one sample. Taxonomic assignment of each OTU is at species level, except in case where OTUs were not identified below the genus level (*). Microbial communities of bilateral MEEFs are similar and consisted mainly of *Alloiococcus otitis*, *Pseudomonas tolaasii*, and *Staphylococcus epidermidis* in addition to other bacterial species.

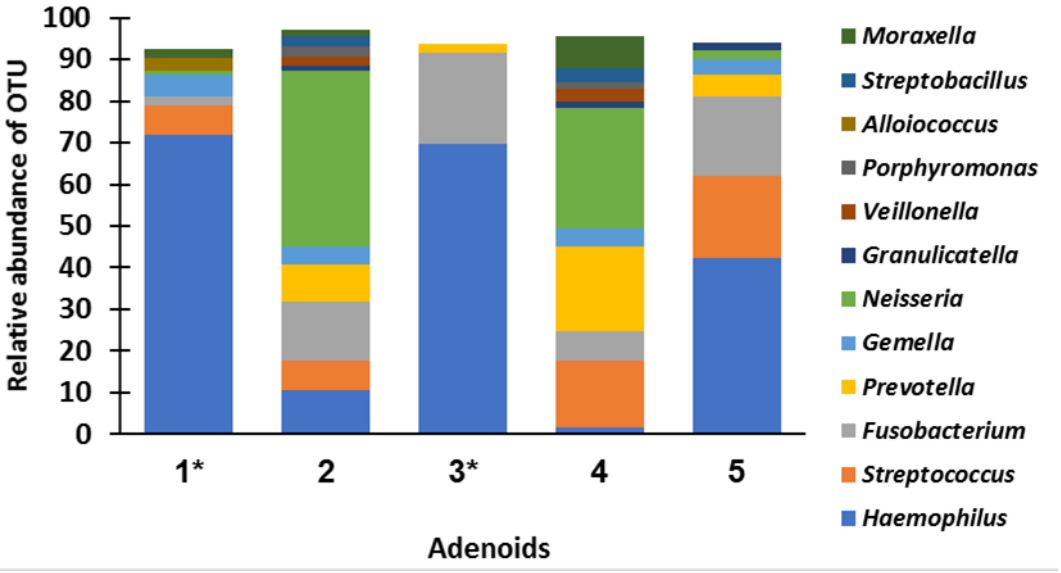


Figure 4. 10 The microbiota present in the pilot set of adenoid samples derived from patients with COME (*) and without COME.

Data represent OTUs that were present at a relative abundance (%) of ≥ 1% in at least one sample. Taxonomic assignment of each OTU is at genus level. Microbial profiles of adenoids were similar, less diverse in patients with COME (*) when compared with control adenoids without COME and they were mostly dominated by *Haemophilus sp*.

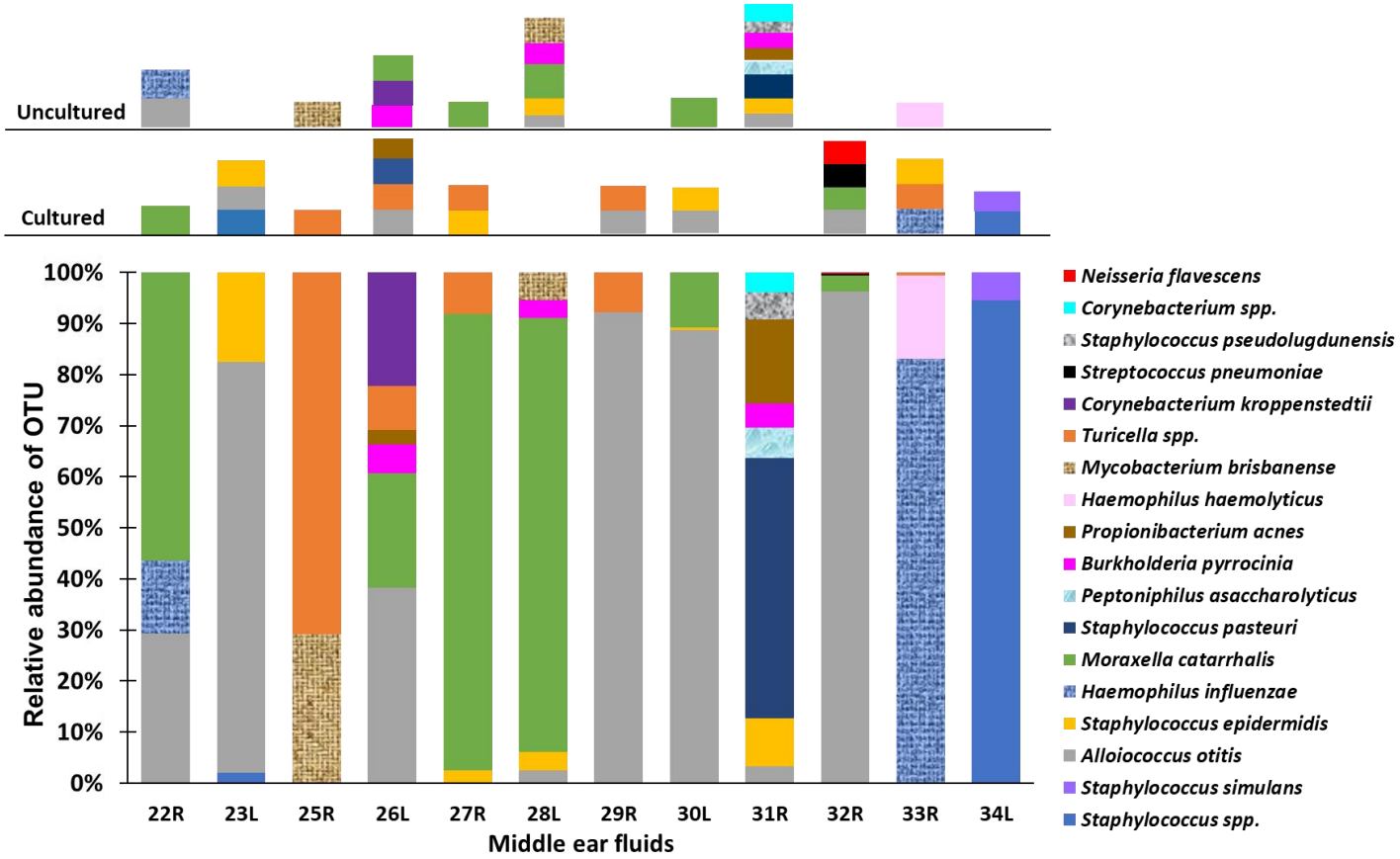


Figure 4. 11 Characterisation of bacterial populations present in MEEFs of patients with COME using both 16S rRNA gene sequencing and the improved culture methods.

The bar chart shows the bacterial taxa identified per MEEF sample compared with bacterial species cultured and isolated by the improved culture methods. Data represent OTUs that were present at a relative abundance (%) of ≥ 1% in at least one sample. Taxonomic assignment of each OTU is at species level. *Alloiococcus otitis*, *Moraxella catarrhalis*, *Turicella spp.*, and *Staphylococcus epidermidis* were the most frequently detected species from MEEFs and the "improved" cultured method was successful in isolating the majority of representative bacteria present in MEEFs when compared to those identified by 16S rRNA gene sequencing.

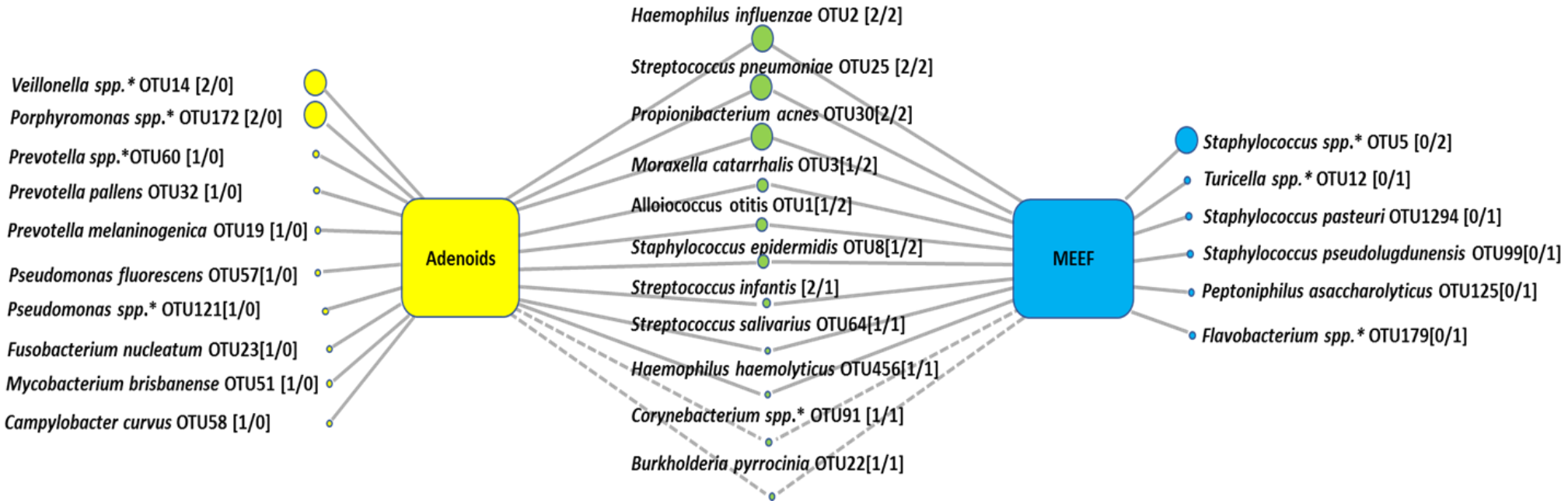


Figure 4. 12 Comparison of microbiota present in 3 matched MEEF and adenoids samples of patients with COME.

Taxonomic assignment of each OTU is at species level, unless the OTU was not classified below the genus level (*). OTUs with ≥ 1% relative abundance in at least on sample were included in visual differentiation between both types of samples. Circle sizes and bracketed numbers [adenoid/MEEF] represent the number of samples that contain the presented OTU. Similar OTUs are presented in the middle and matched by a solid line when the identified OTU in both samples was obtained from the same patient or by a dashed line if it is derived from different patient. The figure shows that matched adenoids and MEEFs of patients with COME were concurrently colonised with 9 similar OTUs that were dominated mainly by classical otopathogens (*Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis*).

4.9 Discussion

In this chapter, the study aimed to characterise bacterial populations present in the MEEFs of COME patients. Bacterial species present in the MEEFs of patients with COME were isolated using conventional culture methods and the potential impact of culturing techniques on the isolation of different types of microorganisms was explored. The efficacy of NucB to improve the culture of bacteria from MEEFs was also investigated and finally 16S rRNA gene sequencing of MEEFs and adenoid microbiota was used to verify the efficacy of the culture methods to capture the correct microorganisms present in MEEFs of COME patients and to explore the potential co-occurrence of COME-related bacteria in the middle ear and adenoids of the same patients.

Initially, the pilot analysis of MEEF samples derived from 6 patients with bilateral COME using the standard culture methods recommended by the UK Standards for Microbiology Investigations (UKSMI) for ear infections and associated specimens was not successful in capturing most of the fastidious microorganisms, including *A. otitis*, *H. influenzae*, *M. pneumoniae*, and *P. acnes* that were identified in the same sample set using 16S rRNA gene sequencing. For this reason, several modifications to culture methods were applied to improve capturing of fastidious and slow-growing bacterial species. These modifications included immediate processing of the MEEF samples within 1 h following sample collection, culturing larger volumes of up to 50 µl of MEEF sample per growth medium used, extending the incubation period of the MEEF samples culture at 37˚C with 5% CO_2 to up to 2 weeks, and adopting stringent sterilisation precautions to reduce contamination. The percentage of culture positive MEEFs increased from just under 58% to 90.5% using the improved traditional culture methods of this study. The most frequently isolated microorganisms were *T. otitidis (*52.4%), *A. otitis* (38.1), and CoNS (76.2%) including *S. epidermidis* (28.6%), and *S. auricularis* (19%).

The exact role of *T. otitidis, A. otitis*, and CoNS in the aetiology of COME is still debated. They are thought to be part of the commensal microbiota that may be translocated from external ear canal to the middle ear cavity following tympanic membrane perforation (Chan et al., 2017b, Harimaya et al., 2006, von Graevenitz and Funke, 2014). On the other hand, *A. otitis*, and *T. otitidis* are the most prevalent bacterial species repeatedly identified in COME patients, and there is evidence that *A. otitis* has a considerable immune stimulating ability, at least *in vitro* (Himi et al., 2000, Harimaya et al., 2007). Furthermore, *A. otitis* has a capability to produce both single-species and polymicrobial biofilms with *H. influenzae.*

When *A. otitis* was co-cultured with *H. influenzae* in multi-species biofilms, both survival and persistence of *H. influenzae* were enhanced compared with monospecies controls (Chan et al., 2017a). As with other studies, CoNS, *S. auricularis,* and *S. aureus* were also the frequently isolated microorganisms from most MEEF samples (Daniel et al., 2012b, Yoo et al., 2018). Most of these CoNS have an ability to produce biofilms and have a recognised pathogenic role in medical implant-associated infections, and possess genetic and phenotypic features that would allow them to form biofilms *in vivo*, highlighting their pathogenic potential to induce otitis media (Paluch-Oles et al., 2011).

The traditionally-considered otopathogens *H. influenzae, M. catarrhalis, and S. pneumoniae* were found in only 19% of MEEF samples (9.5%, 4.8%, and 4.8% respectively). These pathogens have been long considered to be the most common species implicated in the aetiology of COME (Holder et al., 2015, Lundgren and Rundcrantz, 1976). Our findings are broadly consistent with the other observations that found *H. influenzae* in 3-9%, *M. catarrhalis* 3-3.8%, and *S. pneumoniae* in around 6.5% of samples (Papp et al., 2016, Daniel et al., 2012b). However, the findings of this study are contrary to those of Van Hoecke et al. (2016) who cultured *H. influenzae* in 35% of samples. It is likely that the difference is due to the use of both aerobic and anaerobic conditions for incubation of chocolate agar in the study by Van Hoecke *et al*. This approach was not used here because it was considered more appropriate to employ the standard culture methods recommended by UKSMI for ear infections and associated specimens in choosing types of growth media and growth conditions. The current study used a variety of different types of agar designed to isolate a broad range of species. It should be noted that there is no perfect culture method that can accurately isolate all the representative bacteria present in MEEFs of COME patients. Indeed, many other microorganisms that were cultured in this study were overlooked in Van Hoecke *et al's* paper, which exclusively employed Chocolate agar for microbial isolation. Other less frequently identified microorganisms including *S. oralis, S. pyogenes, S. mitis, S. salivarius, P. acnes, A. odontolyticus* and *N. flavescens* were also less frequently identified in most other studies that used traditional culture methods (Daniel et al., 2012b, Yoo et al., 2018, Poetker et al., 2005).

Although only 21 MEEF samples were analysed using the improved culture methods, the proportion of culture positive MEEFs (90.5%) was higher compared to other studies, where up to 60% of samples were positive for at least one type of bacteria (Poetker et al., 2005, Daniel et al., 2012b, Gok et al., 2001, Papp et al., 2016). Nevertheless, our finding is in agreement with that of Daniel et al. (2012b) who found live bacteria in more than 90% of

MEEF samples from children with COME using extended culture techniques and CLSM imaging. Although the MEEFs were cultured in the present study for a longer duration (with the aim to capture fastidious and slow-growing bacteria) compared to that in the standard culture approach used in diagnostic laboratories, it is unlikely that these results represent contamination as utmost care was taken during MEEF sample collection to avoid contact with the external ear canal. For example, the suction catheter used occasionally to clean external ear canal wax was exchanged with a new sterile suction catheter to aspirate MEEFs following myringotomy. Furthermore, the range of bacterial species identified in this study is similar to those identified in the other studies where EEC was decontaminated with 70% ethanol prior to MEEF sample collection (Daniel et al., 2012b, Papp et al., 2016). The most likely explanation for the higher number of culture positive MEEFs in this study is the adoption of the improved culture approaches as previously described, which were successful in culturing a wide range of microorganisms including fastidious and slow growing bacterial species such as *T. otitidis, A. otitis, H. influenzae, A. odontolyticus,* and *P. acnes*. These may have been missed by other studies adopting a more limited conventional culturing approach. The higher culture rate here could also be explained by the younger age of patients included in this study (the majority were below 10 years old) in comparison to previous studies that also recruited adults and older children. It has been shown that the proportion of culture positive MEEF samples was much higher in younger children than those in adults with COME (Gok et al., 2001, Poetker et al., 2005). Furthermore, NICE guidelines in UK for the treatment of COME recommend drainage of MEEF and grommet tube insertion in patients with persistent COME following 6 months of watchful-waiting during which no systemic antibiotics are used. The lack of antibiotic usage by these patients may also explain the greater number of culture positive MEEF samples compared to other studies where antibiotics were used before MEEF sample collection (Gok et al., 2001, Poetker et al., 2005).

Based on the hypothesis that bacteria associated with COME are present within biofilms, the efficacy of NucB in disturbing these biofilms and improving the recovery of bacteria from MEFFs was investigated. The results obtained from the pilot set of MEEF samples treated with NucB showed inconsistent efficacy of NucB at improving the recovery of bacteria from these samples. This finding may be explained by the addition of NucB with inconsistent activity and also lack of appropriate control MEEF samples that were treated exactly the same as NucB treated samples (see Table 4. 4 and Table 4. 5). Addition of a constant amount (mass) of NucB to each sample does not necessarily mean addition of NucB with a constant nuclease activity. In this study, nuclease activity of NucB was shown to be

different between different preparations of NucB and was affected by storage of NucB including repeated freezing and thawing (data not shown). Following this, methods were optimised to measure NucB specific activity in order to be able to add consistent amount of NucB in the subsequent experiments. Consistent with a previous study, NucB was able to completely degrade high molecular weight CTDNA into low molecular weight digestion products within 60 minutes (Basle et al., 2018). However, the calculated specific activity of NucB in this study (1.4×10^{-5} units mg^{-1}) was approximately 44% less than that calculated by Basle et al. (2018)(2.5×10^{-5} units mg^{-1}). Furthermore, semi-quantitative analysis of NucB nuclease activity in 0.9% saline solution and TSB medium showed that NucB had retained approximately 50% of its activity compared to its activity in the optimal buffer.

Overall, NucB did not produce a significant increase in the total number of microorganisms recovered on blood or FA agar compared to control MEEFs with no NucB, even though 20% of samples treated with NucB showed a 2-fold or more increase in the number of bacteria cultured on blood agar in comparison with the negative controls. The finding in this study is contrary to that of Shakir et al. (2012) who found that NucB was effective in releasing higher number of microorganisms from fouled Tracheoesophageal speech valves (TESVs) compared to PBS treated controls. A possible explanation for this might be due to the low biomass of bacterial biofilms present in the MEEF compared to the higher load of microbial biofilms present on the surface of TESV. Another possible explanation is that there was a degree of sample heterogenesity when MEEFs were divided between different treatment groups, and so bacterial biofilms might be localised to one part of the MEEF. Surprisingly, incubation of MEEF samples in the absence of NucB for 1 h at 37˚C aerobically was associated with a significant reduction ($p = 0.007$) in the total number of viable bacteria cultured under 5% CO_2 and anaerobic growth conditions compared to control MEEFs that were cultured immediately without aerobic incubation for 1 h. A possible explanation for this finding might be that a low oxygen conditions, which resemble the natural environment of the middle ear cavity in patients with COME, is the favourable environment for the survival of microorganisms present in MEEFs. Thus, incubation of microorganisms aerobically adversely affected their viability or their ability to regrow on culture media. This is consistent with clinical practice, where grommet insertion frequently resolves middle ear effusion in patients with COME by improving middle ear ventilation. It should be noted that there was no statistically significant reduction in total viable counts recovered from the biofilm when MEEFs were incubated in the presence of NucB for 1 h at 37˚C compared with controls that were cultured immediately ($p = 0.08$). It is possible that the

negative impact of incubation on recovery of microorganisms was counteracted to some extent by the action of NucB in releasing microbial cells from biofilms. Therefore, future studies should focus on employing shorter incubation times with NucB to optimise the benefits of NucB treatment while minimising problems associated with extended processing times.

To verify the efficacy of traditional culture methods in identifying the bacterial populations present in the MEEFs of patients with COME, 16S rRNA gene sequencing was also used to study microbiota present in these MEEFs. The results showed that the traditional culture methods recommended by UKSMI was not successful in culturing most of the microorganisms representing members of bacterial communities present in MEEFs compared to the 16S rRNA gene sequencing data of the same samples. Thus, the culture methods were subsequently optimised. Also results from the pilot set of samples showed that culture-negative MEEFs, which are likely contain low microbial biomass, had higher proportions of DNA from environmental bacteria compared to culture-positive samples (see Table 4. 9). This finding is in accord with recent studies indicating that contaminanting DNA from environmental sources is common in microbiome studies involving low microbial biomass samples and have a significant negative impact on the interpretation of microbiome data (Salter et al., 2014, Eisenhofer et al., 2019). In general, two kinds of contamination can be introduced in microbiome studies: contaminant DNA and cross-contamination (Eisenhofer et al., 2019). Contaminant DNA can arise from different sources despite stringent precautions and care during sample collection and preparation, including sampling and laboratory environments, human commensals on laboratory workers, laboratory consumables, DNA extraction kits and laboratory reagents. On the other hand, cross-contamination arises when DNA is transferred from other samples and sequencing runs from adjacent wells or tubes, resulting in ‘batch-effects’ during microbiome sample processing. To date, more than 60 common contaminant taxa have been detected in DNA extraction blank controls and no-template controls in many microbiome studies. Most of the contaminant taxa, that were identified and removed in this study (see Table 4. 9), were listed within these 60 common contaminant taxa and the majority of these taxa were soil, water, or non-human microorganisms (Eisenhofer et al., 2019, Salter et al., 2014). The presence of low levels of microbial DNA within low microbial biomass samples including MEEF and blood can result in the signals generated by contaminant DNA and cross-contamination to be perceived as biological signals since the contaminant sequences can easily dominate the true biological signals within the samples. It is important to use negative sequencing controls concurrently

with samples in these cases.Therefore, a DNA extraction blank control (negative control) was included when the second set of MEEFs was processed for16S rRNA gene sequencing. The dominant bacterial species identified in 3 out of 4 patients with bilateral COME, were similar, and there was a high degree of similarity in the overall composition of the bacterial communities identified (see Figure 4.9). Similar findings have also been reported in another microbiome study of bilateral COME, although the comparison was limited to the genus level (Jervis-Bardy et al., 2015). Therefore, it was decided to focus on analysing unpaired MEEF samples from more patients.

Species-level microbiome analysis of 12 unpaired MEEFs showed that the improved culture techniques adopted in this study in combination with MALDI-TOF MS were successful in capturing most of the representative members of bacterial communities identified in MEEFs by 16S rRNA gene sequencing, including *A. otitis, T. otitidis, S. pneumoniae, H. influenzae,* and most *Staphylococcus* spp. including CoNS. *M. catarrhalis* was the most common bacterial species that was identified by 16S rRNA gene sequencing and not by the improved culture methods. In the present study, MEEFs were cultured under 5% CO_2 and anaerobic conditions only, which is not well suited to culture the obligate aerobe *M. catarrhalis*. which may explain the low number of *M. catarrhalis* cultured in these samples. Other less frequently identified bacteria such as *B. pyrrocinia, M. brisbanense, C. kroppenstedtii, H. haemolyticus,* and *P. asaccharolyticus* were also not cultured by the improved culture methods. Indeed, it has been estimated that below 2% of bacteria present on the environment can be cultured on the artificial culture media, though the percentage of culturable bacteria present in human body is higher (Wade, 2002). Nevertheless, traditional culture methods combined with MALDI-TOF MS are still considered a convenient microbiological technique to identify bacterial isolates because they are quick and cost-effective (Navrátilová et al., 2016).

Of 12 MEEFs analysed by16S rRNA gene sequencing, *A. otitis, S. epidermidis, M. catarrhalis, T. otitidis,* and *H. influenzae* were the most commonly detected bacterial species, and were present in 66%, 50%, 50%, 41.6%, and 25% of samples, respectively. Approximately 75% of MEEF samples were dominated by one of four species: *A. otitis, M. catarrhalis, H. influenzae,* and *T. otitidis,* which were present at 58-97% relative abundance. Similar bacterial profiles have also been reported in other recent microbiome studies of COME, such as Boers et al. (2018), who detected *Alloiococcus* spp., *Turicella* spp., *Haemophilus* spp., and *Staphylococcus* spp. in 63%, 57%, 47%, and 36.8% of MEEF samples respectively. Furthermore, Jervis-Bardy et al. (2015) also detected *Alloiococcus* spp.,

Turicella spp., *Haemophilus* spp., *Staphylococcus* spp. in 63%, 27%, 63%, and 27% of samples respectively. In this study, bacterial communities dominated by *Alloiococcus* spp., or *Haemophilus* spp. were also detected at relative abundances ranging between 57-95% in 90% of MEEF samples from patients with COME (Jervis-Bardy et al., 2015). *S. pneumoniae*, the third most common otopathogen previously implicated in the aetiology of COME following *H. influenzae*, and *M. catarrhalis*, was found in only 16.7% (2 out of 12) of samples. This finding is consistent with the other microbiome studies of COME that detected *Streptococcus* spp. in 9-31.5% of samples. Variations in *S. pneumoniae* prevalence between studies could be due to the pattern of vaccination that has be introduced against *S. pneumoniae*. A recent systematic review, assessed the global prevalence of the three common pathogens implicated in otitis media, and found that patterns of *S. pneumoniae* colonisation have changed in response to the introduction of vaccination (Ngo et al., 2016). However, the microbiome identified in the present study was different from previous reports to some extent. For example, *M. catarrhalis* was detected in higher numbers of the MEEF samples here (50%) compared to other studies, where *M. catarrhalis* was identified in only 5-18% of samples (Boers et al., 2018, Jervis-Bardy et al., 2015).

Finally, correlations between the microbiome of adenoid tissue and the microbiome present in the MEEFs of patients with COME was investigated. Consistent with the other studies, higher bacterial diversity was detected in adenoids compared to MEEF samples (Boers et al., 2018, Jervis-Bardy et al., 2015). In general, the core microbiota of adenoids from patients with or without COME consisted of mainly *Haemophilus* spp., *Prevotella* spp., *Streptococcus* spp., *Fusobacterium* spp., *Veillonella* spp., and *Porphyromonas* spp. This finding is in agreement with previous studies that showed similar bacterial profiles on adenoids or nasopharyngeal swabs of patients with COME (Boers et al., 2018, Chan et al., 2017b, Jervis-Bardy et al., 2015). Comparison of the microbiome present in 3 matched adenoids and MEEF samples from 3 patients with COME showed both matched sample types were concurrently colonised with similar bacterial OTUs consistent with common paediatric otopathogens such as *H. influenzae, S. pneumoniae,* and *M. catarrhalis*. Other bacterial species including *P. acnes, A. otitis, S. epidermidis, S. infantis, S. salivarius*, and *H. haemolyticu*s were also identified in both sample types. This finding is consistent with several other studies (Boers et al., 2018, Chan et al., 2017b, Jervis-Bardy et al., 2015).

However, many bacterial species were specific to one or other anatomical site or were present at markedly different proportions of the total microbiome. For example, *A. otitis* was detected on MEEF and adenoid tissue of 1 patient, at a relative abundance of 90% and 1.2%

respectively. *A. otitis* has also been identified in the nasopharyngeal swabs of patients with COME (Harimaya et al., 2006). In a different study, *Alloiococcus* spp., *Staphylococcus* spp., and *Pseudomonas* spp. were detected in higher abundance in the EEC of patients with COME than in healthy volunteers but they were absent in adenoids, suggesting that the EEC can serve as a reservoir for middle ear pathogens (Chan et al., 2017b). In this study, *T. otitidis*, *A. otitis*, and *Staphylococcus* spp. that were detected in higher number of MEEF samples might also have been introduced to the middle ear cavity from the EEC. It is estimated that around 80% of children will have at least one attack of acute otitis media by the age of 3 years (Teele et al., 1989), and around 30% of these children are reported to have tympanic membrane rupture (Berger, 1989). This episode could be responsible for the introduction of EEC microorganisms shown in the present study. Patients with previous history of grommet insertion were excluded from our study. However, history of previous episodes of AOM and tympanic membrane perforation was not obtained from our patient group. Furthermore, the relative abundance of *Alloiococcus* was significantly higher in patients with a previous history of grommet tube insertion than those without, which increases the possibility of translocating EEC microorganisms to the middle ear cavity (Chan et al., 2017b). Many EEC bacterial flora such as *S. aureus* and *P. aeruginosa* are reported to induce middle ear infections through the perforated tympanic membrane in patients with chronic suppurative otitis media (Mittal et al., 2015). In contrast to *S. aureus* and *P. aeruginosa,* the role of *A. otitis*, *T. otitidis*, and CoNS in the aetiology of COME remains unclear. Further investigations are needed to elucidate their roles in the pathogenesis of COME. Culture analysis of matched MEEF and adenoid samples obtained from the same patient successfully identified *S. oralis* and *S. parasanguinis* that were present in both the adenoid and the middle ear. Genomic comparison of these similar strains showed that they were not genetically identical. In fact, one of the *S. parasanguinis* strains was re-classified as *Streptococcus australis* on the basis of the genomic DNA sequence. This indicates that MALDI-TOF MS identified three of the four oral *Streptococcus* spp. correctly, but failed to identify *S. australis*. Accurate species identification within viridans group *streptococc*i and more specifically within the mitis subgroup is traditionally difficult. It has also been shown that MALDI-TOF MS is not always accurate in species level identification of oral *streptococci* and specifically within the Mitis group (Angeletti et al., 2015). Overall, there was no evidence that precisely the same strains were located in the adenoid and middle ear of a single patient based on the very limited sampling and analysis presented here. The role of adenoids as a reservoir for otopathogens needs further analysis in larger patient cohorts using both traditional culture methods and full genome comparisons.

In conclusion, the improved culture methods adopted in this study were able to detect a wide spectrum of representative bacterial species present in MEEFs of COME patients compared to the conventional culture approach. The presence of these polymicrobial species in the MEEFs of COME may play a crucial role in the aetiopathogenesis of COME. In accordance with the evidence that bacteria associated with COME are present within biofilms and that NucB can disrupt biofilms both alone and in combination with antibiotics, it will be important next to determine whether NucB is effective against *in vitro* biofilms of the representative isolates of COME.

Chapter 5. Efficacy of NucB against biofilms of COME isolates

5.1 Introduction

Bacterial biofilms have been identified in the majority of COME patients, suggesting that biofilms may play a key role in the aetiopathogensis of the disease (Daniel et al., 2012b, Hall-Stoodley et al., 2006, Thornton et al., 2011, Van Hoecke et al., 2016). The otopathogens clasically associated with COME are *H. influenzae*, *S. pneumoniae* and *M. catarrhalis*. All have the ability to form single and multispecies biofilms *in vitro* and *in vivo* (Hall-Stoodley and Stoodley, 2009, Starner et al., 2006, Bakaletz, 2012, Silva and Sillankorva, 2019). Biofilm is defined as surface-associated microbial communities that are embedded in a self-produced matrix, consisting of macromolecules such as polysaccharides, lipids, proteins, and nucleic acids (Flemming et al., 2016). Bacteria within biofilms have evolved a range of complex protective mechanisms against hostile external insults. Therefore, they are often more resistant to antibiotics, cellular and humoral immune actions than planktonic cells of the same species (Gu et al., 2014, de la Fuente-Nunez et al., 2013). Increasing evidence has accumulated that eDNA is a ubiquitous structural component in the EPS of biofilms formed by many Gram-positive and Gram-negative bacteria (Jakubovics et al., 2013, Okshevsky et al., 2015). The eDNA plays a critical role in maintaining the structural integrity of biofilms, facilitating initial adhesion of bacterial cells, protecting biofilms against antimicrobial and immune actions, facilitating genetic material exchange, and acting as a nutrient source.

The current treatment options for persistent COME involve myringotomy and grommet insertion or hearing aids in patients who are unfit for surgery. Occasionally, insertion of grommets is combined with adenoidectomy in patients with recurrent or persistent upper respiratory tract infections (NICE, 2016, Rosenfeld et al., 2016). However, the current treatment options for COME are far from ideal. Several complications have been reported with grommet insertion such as purulent ear discharge, tympanic membrane (TM) scarring, and permanent TM perforation. In addition, further grommet insertion is required in about 25% of cases within 2 years (Kubba et al., 2000, Vlastarakos et al., 2007, Rosenfeld et al., 2013). This significant rate of recurrence is consistent with the essential role of bacterial biofilms in the pathogenesis of COME because grommet insertion likely will only drain MEEF that is produced secondary to the middle ear mucosal inflammation and will not treat the underlying biofilm, which may subsequently grow again following the grommet extrusion (Daniel et al., 2012b).

The possibility that biofilms are central to the pathogenesis of COME opens potential new avenues for the development of better treatment approaches. With the realisation that eDNA plays critical roles in bacterial biofilms, targeting eDNA within the biofilm matrix by DNases could potentially provide a new strategy to reduce the present high rate of revision surgery required for COME patients.

5.2 Aims and objectives

Work presented in the previous section clearly showed the presence of multiple species of bacteria in the MEEFs of patients with COME. In accordance with the increasing evidence that bacteria present in COME are organised in biofilms, the aim of this chapter was to investigate the sensitivity of *in vitro* biofilms of representative COME isolates to treatment with the DNase NucB from *B. licheniformis*. The objectives were as follow:

1. Assess biofilm formation capacity of representative species isolated from COME.
2. Test the efficacy of NucB to disperse and inhibit *in vitro* biofilms formed by COME isolates.
3. Analyse changes in the structure of biofilms formed by the selected NucB-sensitive COME isolates using CLSM imaging.

5.3 Biofilm formation of COME isolates

The biofilm formation capacity of 23 bacteria, isolated from MEEFs of patients with COME, were investigated. These bacteria were selected as representative strains of most bacterial species that were isolated from MEEF aspirates including the most frequently detected bacterial species and the classical otopathogens implicated in COME (see Table 5. 1). *Alloiococcus otitis* strains were excluded from this analysis because they did not grow on rich liquid culture media such as THYE or BHYE. Bacteria were cultured in a 96-well MTPs and the extent of biofilm formation was assessed using the CV staining assay (see Materials and Methods 2.8.1). The cut off absorbance (A_{570C}) for biofilm formation was defined as the mean absorbance (A_{570}) plus three times the standard deviation (SD) of a negative control consisting of just organism-specific growth medium with no cells (Vuotto et al., 2017). Bacterial strains were classified on the basis of their biofilm formation capacity into the following categories: no biofilm formation ($A_{570} \leq A_{570C}$), weak biofilm formation ($A_{570C} < A_{570} \leq 2 \times A_{570C}$), moderate biofilm formation ($2 \times A_{570C} < A_{570} \leq 4 \times A_{570C}$), strong biofilm formation ($4 \times A_{570C} < A_{570}$).

In total, 21 out of 23 (91.3%) bacterial strains produced biofilms that were detectable by CV staining assay (Table 5. 1). On the basis of the A_{570C} cut off = 0.2, 69.5% (16/23) of the isolates showed strong biofilm formation (SBF) ability, 17.4% (4/23) showed moderate biofilm formation (MBF) ability, 4.3% (1/23) showed weak biofilm formation (WBF) ability, and 8.7% (2/23) did not form biofilms (NBF) *in vitro* (see Table 5. 1). In general, there was considerable variation in the extent of biofilm formation between different species, and between different strains of the same species. As an example, out of 11 strains of *T. otitidis* assayed, five strains were strong biofilm producers, two were moderate biofilm producers, whereas *T. otitidis* 18 and 26 produced weak or no biofilm *in vitro*, respectively. All the classical otopathogens such as *S. pneumoniae* and *H. influenzae* were strong biofilm producers except *M. catarrhalis* 21, which did not produce detectable biofilm *in vitro*. Similarly, all *Staphylococcus* species showed strong ability to produce biofilms, whereas strains of other less frequently detected species such as *S. pyogenes* 11 and *S. mitis* 20 were moderate biofilm producers.

Table 5. 1 Biofilm formation of selected isolates from MEEFs of COME patients quantified by CV assay

Species	Strain [c]	Mean A_{570}(SE)[a]	Biofilm formation capacity[b]
S. aureus	18	2.14 ± 0.05	SBF
	21	2.52 ± 0.16	SBF
S. auricularis	16	1.6 ± 0.15	SBF
	12	2.1 ± 0.03	SBF
T. otitidis	8	1.57 ±0.14	SBF
	11	0.35 ± 0.01	MBF
	18	0.2 ± 0.01	WBF
	19	0.4 ± 0.03	MBF
	24	1.61 ± 0.06	SBF
	25	2.82 ± 013	SBF
	26	0.16 ± 0.02	NBF
	27	2.3 ± 0.2	SBF
	29	1.82 ± 0.04	SBF
S. pneumoniae	11	2.43 ± 0.13	SBF
	32	1.01 ± 0.03	SBF
S. oralis	14	4.67 ± 013	SBF
	20	2.27 ± 0.48	SBF
S. pyogenes	11	0.45 ± 0.06	MBF
S. mitis	20	0.27 ± 0.1	MBF
H. influenzae	21	1.31 ±0.1	SBF
	33	1.33 ± 0.3	SBF
M. catarrhalis	22	0.17 ± 0.01	NBF
A. odontolyticus	14	3.18 ± 0.07	SBF

[a]Data were generated from at least three independent experiments. [b]SBF= strong biofilm formation, MBF= moderate biofilm formation, WBF= weak biofilm formation, NBF= no biofilm formation, SE= standard error. Most of the COME isolates were strong biofilm formers. [c]Strain designations are explained in more detail in Table 2.2.

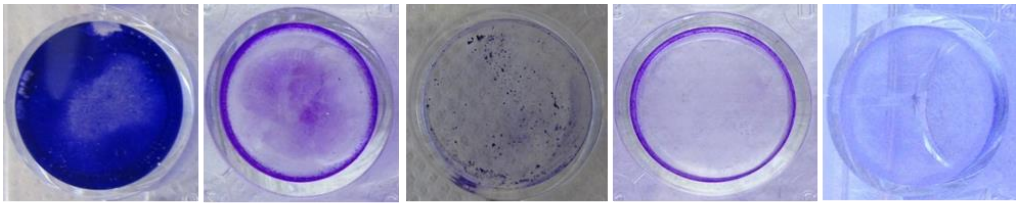


Figure 5. 1 Categories of crystal-violet stained biofilms formed by COME isolates.

Biofilms were grown in 12-well plates for 24-48 h and stained with CV to assess biofilm formation capacity of COME isolates. (A) *A. odontolyticus* 14 represents SBF bacteria, (B) *T. otitidis* 11 represents MBF bacteria, (C) *T. otitidis* 18 represents WBF bacteria, (D) *T. otitidis* 26 represent NBF bacteria, and (E) represents negative control (contains growth medium only). The intensity of CV staining was proportional to the biofilm formation capacity of the microorganism.

5.4 Efficacy of NucB against *in vitro* biofilms of COME isolates

All SBF bacteria and 2 MBF isolates were treated with NucB to assess the role of eDNA in promoting biofilm formation and in maintaining the structural stability of pre-formed biofilms.

5.4.1 Effects of NucB on biofilm formation of COME isolates

As previously stated, eDNA plays an important role in promoting the initial attachment of bacterial cells to living or inert surfaces during the early stages of biofilm formation (Whitchurch et al., 2002). To investigate the inhibitory effect of NucB on biofilm formation, 100 units ml^{-1} of NucB with a specific activity of 1.4 $x10^{-5}$ units mg^{-1} were included with the culture media and the inoculum during biofilm formation for 24-48 h in the 96-well or 12-well plate model (see Material and Methods 2.8.2). Biofilms of *T. otitidis*, *H. influenzae*, *S. pneumoniae*, and *A. odontolyticus* were grown for 48 h, whereas other bacterial biofilms were grown for 24 h in the appropriate culture media and conditions (see Materials and Methods 2.4.5).

In general, biofilm formation by 14 out of 18 (77.8%) COME isolates was significantly inhibited by NucB ($p<0.05$, n=3; paired two samples *t*-test) (see Figure 5. 2). Biofilm formation by all isolates of the closely related species *S. aureus* and *S. auricularis* was significantly reduced by NucB. The amount of reduction on average was just above 50% compared to control biofilms. In the presence of NucB, biofilm formation by 4 out of 6 *T. otitidis* strains was significantly impaired by 47-65% in comparison with controls. Biofilms formed by *T. otitidis* 29 were the most strongly inhibited. Interestingly, all the SBF classical

otopathogens of COME (*H. influenzae*, and *S. pneumoniae* strains) were sensitive to NucB treatment, and biofilm formation was decreased by approximately 50%. *A. odontolyticus* 14 was the most sensitive microorganism to NucB treatment; biofilm formation was reduced by 80%. *S. oralis* 20 was less sensitive to NucB treatment, but biofilm formation was still impaired significantly by 28% ($p = 0.02$, n=3; paired two samples *t*-test). However, NucB treatment did not significantly inhibit biofilm formation by *T. otitidis* 24, *T. otitidis* 11, *S. pyogenes* 11, or *S. oralis* 11.

5.4.2 Efficacy of NucB against pre-formed biofilms of COME isolates

With the realisation over the past few years that eDNA within biofilm matrix of many microorganisms has an essential role in maintaining the structural integrity of biofilms, the efficacy of NucB against pre-formed biofilms of COME isolates was assessed. For these experiments, mature biofilms of *T. otitidis*, *H. influenzae*, *S. pneumoniae*, and *A. odontolyticus* were grown for 48 h, whereas other bacterial biofilms were grown for 24 h in the appropriate culture media and conditions (see Materials and Methods 2.5.1). These pre-formed biofilms were again treated with 100 units ml^{-1} of NucB with a specific activity of 1.4 $x10^{-5}$ unit mg^{-1} for 1 h at 37°C in ambient air (see Materials and Methods 2.8.2). Biofilms formed by 14 out of 18 (77.8%) COME isolates were significantly dispersed by NucB compared to buffer treated controls (see Figure 5. 3). Pre-established biofilms of *A. odontolyticus* 14 were again remarkably sensitive to NucB treatment, and biofilm biomass was significantly reduced by more than 70% compared with control biofilms. Similarly, pre-formed biofilms of all *S. aureus* and *S. auricularis* isolates were significantly dispersed by NucB treatment. On average, the amount of reduction in biofilm biomass of these species was approximately 50%. Biofilms formed by 5 out of 6 *T. otitidis* strains were significantly dispersed by NucB, and the extent of biofilm decreased by 31.5-62.7% compared with negative controls that lacked enzyme. Biofilms of all classical otopathogens were dispersed by more than 56% in the presence of NucB, except *H. influenzae* 33 biofilms in which the dispersal effect of NucB (37% reduction) was lower than its inhibition effect (49% reduction) on biofilm formation. In contrast, the dispersal efficacy of NucB was higher than its inhibitory efficacy on biofilm formation by *T. otitidis* 7, 24, 25, 27, *S. pneumoniae* 11, 32, and *S. oralis* 20. Reductions in biomass of *A. odontolyticus* 14, *H. influenzae* 21, *T. otitidis* 29, and all *S. aureus* and *S. auricularis* isolates were broadly similar in assays of inhibition of biofilm formation and biofilm dispersal (see Figure 5. 2 and Figure 5. 3). As had been observed with assays for inhibition of biofilm formation, NucB did not significantly dispersed mature

biofilms formed by *T. otitidis* 11, *S. pyogenes*11, and *S. oralis* 11. In conclusion, NucB efficiently dispersed pre-formed biofilms and inhibited biofilm formation by more than 75% of bacteria isolated from MEEFs of COME patients.

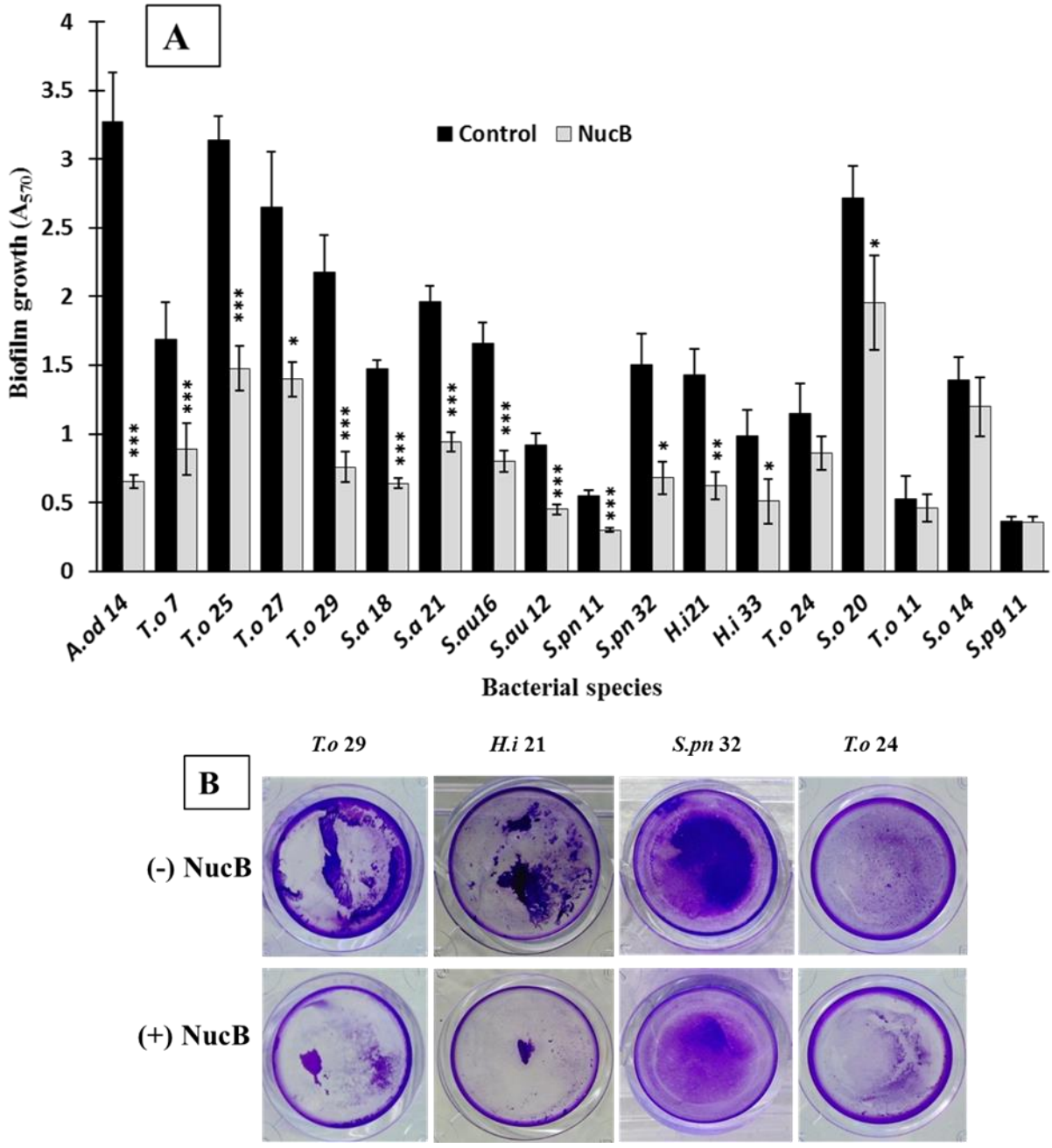


Figure 5. 2 NucB-mediated inhibition of biofilm formation by COME isolates.

Biofilms were grown in 96 or 12 well plates in the presence or absence of 100 units ml^{-1} of NucB for 24-48 h. Biofilms were stained by crystal violet and the extent of biofilm formation was quantified by measuring A_{570}. (A) Bars represent mean data from at least three independent experiments and standard errors are presented. (B) Representative images of crystal violet stained biofilms grown in 12-well plates without or with 100 units of NucB treatment. Biofilms formed by the majority of COME isolates were significantly inhibited by NucB treatment (*p < 0.05, **p< 0.01, ***p< 0.001; paired two samples *t*-test). *T.o*= T. *otitidis, H.i= H. influenzae, S.pn= S. pneumoniae, A.od= A. odontolyticus, S.a= S. aureus, S.au= S. auricularis, S.o= S. oralis, S.pg= S. pyogenes.*

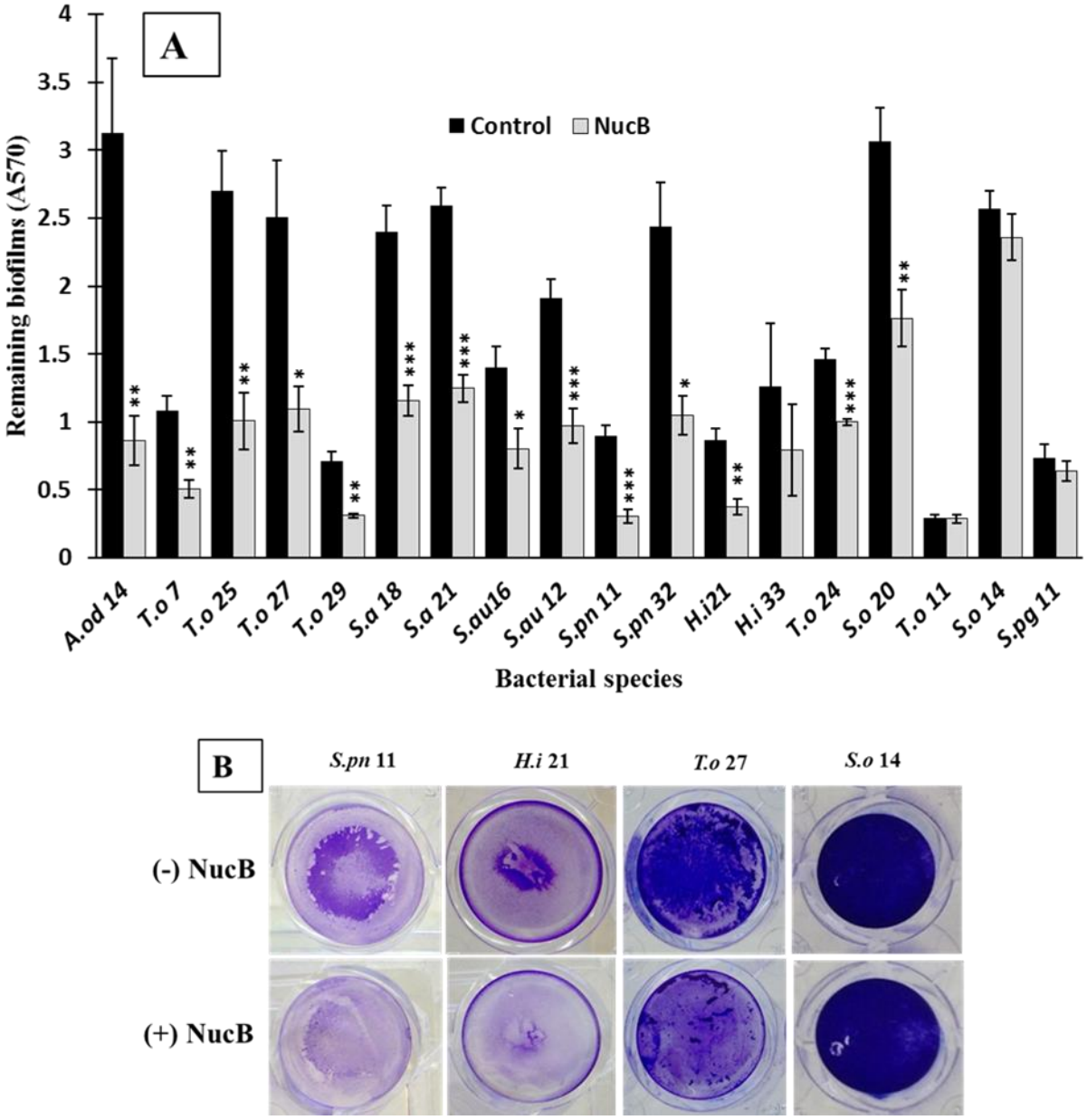


Figure 5. 3 Dispersal of pre-formed biofilms of COME isolates by NucB treatment.

Biofilms of COME isolates were grown in 96- or 12-well plates for 24-48 h. Biofilms were treated with 100 units ml^{-1} of NucB for 1 h at 37°C aerobically and quantified by CV staining. (A) Bars represent mean data from at least three independent experiments and standard errors are presented. (B) Representative images of crystal violet stained pre-formed biofilms treated with 100 units ml^{-1} of NucB in Tris buffer (+) or with Tris buffer only (-) for 1 h at 37°C aerobically. NucB significantly reduced the biomass of pre-formed biofilms of the majority of COME isolates compared to controls treated with buffer only (*$p < 0.05$, **$p< 0.01$, ***$p< 0.001$; paired two samples *t*-test). *T.o= T. otitidis, H.i= H. influenzae, S.pn= S.pneumoniae, A.od= A. odontolyticus, S.a= S. aureus, S.au= S. auricularis, S.o= S. oralis, S.pg= S. pyogenes.*

5.5 Effects of NucB on biofilm structure of selected COME isolates

Crystal violet assay data provided a quantitative measure of the inhibitory effects of NucB against biofilms, but did not give information on the structure of the biofilms formed. To understand the effects of NucB on biofilm structure, biofilms of selected bacteria were grown on the surface of glass coverslips and analysed using LIVE/DEAD® BacLight™ stain and CLSM imaging (see Materials and Methods section 2.12.1). As the imaging is time-consuming and labour-intensive, it was not practical to analyse all strains. Therefore, this work focused on representative species of the most commonly isolated bacterial genera from COME such as *Turicella* and *Staphylococcus,* as well as classical otopathogens of COME, which were shown to be sensitive to NucB treatment. *T. otitidis* 27, *T. otitidis* 29, *S.aureus* 18, *S. pneumoniae* 11, *H. influenzae* 21, and *A. odontolyticus* 14 were selected for this analysis. Visualisation of 3D images of CLSM showed that biofilms formed by all these bacteria in the absence of NucB generally had a complex structure in which dense, dome-shaped microcolonies consisting of several layers of cells covered most of the glass surface, occasionally separated by poorly colonised zones (see Figure 5. 4 and Figure 5. 5). There was considerable variation in biofilm thickness and structure between different species, and between different strains within the same species. For example, biofilms formed by *A. odontolyticus* 14 were more extensive and thicker than those formed by other bacterial species and consisted of unorganised, multicellular layers of dense microcolonies covering most of the surface with an average thickness of 51.6 μm. Biofilms produced by *T. otitidis* 27 were more extensive, thicker and structurally different than those produced by *T. otitidis* 29. Both live (green) and dead (red) cells were visualised when biofilms were stained by LIVE/DEAD® BacLight™ stain, and in general the proportions of live cells were far greater than dead/compromised cells within the biofilms of all bacterial species.

Consistent with the CV quantification of NucB efficacy against COME isolates, biofilm formation by all tested species was significantly inhibited in the presence of 100 units ml^{-1} of NucB compared with negative controls (Figure 5. 4). Observation of 3D reconstructed CLSM images showed that biofilms developed in the presence of NucB by all tested species were substantially less extensive than those of the untreated controls, and they generally consisted of randomly distributed single cells or very small clusters of cells on the surface. Again, inspection of the CLSM images showed that proportions of live cells to dead/compromised cells in biofilms formed in the presence of NucB were almost equal to

those formed in the absence of NucB, confirming that NucB has no obvious killing effect on bacterial cells within biofilms.

Visualisation of 3D rendered CLSM images showed that the structure of each biofilm treated with NucB was different from that of the negative controls. To obtain quantitative information about these structural changes, COMSTAT was used to determine the important structural parameters such as biomass, average thickness and roughness coefficient of the biofilm (Table 5. 2). Generally, the average biomass of biofilms reflected the visual observations, and significant reductions ($p < 0.05$) of between 60-85% were observed in biofilms of all tested species formed in the presence of NucB compared with controls. Biomass levels of *S. pneumoniae* 11 and *S. aureus* 18 biofilms were the most strongly reduced by NucB, and each was decreased by more than 80% in the presence of the enzyme. By contrast, *T. otitidis* 27, *T. otitidis* 29, and *H. influenzae* 21 biofilm biomasses were decreased on average by 65% compared with untreated controls. Also consistent with visual observations, the average thickness of biofilm formed by all tested species was significantly ($p < 0.05$) reduced by 50-80% in the presence of NucB. Biofilms of *S. pneumoniae* 11 and *S. aureus* 18 were also strongly reduced in thickness by 75-85%, whereas the thickness of biofilms formed by *T. otitidis* 27, *T. otitidis* 29, and *H. influenzae* 21 was reduced on average by 60%. The roughness coefficient of biofilms formed by all tested COME isolates in the presence of NucB was significantly changed compared to biofilms formed in the absence of NucB, once again consistent with a shift in biofilm architecture upon treatment with NucB.

Again in agreement with CV data, visualisation of CLSM images further showed that 48 h old pre-formed biofilms of the selected COME isolates were substantially dispersed by the addition of 100 units of NucB in Tris buffer, for 1 h at 37°C aerobically, compared with controls treated with Tris buffer with no NucB (see Figure 5. 5). Generally, NucB treated pre-established biofilms of all tested COME isolates were less extensive than the control biofilms, and consisted of either irregularly scattered isolated cells with very small clumps of cells or a single layer of cells containing many cell-free zones. Again, no differences in the viability of biofilm cells were detected between NucB treated biofilms and control biofilms. Quantitative analysis of the CLSM images by COMSTAT software showed that the average biomass and average thickness of the pre-established biofilms formed by the selected COME isolates were significantly ($p < 0.05$) reduced, by 55-80%, when they were incubated with 100 units ml^{-1} of NucB compared to the control biofilms (Table 5. 3). Biofilms of *A. odontolyticus* 14 and *H.influenzae* 21 were remarkably sensitive to NucB treatment. In both cases, the average biomass and thickness of biofilms was reduced by more than 76% compared to the untreated

controls. Consistent with CV data and visual observations of the CLSM images, the average biomass and thickness of the pre-formed biofilms of *T. otitidis* 27, *T. otitidis* 29, *S. pneumoniae* 11 and *S.aureus* 18 were also significantly decreased by 55-70 % compared to the negative controls. Finally, pre-established *T. otitidis* 27, *T. otitidis* 29, and *S. aureus* 18 biofilms exposed to NucB were significantly altered in roughness coefficient compared to the untreated controls. However, in the case of *A. odontolyticus* 14, *H. influenzae* 21, and *S. pneumoniae* 11 no significant changes in biofilm roughness were evident even though there were clear differences in biofilm architecture (see Figure 5. 5).

In conclusion, quantitative data obtained by COMSTAT support the qualitative data obtained from the observation of 3D rendered CLSM images and show that the architecture of biofilms formed by selected COME isolates is significantly different following exposure to NucB during or after biofilm formation compared with controls lacking enzyme. By contrast, no detrimental effects on the viability of cells within biofilms were observed following NucB treatment.

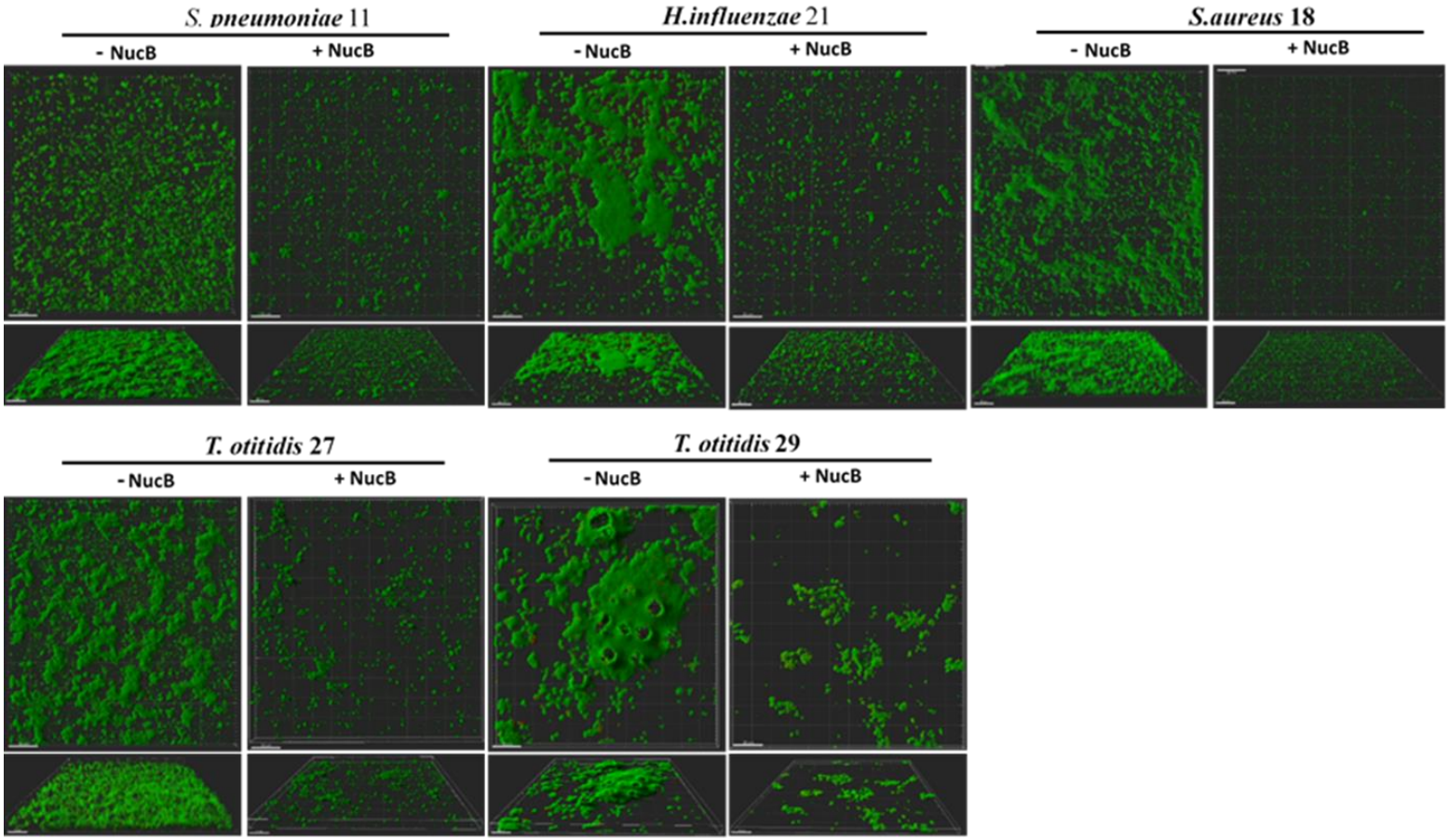


Figure 5. 4 Effects of NucB on biofilm structure and formation by COME isolates observed by CLSM.

Biofilms were grown on glass surfaces in the presence or absence of 100 units ml^{-1} of NucB for 24-48 h and visualised with CLSM using LIVE/DEAD® BacLight™ stain. Cells with green signals (Syto9) are alive and cells with red signals (propidium iodide) are dead or damaged. Upper lines represent x–y plane, lower lines represent 3D reconstruction of a z stack. The scale bars represent 20 µm for upper lines, and 30 µm for lower lines. Images show that biofilms formed by the selected COME isolates in the presence NucB were remarkably less extensive than that of untreated controls.

Table 5. 2 COMSTAT analysis of biofilm formation by the COME isolates in the presence or absence of NucB (100 units ml^{-1}).

Species	Treatment Group	Biomass ($\mu m^3/\mu m^2$)[a]	Average thickness (μm)[a]	Roughness coefficient[a]
S. pneumoniae 11	(-) NucB	2.8 (0.4)	9.5 (1.5)	0.5 (0.2)
	(+) NucB	0.4 (0.0)**	1.8 (0.3)**	1.5 (0.1)**
H. influenzae 21	(-) NucB	3.3 (0.7)	12.9 (2.3)	0.8 (0.2)
	(+) NucB	0.9 (0.3)**	5.7 (2.7)*	1.4 (0.1)*
S. aureus 18	(-) NucB	3.3 (0.5)	7.7 (1.1)	0.6 (0.1)
	(+) NucB	0.6 (0.1)**	2 (0.3)**	1.3 (0.1)**
T. otitidis 27	(-) NucB	4.8 (0.6)	10.9 (1.4)	0.8 (0.1)
	(+) NucB	1.9 (0.7)*	5.7 (1.9)*	1.5 (0.2)*
T. otitidis 29	(-) NucB	2 (0.5)	9.7 (2.5)	0.8 (0.1)
	(+) NucB	0.6 (0.1)*	3.1 (0.8)*	1.5 (0.1)**

[a]Data [mean (SE)] were generated from images of three randomly selected areas on each coverslip from at least three independent experiments. NucB significantly altered the structure of biofilms formed by the selected COME isolates, leading to significant reductions in biofilm biomass, average thickness, and roughness coefficient compared to no NucB controls (*$p < 0.05$, **$p< 0.01$, ***$p< 0.001$; One-Way ANOVA test with Tukey's HSD *post-Hoc* comparison).

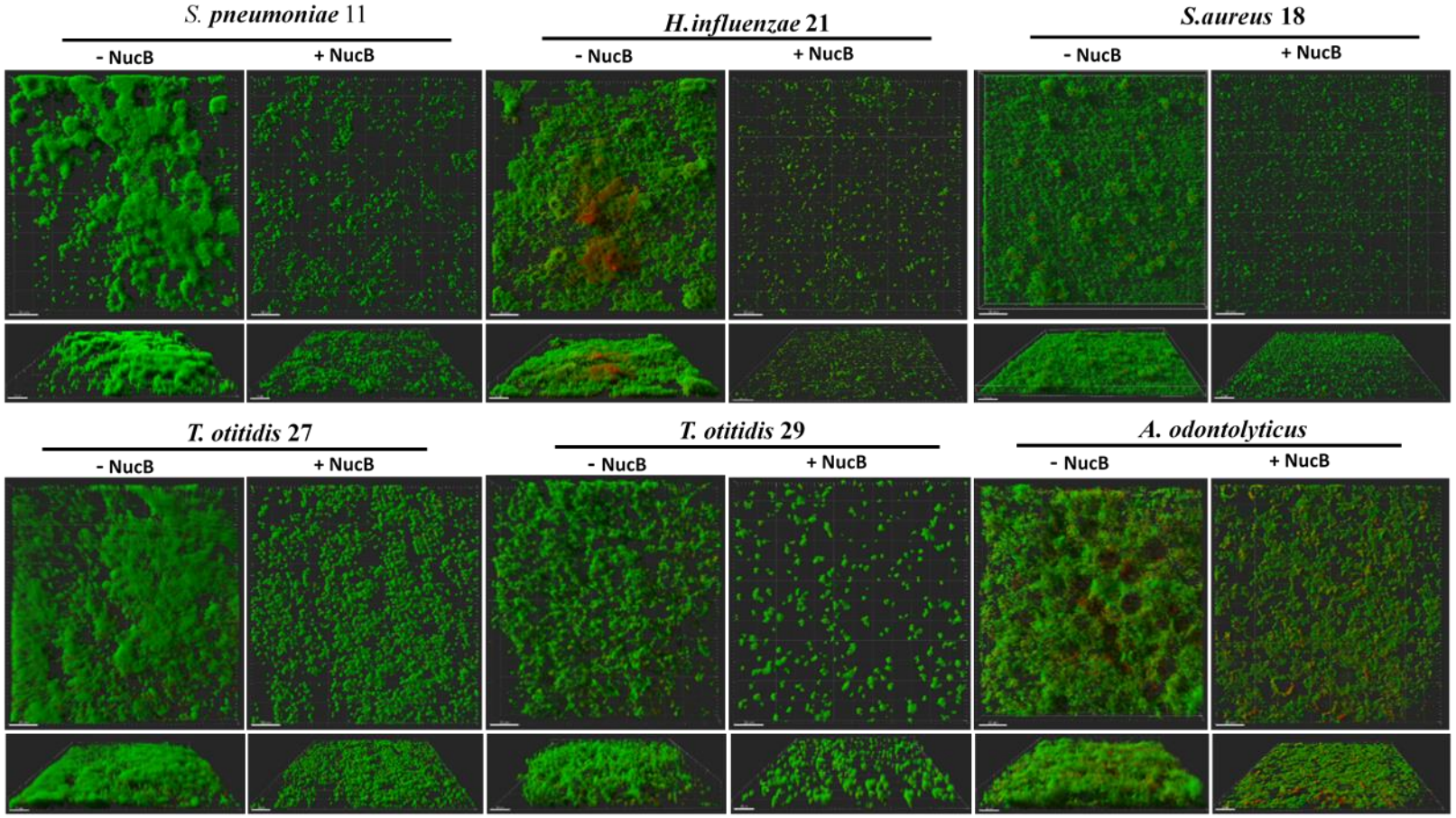


Figure 5. 5 Effects of NucB on the structure of established biofilms of COME isolates observed by CLSM.

Biofilms were grown on glass surfaces for 48 h, treated with NucB (100 units ml^{-1}) for 1 h at 37°C and visualised with CLSM using LIVE/DEAD® BacLight™ stain. Cells with green signals (Syto9) are alive and cells with red signals (propidium iodide) are dead or damaged. For each biofilm, upper panels represent x–y plane, lower panels represent 3D reconstruction of a z stack. The scale bars represent 20 µm for upper panels, and 30 µm for lower panels. Images show that NucB substantially dispersed pre-formed biofilms of selected COME isolates compared to control biofilms.

Table 5. 3 COMSTAT analysis of COME isolates biofilm dispersal by NucB (100 units ml^{-1}).

Species	Treatment Group	Biomass ($\mu m^3/\mu m^2$)[a]	Average thickness (μm)[a]	Roughness coefficient[a]
S. pneumoniae 11	(-) NucB	8.4 (1.7)	24.7 (4.1)	0.6 (0.2)
	(+) NucB	3.0 (1.1)**	11.8 (2.9)*	1.0 (0.2)
H.influenzae 21	(-) NucB	6.2 (1.1)	21.6 (3)	0.7 (0.1)
	(+) NucB	1.0 (0.2)***	5.2 (1.7)***	1.1 (0.2)
S.aureus 18	(-) NucB	4.8 (1.1)	21.6 (3.2)	0.4 (0.1)
	(+) NucB	2.3 (0.6)*	9.0 (2.6)**	1.3 (0.1)***
T. otitidis 27	(-) NucB	9.6 (1.6)	23.9 (1.6)	0.5 (0.1)
	(+) NucB	4.4 (1.2)*	8.7 (1.3)***	1.2 (0.1)***
T. otitidis 29	(-) NucB	4.3 (0.7)	8.4 (1.6)	1 (0.1)
	(+) NucB	1 (0.1)***	3.5 (0.4)*	1.3 (0.1)*
A. odontolyticus 14	(-) NucB	10.7 (2.3)	51.6 (10.6)	0.6 (0.1)
	(+) NucB	2.0 (0.26)***	9.4 (1.38)***	0.9 (0.1)

[a]Data [mean (SE)] were generated from images of three randomly selected areas on each coverslip from at least three independent experiments. NucB greatly altered pre-formed biofilms structure by significantly reducing biomass and average thickness of biofilms compared to the untreated controls (*$p < 0.05$, **$p< 0.01$, ***$p< 0.001$; One-Way ANOVA test with Tukey's HSD *post-Hoc* comparison).

5.6 Discussion

Using the 96-well MTP model and CV staining assay, more than 90% of the COME isolates that were assayed in this Chapter formed biofilms *in vitro*, and around 70% of them were strong biofilm forming (SBF) strains. This is not surprising as it has been suggested that more than 99% of microorganisms can demonstrate the phenotypic characteristic of biofilm formation (Costerton et al., 1978). Although no previous studies have systematically screened COME isolates for their biofilm formation capacity as performed in this study, several studies have focused on *in vitro* and *in vivo* biofilms formed by classical otopathogens implicated in the aetiology of COME including *S. pneumoniae* and *H. influenzae* (Hall-Stoodley and Stoodley, 2009, Starner et al., 2006, Van Hoecke et al., 2016). All strains of *S. pneumoniae, H. influenzae,* and *S. aureus* that were isolated in this study also showed strong biofilm formation ability. *T. otitidis* and CoNS are frequently isolated from MEEFs of children with COME (Daniel et al., 2012b, Gomez-Garces et al., 2004, Holzmann et al., 2002, Papp et al., 2016). Their roles in the aetiology of COME are debated as they also have been cultured from the external ear canal of healthy individuals, although the frequency of *T. otitidis* detection in patients with COME appears to be higher than in controls (Funke et al., 1994, Gomez-Garces et al., 2004, Holzmann et al., 2002). Interestingly, most of the *T. otitidis* strains that were assayed formed robust biofilms *in vitro* under the conditions employed. Furthermore, in agreement with this study, CoNS isolated from patients with COME and CRS also showed phenotypic and genotypic characteristics compatible with the formation of biofilms *in vitro* and *in vivo* (Paluch-Oles et al., 2011). The ability to form biofilms may potentially protect these species against hostile external insults such as antimicrobials and host immune actions (Longauerova, 2006). Furthermore, *T. otitidis* and CoNS have also been found to be associated with other infection-related conditions such as medical implant-associated infections for CoNS, and mastoiditis, auricular and a cervical abscess caused by *T. otitidis* (Paluch-Oles et al., 2011, von Graevenitz and Funke, 2014). It is likely that these microorganisms are opportunistic pathogens.

Consistent with the roles of eDNA in facilitating initial microbial adhesion during early biofilm formation and in maintaining the structural integrity of established biofilms, NucB was effective in inhibiting biofilm formation and disrupting established biofilms in more than 75% of the COME isolates. *Staphylococci* and *T. otitidis* were the most frequently cultured bacteria in MEEFs of COME patients and the majority of strains investigated here were sensitive to NucB treatment. *S. aureus* and CoNS including *S. epidermidis*, which is

closely related to *S. auricularis*, have been shown to be sensitive to rhDNase (Dornase alfa), DNase I and NucB in previous studies (Qin et al., 2007a, Shields et al., 2013, Tetz et al., 2009, Kaplan et al., 2012, Sugimoto et al., 2018), indicating a critical importance of eDNA within the biofilm matrix of these species. Importantly, biofilms formed by *S. pneumoniae* and *H. influenzae*, which are two of the most commonly implicated pathogens in COME, were significantly inhibited and dispersed by NucB treatment. Evidence from previous studies clearly indicates that eDNA is a key structural component within the biofilm matrix of *S. pneumoniae* and *H. influenzae* (Domenech and Garcia, 2018, Hall-Stoodley et al., 2008, Jurcisek et al., 2017, Tetz et al., 2009), which may explain their significant sensitivity to NucB. Several distinct mechanisms have been reported to be responsible for the accumulation of eDNA within the biofilm matrix of *S. pneumoniae* and *H. influenzae* including cellular autolysis, the formation of extracellular vesicles, and active release of DNA from the bacterial cytoplasm to the extracellular environment through an inner-membrane pore complex (Jurcisek et al., 2017, Domenech and Garcia, 2018).

A. odontolyticus is a commensal of the oral cavity that can cause serious infections in individuals with defects in the mucosal barrier and/or with immune deficiency. Although *A. odontolyticus* has been reported to be associated with about 25 cases of invasive pathology over last 50 years including cardiopulmonary, mediastinal, bacteraemia, and soft tissue infections (Broly et al., 2016), it has not been isolated in middle ear disease. Therefore, it was considered interesting to study the biofilm formation capacity and sensitivity to NucB treatment of this strain. NucB had a substantial effect on *A. odontolyticus* biofilms. In our laboratory, we have also observed that *Actinomyces oris* MG1, an isolate originally from the oral cavity, also forms biofilms that are markedly sensitive to treatment with DNases such as NucB or DNase I (N. Jakubovics, unpublished data). Therefore, a reliance on eDNA for biofilm formation may be a characteristic of *Actinomyces* species. NucB also significantly reduced *S. pneumoniae* biofilms. However, the sensitivity of biofilms formed by other streptococcal species to NucB was variable. *S. oralis* 20 biofilms were significantly dispersed and inhibited by NucB, but *S. oralis* 14 and *S. pyogenes* 11 were not significantly sensitive to NucB. This finding is consistent with other research which also reported marked differences in DNase sensitivity between different species of bacteria or occasionally, between different strains of the same species (Lappann et al., 2010, Shields et al., 2013). It is not clear why some strains are more sensitive to NucB than others. This could be due to the differences in the pathways of eDNA production or in the dependence for eDNA to maintain biofilm structural stability. Genomic DNA sequencing of these different strains may help to reveal the

genes responsible for release of eDNA into the biofilm matrix. *T. otitidis* would be an interesting target for this investigation, because marked variations in NucB sensitivity were detected between different strains of *T. otitidis*. Apparently eDNA production and accumulation is strictly regulated and relies on several factors such as growth phase and the availability of nutrient source. Other factors such as growth conditions including temperature and pH, differences in the structure of eDNA, production and accumulation of inhibitors, differences in the nuclease activity of DNases against eDNA within biofilm matrix, and the type of surfaces used to grow bacterial biofilms were also found to have significant impact on DNase-mediated biofilm control (Lappann et al., 2010, Shields et al., 2013).

Using CLSM and quantitative image analysis by COMSTAT, significant alterations in the structure of biofilms formed by six different strains could be observed (*T. otitidis* 27, *T. otitidis* 29, *S.aureus* 18, *S. pneumoniae* 11, *H. influenzae* 21, and *A. odontolyticus* 14). This was displayed by substantial reductions in biomass and average thickness of the biofilms when compared with the negative controls, suggesting that degradation of eDNA by NucB within the matrix of established biofilms or during biofilm formation by these isolates had weakened the biofilm architecture and led to biofilm collapse, or prevented the eDNA from enabling biofilm formation in the first place. Consistent with the present study, established biofilms of *S. pneumoniae* clinical isolates treated with rhDNase (Dornase alfa) showed significant reductions in biomass and average thickness in a dose-dependent manner regardless of their biofilm formation capacity (Hall-Stoodley et al., 2008). These studies highlight the important role of eDNA in maintaining structural integrity of biofilm in this species. Similarly, eDNA is considered one of the key structural constituents of the biofilm matrix of *H. influenzae* and has been shown to exhibit an important role in protecting *in vivo* biofilms of *H. influenzae* against the antimicrobial action of human β-defensin-3 (hBD-3), an important antimicrobial host defence peptide which is critical in innate immunity of the middle ear mucosa (Jones et al., 2013). Interestingly, degradation of eDNA within the *in vitro* biofilm matrix of *H. influenzae* enhanced the antimicrobial activity of recombinant hBD-3 and markedly reduced overall biomass and thickness of biofilms. These observations are in agreement with the high sensitivity of *H. influenzae* strains to NucB treatment that was observed in this study.

Quantitative analysis of biofilms using CLSM imaging and COMSTAT analysis showed higher levels of dispersal and inhibition of biofilms formed by COME isolates than

those observed in the 96-well MTP model. For example, when testing biofilm inhibition, NucB significantly reduced biofilm biomass of, *S. aureus* 18, *S. pneumoniae* 11, *H. influenzae* 21, *T. otitidis* 27, and *T. otitidis* 29 by 56.5%, 45%, 56.3%, 47.3%, and 65%, respectively, as quantified using the 96-well MTP biofilm model and CV staining. By comparison, biomass of biofilms formed by the same bacteria was significantly reduced by 81.8%, 85.7%, 72.7%, 60.4%, and 70%, respectively, as quantified using combined CLSM and COMSTAT analysis. A similar finding was shown with *S. aureus* SB14 and SB17 biofilms in Chapter 3. As discussed previously, growing biofilms on different surfaces, glass in the case of the CLSM imaging and polystyrene for the 96-well MTP and CV staining model, may cause variability in the reliance of biofilms on eDNA for the structural support. Second, the CLSM approach shows bacterial cells only, whereas the CV staining technique quantifies overall biofilm biomass including cells and extracellular matrix, which may have affected the measurements of dispersal and inhibition of biofilm formation by NucB.

Although the underlying aetiology of COME is unclear, there is increasing evidence that bacterial biofilms are the key stimulus of the chronic inflammatory process that leads to overproduction and accumulation of effusion fluid in the middle ear of COME patients. There is a clear deficiency in the current treatment of COME as evidenced by the significant rate of recurrence following myringotomy and grommet insertion (Kubba et al., 2000, Vlastarakos et al., 2007). Therefore, there is pressing need for the development of better treatment techniques that can be used to improve the current treatment options. This study showed that most COME-associated microorganisms formed robust biofilms *in vitro* that were efficiently inhibited and dispersed by NucB treatment, indicating that NucB may be useful in the therapy of COME. Further validation of these *in vitro* data is required in a more realistic animal model of COME and ultimately in human clinical trials.

Chapter 6. General Discussion

The most important inherited challenge in targeting otopathogens associated with otitis media including COME is the ability to isolate and identify the microbial population, and monitor the abundance of each species (Stol et al., 2013, Rettig and Tunkel, 2014). Traditionally, *S. pneumoniae, H. influenzae* and *M. catarrhalis* are the most frequently isolated otopathogens in approximately 40% of patients with COME using conventional culture methods (Ngo et al., 2016). More fastidious microorganisms including *A. otitis*, *T. otitidis* and *P. acnes* are also often isolated, though their role in infection is not so clear (Leskinen et al., 2002, Harimaya et al., 2006, von Graevenitz and Funke, 2014, Ngo et al., 2016, Barron et al., 2019). Initial attempts to replicate laboratory procedures recommended by UKSMI (PHE, 2014) had limited success for isolating fastidious microorganisms including *A. otitis*, *H. influenzae*, and *Propionibacterium acnes*, even though these were detected using culture independent methods. Culturing techniques were improved by processing MEEF samples immediately following sample collection, extending the incubation of initial cultures for up to 2 weeks, and adopting stringent precautions to prevent contamination. These optimisation steps dramatically increased the diversity of species that were cultured. Overall,16 different species were isolated from 21 MEEF samples collected from 12 patients on three different agar media. The microbial communities present in MEEFs of COME patients were dominated by *T. otitidis, A. otitis,* and CoNS including *Staphylococcus epidermidis* and *Staphylococcus auricularis*. Bacterial strains identified in our cohort were broadly consistent with those previously observed in other studies (Daniel et al., 2012b, Papp et al., 2016, Min et al., 2019, Barron et al., 2019, Harimaya et al., 2006, von Graevenitz and Funke, 2014, Bosley et al., 1995). However, *T. otitidis* and *A. otitis* were identified in our cohort at higher frequencies (52.4% and 38.1%, respectively) than reported in these studies (0-10% and 0-4.7 % respectively). It is possible that the adoption of the improved culture techniques in this study, particularly the extended incubation period, may have been responsible for the increased isolation frequency.

Recently, the development of culture-independent technologies such as 16S rRNA gene sequencing have allowed a non-selective, quantitative characterisation of the microbial communities present in complex samples such as MEEFs. It is important to note that 16S rRNA gene sequencing has a number of limitations, particularly for low biomass samples. Many of the extraction kits are contaminated with microbial DNA and it is important to include negative controls. In this study, many of the major contaminating sequences were

identified and removed by sequencing and analysing DNA extraction blanks. However, since this work was done, new guidelines have been published that recommend the use of multiple negative controls (Eisenhofer et al., 2019). Overall, the 16S rRNA gene sequencing performed here identified *A. otitis*, CoNS, *Moraxella catarrhalis*, *T. otitidis*, and *Haemophilus influenzae* as the most common species in MEEFs of patients with COME. Importantly, there was excellent agreement between microbial culture and culture-independent analysis, providing confidence in the microbiological analyses. Consistent with this study, recently published papers using both traditional culture dependent and 16S rRNA gene sequencing techniques have found that six main species or genera of bacteria as the most common microorganisms in COME: *A. otitis, H. influenzae, staphylococci* (mainly CoNS), *T. otitidis, Moraxella catarrhalis* and *S. pneumoniae* (Van Hoecke et al., 2016, Boers et al., 2018, Chan et al., 2017b, Min et al., 2019, Kolbe et al., 2019, Papp et al., 2016). The use of culture-independent methods has shown that *A. otitis* and *T. otitidis* have a high prevalence in COME, and are consistent with both our culture and 16S rRNA sequencing data (Ari et al., 2019, Kolbe et al., 2019, Boers et al., 2018, Chan et al., 2017b). *A. otitis* appears to be more dominant than *T. otitidis* in the previously published literature, likely due to lack of appropriate primer pairs for the identification of *T. otitidis* (von Graevenitz and Funke, 2014*).* It is difficult to differentiate *T. otitidis* from closely-related commensal *Corynebacterium* species, and in fact it has recently been propsed that *T. otitidis* should be reclassified as *Corynebacterium otitidis* (Baek et al., 2018, von Graevenitz and Funke, 2014).

The role of *T. otitidis, A. otitis* and *staphylococci* in the pathogenesis of COME is still unclear due to their high abundance in EEC (Stroman et al., 2001, Chan et al., 2017b), which is a potential source of contamination of samples. Even when proper procedures for collecting MEEF samples are followed, including sterilisation of EEC and adopting measures to avoid contact with it during sampling, the possibility of a sample becoming contaminated with a EEC commensal flora cannot be eliminated (Buzatto et al., 2017, Chan et al., 2017b, Daniel et al., 2012b). Evidence supporting the potential pathogenicity of *T. otitidis, A. otitis* and staphylococci, and the possible mechanism of transmission into the middle ear through retrograde ascension of the Eustachian tube from the adenoids was considered in chapter 4 and chapter 5. This retrograde ascension is thought to be important for transmission of three major otopathogens (*S. pneumoniae, H. influenzae* and *M. catarrhalis*) to the middle ear, since these species are commonly increased in the adenoids when COME is present (Marchisio et al., 2003, Chan et al., 2017b, Van Hoecke et al., 2016). *T. otitidis, A. otitis* and staphylococci (mainly CoNS) are rarely detected in the adenoid. The pattern of high

abundance of *T. otitidis, A. otitis* and staphylococci in the EEC and middle ear and low abundance in adenoids suggests that EAC can also serve as a reservoir for these species to reach the middle ear, possibly through a perforated tympanic membrane (whether from spontaneous rupture or in the presence of grommet tube) as discussed in chapter 4 (Lappan et al., 2018, Chan et al., 2017b). Although I did not investigate the microbial species in the EEC, comparisons were made between the middle ear microbiome and that of adenoids. In general, the microbial profiles of adenoids were more diverse than those observed in MEEFs of patients with COME. Consistent with recent microbiome studies, the microbiome of adenoids in our cohort consisted mainly of *Haemophilus* spp., *Prevotella* spp., *Streptococcus* spp., *Fusobacterium* spp., *Veillonella* spp., and *Porphyromonas* spp. (Boers et al., 2018, Chan et al., 2017b, Johnston et al., 2019, Jervis-Bardy et al., 2015). However, the microbiome of adenoids of patients with COME appeared to be less diverse than those of patients without COME (controls) and they were concurrently colonised by the three classical otopathogens (*H. influenzae, S. pneumoniae,* and *M. catarrhalis*), in addition to other bacterial species that were detected at lower levels in MEEFs of the same patients. This finding supports the notion that the co-colonisation of adenoid with otopathogens is an important predisposing factor for the development of COME.

I attempted to directly demonstrate translocation events by sequencing the genomes of two paired species from adenoids and MEEFs, with the idea that identification of identical clones would be strong evidence for transmission between the two environments. However, all strains were distinct from one another and direct evidence for translocation through the Eustacian tube is therefore still lacking. On the other hand, commensal genera such as *Corynebacterium* and *Dolosigranulum*, that were observed at very low relative abundance in our cohort, have been detected at high abundance in the nose and adenoids of healthy controls and at low relative abundance in the nasopharynx of children with middle ear infections (Laufer et al., 2011, Man et al., 2019, Pettigrew et al., 2012b). This suggests that either the risk for middle ear infections including COME increases by a reduction in the abundance of these key adenoid commensals. Alternatively, middle ear infections themselves or antibiotic treatment may result in the loss of these important commensals creating a state of dysbiosis. One implication is that probiotic bacteria may be an efficient alternative approach to prevent the onset or recurrence of otitis media (Marchisio et al., 2015). The work from chapter 4 provided an overview of the overall bacterial communities present in the MEEFs and adenoids of patients with COME. Most importantly, a wide range of representative fresh clinical isolates, which included both classical otopathogens (*H. influenzae, S. pneumoniae,*

and *S. aureus*) and novel potential otopathogens (*A. otitis*, *T. otitidis*, and *CoNS* (, *S. auricularis*)), were obtained that could be used in *in vitro* biofilm models to assess the anti-biofilm activity of NucB in Chapter 5.

The majority of COME isolates exhibited a strong capacity to form biofilms *in vitro*, although the clinical isolates of *A. otitis* were excluded from the analysis due to their fastidious growth requirements. Previous studies have studied the biofilm-forming ability of the classical otopathogens implicated in COME including *S. pneumoniae*, *H. influenzae*, and *S. aureus* (Daniel et al., 2012a, Hall-Stoodley et al., 2008, Jones et al., 2013). However, the ability of other COME isolates has not been thoroughly explored. It appears that eDNA was critical for more than 75% of biofilms formed by the COME isolates tested since NucB significantly inhibited biofilm formation and dispersed established biofilms. In particular, all strains of *S. pneumoniae*, *H. influenzae* and *S. aureus* were sensitive to NucB. *S. aureus* strains isolated from two cases of COME exhibited similar behaviour to that shown by CRS clinical isolates assayed in chapter 3, since they formed robust *in vitro* biofilms that were substantially susceptible to NucB treatment. Consistent with these findings, a recent study also found that CoNS were the most frequently cultured bacteria from MEEFs of children with COME and the detection rate of Methicillin resistant *S. aureus* (MRSA) was significantly higher in children with recurrent COME than those with non-recurrent COME (Min et al., 2019). This work further supports my decision to select *S. aureus* as a model microorganism relevant to COME in chapter 3. The finding that eDNA was frequently important in biofilm formation was also consistent with previous research. For example, *in vitro* and *in vivo* biofilms formed by *S. pneumoniae*, *H. influenzae* and *S. aureus* have been shown to contain significant amounts of eDNA and were susceptible to DNase treatment (Hall-Stoodley et al., 2008, Jones et al., 2013, Cavaliere et al., 2014a, Jurcisek and Bakaletz, 2007, Sugimoto et al., 2018). Overall, there is accumulating evidence that DNase enzymes such as NucB may potentially be effective for controlling biofilms associated with COME.

In accordance with previously published studies (Lappann et al., 2010, Shields et al., 2013, Sugimoto et al., 2018), the results here also demonstrated differences in DNase sensitivity between different species of bacteria or occasionally, between different strains of the same species. There was variability in the efficacy of NucB against biofilms formed by different *Streptococcus* spp. and *T. otitidis*, for example. It is not clear why some strains are more sensitive to DNase than others. This could be due to differences in the pathways of

eDNA production or in the adhesion of cells to the eDNA matrix. It appears that eDNA production and accumulation is strictly regulated and relies on several factors such as growth phase and the availability of nutrient source (Zetzmann et al., 2015b, Windham et al., 2018) . It has been found that eDNA plays an important role during early stages of *H. pylori* biofilm formation but does not play a significant structural role in mature biofilms (Windham et al., 2018, Grande et al., 2012). The authors have attributed this shift to the contribution of other biofilm matrix components that may take on the role of maintaining structural stability as the biofilm matures. Furthermore, Zetzmann et al. (2015b) have shown that *Listeria monocytogenes* forms DNase-sensitive biofilms in diluted complex medium and these biofilms contained abundant eDNA within the matrix. By contrast, in full strength medium biofilms were resistant to DNase I treatment and lacked eDNA within their matrix, suggesting that low nutrient medium may favour bacterial cell lysis and the release of chromosomal DNA. Other factors such as growth conditions including temperature and pH, interspecies and intraspecies variability in the composition of the extracellular matrix of the biofilm, differences in the structure of eDNA, and type of surfaces used to grow bacterial biofilms were also found to have significant impacts on DNase-mediated biofilm control (Zetzmann et al., 2015b, Sugimoto et al., 2018, Windham et al., 2018). It has been shown that the structural role of eDNA is not proportional to its level within the matrix of many DNase sensitive *S. aureus* biofilms. However, in a small number of *S. aureus* strains where polysaccharide-intracellular adhesins or proteins were present in large amounts within the extracellular matrix (ECM), biofilms were insensitive to DNase I treatment. It is likely that these dominant components of the ECM may either prevent DNase enzyme from acting on eDNA or compensate the structural roles of eDNA within the ECM of these biofilms (Sugimoto et al., 2018). Comprehensive approaches such as transcriptomics or proteomics help to identify the key genes responsible for release of eDNA and other ECM components into the biofilm matrix and explain the differences in eDNA sensitivity between closely related strains of the same species.

6.1 Impact and applications of the study

This study significantly enhances our understanding of the composition of microbial populations associated with COME and the efficacy of a DNase, NucB, against relevant *in vitro* biofilms. There have been several previous investigations studying the anti-biofilm action of DNases, but very few have looked at biofilm-associated ear diseases, particularly COME. Most previous studies have focused on investigating eDNA and DNase-sensitivity of

the classical pathogens implicated in middle ear infections such as *S. pneumoniae* (Hall-Stoodley et al., 2008), *H. influenzae* (Jones et al., 2013, Cavaliere et al., 2014b), *S. aureus* (Mann et al., 2009, Sugimoto et al., 2018), and *P. aeruginosa* (Whitchurch et al., 2002, Swartjes et al., 2013). This study built on this body of previous work and broadened the investigation to a much wider range of microbial strains freshly isolated from children with COME. A key finding was that biofilms formed by the majority of the tested COME isolates were significantly dispersed, inhibited or structurally altered by NucB treatment. These observations add further weight to the hypothesis that eDNA is a key structural component within the ECM of biofilms formed by a wide range of bacteria associated with COME and that NucB has promise as a novel eDNA-targeting approach to control biofilm diseases including COME.

While our study has shown promising results *in vitro*, it is important to determine the extent to which eDNA is important in clinical otitis media including COME. Recently published *in vivo* studies have also identified both host- and bacterial-derived extensive strands of eDNA matrix which are often associated with DNABII proteins such as integration host factor (IHF), and histone-like protein (HU) in the MEEF samples recovered from children with rAOM (Thornton et al., 2013), COME (Barron et al., 2019) and in persistent otorrhea samples retrieved from paediatric patients with tympanostomy tubes (Idicula et al., 2016). Targeting these extensive strands of eDNA directly using Dornase alfa was associated with complete and rapid fragmentation of this DNA matrix in 7 MEEF samples collected from children with rAOM (Thornton et al., 2013). Alternatively, indirect targeting of eDNA using antibody against DNABII proteins has been shown to: 1) induce disruption of biofilms and rapid resolution of the disease in an experimental model of *H. influenzae*-induced otitis media (Goodman et al., 2011), 2) promote dissolution of sputum solids collected from patient with cystic fibrosis (Gustave et al., 2013) and exudate samples retrieved from EEC of children with persistent post-tympanostomy tube otorrhoea (Idicula et al., 2016), 3) prevent development of experimental otitis media in a multispecies model of ascending disease (Novotny et al., 2017), and 4) trigger disruption of *H. influenzae* biofilms in the chinchilla model of experimental otitis media by redirecting the immune response toward immuno-protective domains of DNA-binding (DNABII) proteins (Novotny et al., 2019). Therefore, the presence and ubiquity of eDNA in clinical specimens of the different types of otitis media including COME can serve as an important clinical target for our novel eDNA-directed approach (NucB) to control biofilm associated diseases including COME.

Before an agent such as NucB can be used in clinical applications, it is essential to ensure that they are non-toxic and safe for human use. Our results, though preliminary, have provided evidence that NucB is a safe anti-biofilm agent as it had no cytotoxicity to human bronchial epithelial cells relevant to the middle ear cavity. It is important to further investigate the immunogenic effect of NucB on human cells *in vitro* by measuring the release of inflammatory markers such cytokines, determining glutathione and leukocyte proliferation, before progressing to investigate the safety of NucB using an animal model. There is some evidence from this study and other studies that NucB, as a DNase, would very likely to be safe for the use in the clinical setting (Chan et al., 2018).

If NucB is shown to be safe for use, there are a number of different options available for delivery of the enzyme. Drug formulations such as ear drops generally contain salts at low concentrations and could be administered locally into the middle ear following myringotomy and grommet insertion to reduce recurrence of disease and the need for further surgery. Furthermore, our preliminary results have shown that NucB can be effective in combination with antibiotics. Potentially, NucB could be incorporated with antibiotics either in ear drops or in other drug formulations such as biodegradable modified-release antibiotic pellets (Daniel et al., 2012a). A combination of NucB and antibiotics potentially can also be applied locally to treat persistent post-tympanostomy otorrhoea. In a recent clinical trial, it was shown that Dornase alfa can facilitate the clearing of grommets in 59% of children with clogged grommets, though this effect was not significantly different from treatment with antibiotics (Chan et al., 2018).

Another potential approach for the local delivery of NucB for the treatment of COME is through the transtympanic route. NucB can potentially be incorporated into phage-based therapeutic preparations to pass through the intact tympanic membrane into the middle ear cavity for the treatment of biofilms associated with otitis media including COME. In experimental animal models, phage-based products armed with specific peptide sequences have been used to deliver payload across the tympanic membrane into the middle ear cavity with minimal or no delivery into the inner ear (Kurabi et al., 2018). Other forms of trans-tympanic drug delivery system that potentially can be used to deliver NucB for the treatment of COME include the penta-block copolymer of poloxamer 407–polybutylphosphoester (P407-PBP). This system has been developed to deliver an entire course of antimicrobial therapy to the middle ear when applied once to the tympanic membrane through the EEC (Yang et al., 2016). The product flows easily during application and forms a strong hydrogel on the tympanic membrane. When this therapy was tested on *H. influenzae*-induced

experimental otitis media, it eradicated AOM in the chinchilla model in 100% of cases, whereas only 63% of animals receiving 1% ciprofloxacin alone had cleared infection by day 7. NucB can also potentially be incorporated into mouthwash or nasal spray to control biofilms associated with dental diseases or chronic rhinosinusitis, respectively (Jakubovics and Burgess, 2015, Shields et al., 2013). Alternatively, NucB could be used to coat artificial surfaces such as polymethylmethacrylate (PMMA), stainless steel or titanium, that are commonly used in the manufacture of prosthetic medical devices including dentures, dental implants or orthopaedic implant materials, in order to delay or prevent medical device-related biofilm infections (Swartjes et al., 2013, Jakubovics and Burgess, 2015, Khatoon et al., 2018).

Another important issue necessary for the application of NucB in the clinical setting is to develop systems for the cost-effective production at scales that would be needed for clinical use. Towards this goal, an optimized *B. licheniformis* NucB expression system has been developed that potentially could be scaled up (Rajarajan et al., 2013).

6.2 Limitations of the study and future work

The work presented in this thesis has provided a greater understanding of the composition of microbial populations associated with MEEFs and adenoids in patients with COME and the role of eDNA in maintaining stability of *in vitro* biofilms formed by representative microbial isolates. However, there are a number of limitations that need to be considered.

Here, bacterial communities colonising the middle ear and adenoids of children with COME were characterised using culture-independent 16S rRNA gene sequencing. In general, this approach can provide a comprehensive overview of the composition of microbiome from which DNA is present at the time of sampling. However, it does not provide information on the viability and the function of microorganisms in their environment. Additionally, there are some important biases to consider when conducting these studies (Eisenhofer et al., 2019). The efficacy of DNA extraction methods and amplicon primers can be variable across different bacterial species, which can ultimately result in underrepresentation of some microorganisms. Samples can possibly be contaminated by DNA from different sources including laboratory reagents, people, environment, DNA extraction kits, and cross-contamination from other high biomass samples and sequencing runs from adjacent wells or tubes which can heavily confound true biological signals within low biomass samples or environments (Eisenhofer et al., 2019, Salter et al., 2014). In this study, the use of an optimised microbial identification approach that incorporated both improved culture methods

and culture-independent 16S rRNA gene sequencing techniques enabled a more complete characterisation of microbial communities of COME than either technique alone. This is of particular importance in order to validate that species isolated from MEEFs were representative of the total microbial population present. Together, these methods showed that COME samples are dominated by *T. otitidis, A. otitis*, and CoNS and also harboured a wide range of other bacterial species including *H. influenzae, S. pneumoniae*, and *M. catarrhalis*. Furthermore, preliminary microbiome and genomic comparisons of the co-occurrence of COME pathogens in paired MEEFs and adenoids from 3 patients revealed more complex microbial profiles in adenoids that were concurrently colonised with similar bacterial species to those found in MEEFs, including major paediatric otopathogens such as *H. influenzae, S. pneumoniae, M. catarrhalis* and a variety of other bacterial species. Bacterial genera such as *Haemophilus, Streptococcus*, and *Moraxella* that colonise adenoids may contain both pathogenic species and commensal species. Therefore, there is a need to characterise these genera at the species and even the strain level.

Genome sequencing is the most powerful approach to characterise microbial strains and to explore differences between closely related isolates. Here, genome sequencing was employed in a preliminary attempt to identify identical strains in paired MEEF and adenoid swabs. However, streptococcal species that were detected in both sample types from the same patient were not genetically identical. Therefore, there was no clear evidence that strains had translocated between adenoids and the middle ear. The hypothesis that adenoids act as a source for otopathogens in COME needs further validation. Further microbiome investigations should focus on establishing causal relationships between microorganisms and COME. In particular, it is important to assess the role of controversial pathogens such as *T. otitidis, A. otitis*, and CoNS in addition to the major paediatric otopathogens such as *H. influenzae, S. pneumoniae, M. catarrhalis* as this may lead to new avenues for treatment. This could be addressed by the characterisation of microbial communities present in matched adenoids and MEEFs from a larger cohort of COME patients with appropriate controls using both traditional culture and 16S rRNA gene sequencing techniques, detailed bioinformatics analysis and full genome comparisons of similar species isolated from both the adenoids and middle ear of the same patient. More advanced culture-independent techniques such as metagenomics would enable investigation of the functional roles and characteristics of the microbial community by identifying the entire coding potential of the microbial community. Proteomics could allow characterisation of the expression of proteins, although currently this approach may be limited by the need for relatively large quantities of biomass.

Transcriptomics is more sensitive and would allow characterisation of mRNA expression. Together, these approaches could be applied to further explore the functional role and causal relationships of the middle ear microbiome.

Another limitation to our work is that the *in vitro* biofilm models used in this research may not be closely relevant to the *in vivo* biofilms found in COME patients. A key step will be the development of improved model systems that more closely mimic the *in vivo* environment. For example, a well-controlled *in vitro* model that will simulate epithelial mucosa of the middle ear with its secretions would be of great benefit. This could be performed by incorporating human epithelial cells similar to the approach used by Marks et al. (2012) to model *in vivo* biofilms of *S. pneumoniae* or a 3-D host tissue model. It would be interesting to assess the anti-biofilm action of NucB against biofilms cultured in these more realistic *in vitro* models and ultimately in already established animal models of otitis media and biofilms (Goodman et al., 2011, Novotny et al., 2019). Data from models such as these would provide strong proof-of-concept evidence to support further development and ultimately human clinical trials.

Our results have provided evidence that NucB can potentially increase the efficacy of antibiotics against *in vitro* biofilms. It is important to note that this analysis was limited to only one type of antibiotic (Co-amoxiclav) and a limited number of *S. aureus* clinical isolates. It would be interesting to extend this research, including assessing the efficacy of NucB in combination with other types of penicillin (amoxicillin), macrolides (azithromycin), aminoglycosides (gentamicin), and quinolones (ciprofloxacin). In addition, these combinations could be assessed against biofilms formed by a greater number of relevant clinical isolates from cases of COME. This analysis could be performed using the methods optimised in this thesis such as colorimetric XTT and MBEC assays. It is important to consider including total viable count calculations during determination of the minimum biofilm eradication concentration (MBEC) values of antibiotics when using the MBEC assay in addition to the measurement of absorbance (A_{650}) that was performed in this investigation. If DNases such as NucB are to be used in a clinical setting using the potential formulations that were previously described in section 6.1, it is of particularly important to ascertain their efficacy in treatment solutions. Our preliminary results have shown that NucB efficacy was not affected when it was combined with high concentrations of Co-amoxiclav. Further investigations are required to assess compatibility of NucB with common components of ear drops, nasal sprays or mouthwash products. It will be important to design a formulation that permits optimal activity of NucB if it is to prove useful at disrupting COME biofilms *in vivo*.

6.3 Conclusion

In conclusion, the work presented in this thesis has provided a detailed characterisation of microbial populations associated with COME through incorporating a combination of improved culture and culture-independent techniques. This approach is of particular importance in order to provide a rich resource of representative isolates from COME patients which were shown to have the ability to form robust biofilms *in vitro*. NucB has been shown to be potentially nontoxic on human cells and exhibited a potent anti-biofilm action against the majority of COME isolates. Several clinical characteristics of COME are typical of biofilm infection. Greater understanding of the role of bacterial biofilms in the pathogenesis of COME will allow the development of novel therapeutic approaches to prevent recurrence of the disease. NucB alone or in combination with antibiotics may potentially be a potent and safe agent to control a wide range of biofilm-associated conditions including COME.

References

AARTS, J. W. M., VAN DEN AARDWEG, M. T. A., ROVERS, M. M., BONTEN, M. J. M. & SCHILDER, A. G. M. 2010. Alterations in the nasopharyngeal bacterial flora after adenoidectomy in children: A systematic review. *Otolaryngology - Head and Neck Surgery,* 142**,** 15-20.e1.

AKMATOV, M. K., KOCH, N., VITAL, M., AHRENS, W., FLESCH-JANYS, D., FRICKE, J., GATZEMEIER, A., GREISER, H., GÜNTHER, K., ILLIG, T., KAAKS, R., KRONE, B., KÜHN, A., LINSEISEN, J., MEISINGER, C., MICHELS, K., MOEBUS, S., NIETERS, A., OBI, N., SCHULTZE, A., SIX-MERKER, J., PIEPER, D. H. & PESSLER, F. 2017. Determination of nasal and oropharyngeal microbiomes in a multicenter population-based study - findings from Pretest 1 of the German National Cohort. *Sci. Rep.,* 7**,** 1855-1855.

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. *Mol. Microbiol.,* 59**,** 1114-28.

ALMEIDA, F., WOLF, J. M. & CASADEVALL, A. 2015. Virulence-associated enzymes of Cryptococcus neoformans. *Eukaryotic cell,* 14**,** 1173-1185.

ALONSO, B., CRUCES, R., PÉREZ, A., SÁNCHEZ-CARRILLO, C. & GUEMBE, M. 2017. Comparison of the XTT and resazurin assays for quantification of the metabolic activity of Staphylococcus aureus biofilm. *J. Microbiol. Methods,* 139**,** 135-137.

ANDERSSON, S., DALHAMMAR, G., LAND, C. J. & KUTTUVA RAJARAO, G. 2009. Characterization of extracellular polymeric substances from denitrifying organism Comamonas denitrificans. *Appl. Microbiol. Biotechnol.,* 82**,** 535-43.

ANDREWS, J. M. 2001. Determination of minimum inhibitory concentrations. *J. Antimicrob. Chemother.,* 48 Suppl 1**,** 5-16.

ANGELETTI, S., DICUONZO, G., AVOLA, A., CREA, F., DEDEJ, E., VAILATI, F., FARINA, C. & DE FLORIO, L. 2015. Viridans Group Streptococci clinical isolates: MALDI-TOF mass spectrometry versus gene sequence-based identification. *PLoS One,* 10**,** e0120502.

ARCIOLA, C. R., CAMPOCCIA, D. & MONTANARO, L. 2018. Implant infections: adhesion, biofilm formation and immune evasion. *Nature Reviews Microbiology,* 16**,** 397.

ARI, O., KARABUDAK, S., KALCIOGLU, M. T., GUNDUZ, A. Y. & DURMAZ, R. 2019. The bacteriome of otitis media with effusion: Does it originate from the adenoid? *Int. J. Pediatr. Otorhinolaryngol.,* 126**,** 109624.

ATKINSON, H., WALLIS, S. & COATESWORTH, A. P. 2015. Otitis media with effusion. *Postgrad. Med.,* 127**,** 381-385.

BAEK, I., KIM, M., LEE, I., NA, S. I., GOODFELLOW, M. & CHUN, J. 2018. Phylogeny Trumps Chemotaxonomy: A Case Study Involving Turicella otitidis. *Front. Microbiol.,* 9**,** 834.

BAKALETZ, L. O. 2012. Bacterial biofilms in the upper airway-evidence for role in pathology and implications for treatment of otitis media. *Paediatr. Respir. Rev.,* 13**,** 154-159.

BAO, Y., ZHANG, X., JIANG, Q., XUE, T. & SUN, B. 2015. Pfs promotes autolysis-dependent release of eDNA and biofilm formation in Staphylococcus aureus. *Med. Microbiol. Immunol.,* 204**,** 215-226.

BARAKATE, M., BECKENHAM, E., CUROTTA, J. & DA CRUZ, M. 2007. Bacterial biofilm adherence to middle-ear ventilation tubes: scanning electron micrograph images and literature review. *J. Laryngol. Otol.,* 121**,** 993-7.

BARRON, C. L., KAMEL-ABUSALHA, L. B., SETHIA, R., GOODMAN, S. D., ELMARAGHY, C. A. & BAKALETZ, L. O. 2019. Identification of essential biofilm proteins in middle ear fluids of otitis media with effusion patients. *Laryngoscope*.

BASLE, A., HEWITT, L., KOH, A., LAMB, H. K., THOMPSON, P., BURGESS, J. G., HALL, M. J., HAWKINS, A. R., MURRAY, H. & LEWIS, R. J. 2018. Crystal structure of NucB, a biofilm-degrading endonuclease. *Nucleic Acids Res.,* 46**,** 473-484.

BAUMGARTEN, T., SPERLING, S., SEIFERT, J., VON BERGEN, M., STEINIGER, F., WICK, L. Y. & HEIPIEPER, H. J. 2012. Membrane vesicle formation as a multiple-stress response mechanism enhances Pseudomonas putida DOT-T1E cell surface hydrophobicity and biofilm formation. *Appl. Environ. Microbiol.,* 78**,** 6217-24.

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. *Infect. Immun.,* 80**,** 1634-8.

BEGEROW, D., NILSSON, H., UNTERSEHER, M. & MAIER, W. 2010. Current state and perspectives of fungal DNA barcoding and rapid identification procedures. *Appl. Microbiol. Biotechnol.,* 87**,** 99-108.

BERENDS, E. T., HORSWILL, A. R., HASTE, N. M., MONESTIER, M., NIZET, V. & VON KOCKRITZ-BLICKWEDE, M. 2010. Nuclease expression by Staphylococcus aureus facilitates escape from neutrophil extracellular traps. *J. Innate Immun.,* 2**,** 576-86.

BERGÉ, M. J., KAMGOUÉ, A., MARTIN, B., POLARD, P., CAMPO, N. & CLAVERYS, J.-P. 2013. Midcell Recruitment of the DNA Uptake and Virulence Nuclease, EndA, for Pneumococcal Transformation. *PLoS Pathog.,* 9**,** e1003596.

BERGER, G. 1989. Nature of spontaneous tympanic membrane perforation in acute otitis media in children. *J. Laryngol. Otol.,* 103**,** 1150-3.

BERKMAN, N. D., WALLACE, I. F., STEINER, M. J., HARRISON, M., GREENBLATT, A. M., LOHR, K. N., KIMPLE, A. & YUEN, A. 2013. Otitis media with effusion: comparative effectiveness of treatments.

BERMAN, S. 1995. Otitis media in children. *N. Engl. J. Med.,* 332**,** 1560-1565.

BIDARIAN-MONIRI, A., RAMOS, M.-J. & EJNELL, H. 2014. Autoinflation for treatment of persistent otitis media with effusion in children: A cross-over study with a 12-month follow-up. *Int. J. Pediatr. Otorhinolaryngol.,* 78**,** 1298-1305.

BIOGRAPHIX. 2006. *Biographixmedia* [Online]. Available: http://www.biographixmedia.com/human/ear-anatomy.html [Accessed 24 February 2015 2015].

BLANC, F., AYACHE, D., CALMELS, M. N., DEGUINE, O., FRANÇOIS, M., LEBOULANGER, N., LESCANNE, E., MARIANOWSKI, R., NEVOUX, J., NICOLLAS, R., TRINGALI, S., TESSIER, N., FRANCO-VIDAL, V., BORDURE, P. & MONDAIN, M. 2018. Management of otitis media with effusion in children. Société française d'ORL et de chirurgie cervico-faciale clinical practice guidelines. *Eur. Ann. Otorhinolaryngol. Head Neck Dis.,* 135**,** 269-273.

BLOKESCH, M. & SCHOOLNIK, G. K. 2008. The extracellular nuclease Dns and its role in natural transformation of Vibrio cholerae. *J. Bacteriol.,* 190**,** 7232-40.

BLUESTONE, C. D., STEPHENSON, J. S. & MARTIN, L. M. 1992. Ten-year review of otitis media pathogens. *Pediatr. Infect. Dis. J.,* 11**,** S7-11.

BOERS, S. A., DE ZEEUW, M., JANSEN, R., VAN DER SCHROEFF, M. P., VAN ROSSUM, A. M. C., HAYS, J. P. & VERHAEGH, S. J. C. 2018. Characterization of the nasopharyngeal and middle ear microbiota in gastroesophageal reflux-prone versus gastroesophageal reflux non-prone children. *Eur. J. Clin. Microbiol. Infect. Dis.,* 37**,** 851-857.

BORONAT-ECHEVERRÍA, N., AGUIRRE-MARISCAL, H., CARMOLINGA-PONCE, M., SEVILLA-DELGADO, Y., MICELI-FLORES, R., KENNEDY-PADILLA, A. & MEJÍA-ARANGURÉ, J. M. 2016. Helicobacter pylori detection and clinical symptomatology of gastroesophageal reflux disease in pediatric patients with otitis media with effusion. *Int. J. Pediatr. Otorhinolaryngol.,* 87**,** 126-129.

BOSLEY, G. S., WHITNEY, A. M., PRUCKLER, J. M., MOSS, C. W., DANESHVAR, M., SIH, T. & TALKINGTON, D. F. 1995. Characterization of ear fluid isolates of Alloiococcus otitidis from patients with recurrent otitis media. *J. Clin. Microbiol.,* 33**,** 2876-80.

BOU HAIDAR, N., MARAIS, S., DÉ, E., SCHAUMANN, A., BARREAU, M., FEUILLOLEY, M. G. J. & DUNCAN, A. C. 2020. Chronic wound healing: A specific antibiofilm protein-asymmetric release system. *Materials Science and Engineering: C,* 106**,** 110130.

BOULOS, L., PREVOST, M., BARBEAU, B., COALLIER, J. & DESJARDINS, R. 1999. LIVE/DEAD BacLight : application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *J. Microbiol. Methods,* 37**,** 77-86.

BRADY, A. J., LAVERTY, G., GILPIN, D. F., KEARNEY, P. & TUNNEY, M. 2017. Antibiotic susceptibility of planktonic- and biofilm-grown staphylococci isolated from implant-associated infections: should MBEC and nature of biofilm formation replace MIC? *J. Med. Microbiol.,* 66**,** 461-469.

BRIDGES, A. A. & BASSLER, B. L. 2019. The intra-genus and inter-species quorum-sensing autoinducers exert distinct control over Vibrio cholerae biofilm formation and dispersal. *BioRxiv***,** 713685.

BROLY, E., RISSE, J., MASCHINO, F. & WAHL, D. 2016. Cardiac Tamponade Due to Actinomyces odontolyticus Originating From a Dentigerous Cyst. *J. Oral Maxillofac. Surg.,* 74**,** 2453-2456.

BROWN, E. L., BELOW, J. E., FISCHER, R. S., ESSIGMANN, H. T., HU, H., HUFF, C., ROBINSON, D. A., PETTY, L. E., AGUILAR, D., BELL, G. I. & HANIS, C. L. 2015. Genome-Wide Association Study of Staphylococcus aureus Carriage in a Community-Based Sample of Mexican-Americans in Starr County, Texas. *PLoS One,* 10**,** e0142130.

BROWNING, G. G., ROVERS, M. M., WILLIAMSON, I., LOUS, J. & BURTON, M. J. 2010. Grommets (ventilation tubes) for hearing loss associated with otitis media with effusion in children. *Cochrane Database Syst. Rev.*

BUENO, J. 2014. Anti-biofilm drug susceptibility testing methods: looking for new strategies against resistance mechanism. *J Microbial Biochem Technol S,* 3**,** 2.

BUZATTO, G., TAMASHIRO, E., PROENCA-MODENA, J., SATURNO, T., PRATES, M., GAGLIARDI, T., CARENZI, L., MASSUDA, E., HYPPOLITO, M. & VALERA, F. 2017. The pathogens profile in children with otitis media with effusion and adenoid hypertrophy. *PLoS One,* 12**,** e0171049.

CARRIE, S., HUTTON, D., BIRCHALL, J., GREEN, G. & PEARSON, J. 1992. Otitis media with effusion: components which contribute to the viscous properties. *Acta Otolaryngol.,* 112**,** 504-511.

CARROLO, M., FRIAS, M. J., PINTO, F. R., MELO-CRISTINO, J. & RAMIREZ, M. 2010. Prophage spontaneous activation promotes DNA release enhancing biofilm formation in Streptococcus pneumoniae. *PLoS One,* 5**,** e15678.

CATLIN, B. W. 1956. Extracellular deoxyribonucleic acid of bacteria and a deoxyribonuclease inhibitor. *Science,* 124**,** 441-2.

CATLIN, B. W. & CUNNINGHAM, L. S. 1958. Studies of extracellular and intracellular bacterial deoxyribonucleic acids. *J. Gen. Microbiol.,* 19**,** 522-39.

CAVALIERE, R., BALL, J. L., TURNBULL, L. & WHITCHURCH, C. B. 2014a. The biofilm matrix destabilizers, EDTA and DNaseI, enhance the susceptibility of nontypeable Hemophilus influenzae biofilms to treatment with ampicillin and ciprofloxacin. *Microbiologyopen,* 3**,** 557-67.

CAVALIERE, R., BALL, J. L., TURNBULL, L. & WHITCHURCH, C. B. 2014b. 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. *J. Clin. Microbiol.,* 37**,** 1771-6.

CHALMERS, C., KHEMLANI, A. H. J., SOHN, C. R., SAN LOH, J. M. & PROFT, T. 2017. Streptococcus pyogenes nuclease A (SpnA) mediated virulence does not exclusively depend on nuclease activity. *Journal of Microbiology, Immunology and Infection*.

CHAN, C. L., RICHTER, K., WORMALD, P. J., PSALTIS, A. J. & VREUGDE, S. 2017a. Alloiococcus otitidis Forms Multispecies Biofilm with Haemophilus influenzae: Effects on Antibiotic Susceptibility and Growth in Adverse Conditions. *Frontiers in cellular and infection microbiology,* 7**,** 344.

CHAN, C. L., WABNITZ, D., BARDY, J. J., BASSIOUNI, A., WORMALD, P. J., VREUGDE, S. & PSALTIS, A. J. 2016. The microbiome of otitis media with effusion. *Laryngoscope,* 126**,** 2844-2851.

CHAN, C. L., WABNITZ, D., BASSIOUNI, A., WORMALD, P. J., VREUGDE, S. & PSALTIS, A. J. 2017b. Identification of the Bacterial Reservoirs for the Middle Ear Using Phylogenic Analysis. *JAMA otolaryngology-- head & neck surgery,* 143**,** 155-161.

CHAN, H. K., AU-YEUNG, K. L. & GONDA, I. 1996. Effects of additives on heat denaturation of rhDNase in solutions. *Pharm. Res.,* 13**,** 756-61.

CHAN, K. H., ALLEN, G. C., KELLEY, P. E., STREUBEL, S. O., FRIEDMAN, N. R., YOON, P., GAO, D., RUIZ, A. G. & JUNG, T. T. K. 2018. Dornase Alfa Ototoxic Effects in Animals and Efficacy in the Treatment of Clogged Tympanostomy Tubes in Children: A Preclinical Study and a Randomized Clinical Trial. *JAMA otolaryngology-- head & neck surgery,* 144**,** 776-780.

CHERNY, K. E. & SAUER, K. 2019. Pseudomonas aeruginosa requires the DNA-specific endonuclease EndA to degrade eDNA to disperse from the biofilm. *J. Bacteriol.***,** JB. 00059-19.

CHIANG, W. C., NILSSON, M., JENSEN, P. O., HOIBY, N., NIELSEN, T. E., GIVSKOV, M. & TOLKER-NIELSEN, T. 2013. Extracellular DNA shields against aminoglycosides in Pseudomonas aeruginosa biofilms. *Antimicrob. Agents Chemother.,* 57**,** 2352-61.

CHO, C., CHANDE, A., GAKHAR, L., BAKALETZ, L. O., JURCISEK, J. A., KETTERER, M., SHAO, J., GOTOH, K., FOSTER, E. & HUNT, J. 2015. Role of the nuclease of nontypeable Haemophilus influenzae in dispersal of organisms from biofilms. *Infect. Immun.,* 83**,** 950-957.

CHO, E.-S., KIM, J.-H., YOON, K.-H., KIM, Y.-H. & NAM, S.-W. 2012. Overexpression and Characterization of Bovine Pancreatic Deoxyribonuclease I in Saccharomyces cerevisiae and Pichia pastoris. *Microbiology and Biotechnology Letters,* 40**,** 348-355.

CHOLE, R. A. & FADDIS, B. T. 2002. EVidence for microbial biofilms in cholesteatomas. *Archives of Otolaryngology–Head & Neck Surgery,* 128**,** 1129-1133.

CHONMAITREE, T., JENNINGS, K., GOLOVKO, G., KHANIPOV, K., PIMENOVA, M., PATEL, J. A., MCCORMICK, D. P., LOEFFELHOLZ, M. J. & FOFANOV, Y. 2017. Nasopharyngeal microbiota in infants and changes during viral upper respiratory tract infection and acute otitis media. *PLoS One,* 12**,** e0180630.

COENYE, T., GOERES, D., VAN BAMBEKE, F. & BJARNSHOLT, T. 2018. Should standardized susceptibility testing for microbial biofilms be introduced in clinical practice? *Clin. Microbiol. Infect.,* 24**,** 570-572.

CONOVER, M. S., MISHRA, M. & DEORA, R. 2011. Extracellular DNA is essential for maintaining Bordetella biofilm integrity on abiotic surfaces and in the upper respiratory tract of mice. *PLoS One,* 6**,** e16861.

COSTERTON, J. W., GEESEY, G. G. & CHENG, K. J. 1978. How bacteria stick. *Sci. Am.,* 238**,** 86-95.

COSTERTON, J. W., STEWART, P. S. & GREENBERG, E. P. 1999. Bacterial biofilms: a common cause of persistent infections. *Science,* 284**,** 1318-22.

COTICCHIA, J. M., CHEN, M., SACHDEVA, L. & MUTCHNICK, S. 2013. New paradigms in the pathogenesis of otitis media in children. *Frontiers in pediatrics,* 1**,** 52.

DALL, G. F. 2013. *An investigation, using an in-vitro alginate biofilm model, into locally delivered antibiotic combinations to treat staphylococcal prosthetic infection.* Doctor of Medicine Dissertation, The University of Edinburgh.

DANIEL, M., CHESSMAN, R., AL-ZAHID, S., RICHARDS, B., RAHMAN, C., ASHRAF, W., MCLAREN, J., COX, H., QUTACHI, O., FORTNUM, H., FERGIE, N., SHAKESHEFF, K., BIRCHALL, J. P. & BAYSTON, R. R. 2012a.

Biofilm eradication with biodegradable modified-release antibiotic pellets: a potential treatment for glue ear. *Arch. Otolaryngol. Head Neck Surg.,* 138**,** 942-9.

DANIEL, M., IMTIAZ-UMER, S., FERGIE, N., BIRCHALL, J. P. & BAYSTON, R. 2012b. Bacterial involvement in otitis media with effusion. *Int. J. Pediatr. Otorhinolaryngol.,* 76**,** 1416-22.

DAPUNT, U., HÄNSCH, G. M. & ARCIOLA, C. R. 2016. Innate Immune Response in Implant-Associated Infections: Neutrophils against Biofilms. *Materials (Basel),* 9**,** 387.

DAS, T., KROM, B. P., VAN DER MEI, H. C., BUSSCHER, H. J. & SHARMA, P. K. 2011. DNA-mediated bacterial aggregation is dictated by acid-base interactions. *Soft Matter,* 7**,** 2927-2935.

DAVIES, D. 2003. Understanding biofilm resistance to antibacterial agents. *Nature reviews. Drug discovery,* 2**,** 114-22.

DE BUHR, N., STEHR, M., NEUMANN, A., NAIM, H. Y., VALENTIN-WEIGAND, P., VON KÖCKRITZ-BLICKWEDE, M. & BAUMS, C. G. 2015. Identification of a novel DNase of Streptococcus suis (EndAsuis) important for neutrophil extracellular trap degradation during exponential growth. *Microbiology,* 161**,** 838-850.

DE LA FUENTE-NUNEZ, C., REFFUVEILLE, F., FERNANDEZ, L. & HANCOCK, R. E. 2013. Bacterial biofilm development as a multicellular adaptation: antibiotic resistance and new therapeutic strategies. *Curr. Opin. Microbiol.,* 16**,** 580-9.

DERRE-BOBILLOT, A., CORTES-PEREZ, N. G., YAMAMOTO, Y., KHARRAT, P., COUVE, E., DA CUNHA, V., DECKER, P., BOISSIER, M. C., ESCARTIN, F., CESSELIN, B., LANGELLA, P., BERMUDEZ-HUMARAN, 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. *Mol. Microbiol.,* 89**,** 518-31.

DI LUCA, M., NAVARI, E., ESIN, S., MENICHINI, M., BARNINI, S., TRAMPUZ, A., CASANI, A. & BATONI, G. 2017. Detection of biofilms in biopsies from chronic rhinosinusitis patients: in vitro biofilm forming ability and antimicrobial susceptibility testing in biofilm mode of growth of isolated bacteria. *Advances in Microbiology, Infectious Diseases and Public Health.* Springer.

DOĞRU, M., KURAN, G., HAYTOĞLU, S., DENGIZ, R. & ARıKAN, O. K. 2015. Role of laryngopharyngeal reflux in the pathogenesis of otitis media with effusion. *J Int Adv Otol,* 11**,** 66-71.

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

DOMENECH, M. & GARCIA, E. 2018. Autolysin-Independent DNA Release in Streptococcus pneumoniae in vitro Biofilms. *bioRxiv*.

DONLAN, R. M. & COSTERTON, J. W. 2002. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin. Microbiol. Rev.,* 15**,** 167-93.

DORWARD, D. W. & GARON, C. F. 1990. DNA Is Packaged within Membrane-Derived Vesicles of Gram-Negative but Not Gram-Positive Bacteria. *Appl. Environ. Microbiol.,* 56**,** 1960-2.

ECKHART, L., FISCHER, H., BARKEN, K. B., TOLKER-NIELSEN, T. & TSCHACHLER, E. 2007. DNase1L2 suppresses biofilm formation by Pseudomonas aeruginosa and Staphylococcus aureus. *Br. J. Dermatol.,* 156**,** 1342-1345.

EHRLICH, G. D., VEEH, R., WANG, X., COSTERTON, J. W., HAYES, J. D., HU, F. Z., DAIGLE, B. J., EHRLICH, M. D. & POST, J. C. 2002. Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA,* 287**,** 1710-5.

EISENHOFER, R., MINICH, J. J., MAROTZ, C., COOPER, A., KNIGHT, R. & WEYRICH, L. S. 2019. Contamination in Low Microbial Biomass Microbiome Studies: Issues and Recommendations. *Trends Microbiol.,* 27**,** 105-117.

ELGHARABLY, H., MANN, E., AWAD, H., GANESH, K., GHATAK, P. D., GORDILLO, G., SAI-SUDHAKAR, C. B., ROY, S., WOZNIAK, D. J. & SEN, C. K. 2013. First evidence of sternal wound biofilm following cardiac surgery. *PLoS One,* 8**,** e70360.

ESPELAND, E. M. & WETZEL, R. G. 2001. Complexation, Stabilization, and UV Photolysis of Extracellular and Surface-Bound Glucosidase and Alkaline Phosphatase: Implications for Biofilm Microbiota. *Microb. Ecol.,* 42**,** 572-585.

FARISA BANU, S., THAMOTHARAN, S., GOWRISHANKAR, S., KARUTHA PANDIAN, S. & NITHYANAND, P. 2019. Marine bacterial DNase curtails

virulence and disrupts biofilms of Candida albicans and non-albicans Candida species. *Biofouling*, 1-11.

FERGIE, N., BAYSTON, R., PEARSON, J. P. & BIRCHALL, J. P. 2004. Is otitis media with effusion a biofilm infection? *Clin. Otolaryngol. Allied Sci.,* 29**,** 38-46.

FERNÁNDEZ, L., GONZÁLEZ, S., CAMPELO, A. B., MARTÍNEZ, B., RODRÍGUEZ, A. & GARCÍA, P. 2017. Downregulation of autolysin-encoding genes by phage-derived lytic proteins inhibits biofilm formation in Staphylococcus aureus. *Antimicrob. Agents Chemother.,* 61**,** e02724-16.

FLEMING, D. & RUMBAUGH, K. 2018. The consequences of biofilm dispersal on the host. *Sci. Rep.,* 8**,** 10738.

FLEMING, D. & RUMBAUGH, K. P. 2017. Approaches to Dispersing Medical Biofilms. *Microorganisms,* 5.

FLEMMING, H. C. & WINGENDER, J. 2010. The biofilm matrix. *Nat. Rev. Microbiol.,* 8**,** 623-33.

FLEMMING, H. C., WINGENDER, J., SZEWZYK, U., STEINBERG, P., RICE, S. A. & KJELLEBERG, S. 2016. Biofilms: an emergent form of bacterial life. *Nat. Rev. Microbiol.,* 14**,** 563-75.

FLEMMING, H. C. & WUERTZ, S. 2019. Bacteria and archaea on Earth and their abundance in biofilms. *Nat. Rev. Microbiol.,* 17**,** 247-260.

FLYNN, T., MOLLER, C., JONSSON, R. & LOHMANDER, A. 2009. The high prevalence of otitis media with effusion in children with cleft lip and palate as compared to children without clefts. *Int. J. Pediatr. Otorhinolaryngol.,* 73**,** 1441-6.

FORMÁNEK, M., ZELENÍK, K., KOMÍNEK, P. & MATOUŠEK, P. 2015. Diagnosis of extraesophageal reflux in children with chronic otitis media with effusion using Peptest. *Int. J. Pediatr. Otorhinolaryngol.,* 79**,** 677-679.

FOUHY, F., CLOONEY, A. G., STANTON, C., CLAESSON, M. J. & COTTER, P. D. 2016. 16S rRNA gene sequencing of mock microbial populations-impact of DNA extraction method, primer choice and sequencing platform. *BMC Microbiol.,* 16**,** 123.

FRANK, R. M. A. H., G 1970. An unltrastructural study of human supragingival pdental plaque formation. In Dental Plaque. *In Dental Plaque ed. McHugh*, 85–108.

FREDERIKSEN, B., PRESSLER, T., HANSEN, A., KOCH, C. & HOIBY, N. 2006. Effect of aerosolized rhDNase (Pulmozyme) on pulmonary colonization in patients with cystic fibrosis. *Acta Paediatr.,* 95**,** 1070-4.

FREDHEIM, E. G., KLINGENBERG, C., ROHDE, H., FRANKENBERGER, S., GAUSTAD, P., FLAEGSTAD, T. & SOLLID, J. E. 2009. Biofilm formation by Staphylococcus haemolyticus. *J. Clin. Microbiol.,* 47**,** 1172-80.

FUJIHARA, J., YASUDA, T., KUNITO, T., FUJII, Y., TAKATSUKA, H., MORITANI, T. & TAKESHITA, H. 2008. Two N-linked glycosylation sites (Asn18 and Asn106) are both required for full enzymatic activity, thermal stability, and resistance to proteolysis in mammalian deoxyribonuclease I. *Biosci. Biotechnol. Biochem.***,** 0811051143-0811051143.

FUNKE, G., STUBBS, S., ALTWEGG, M., CARLOTTI, A. & COLLINS, M. D. 1994. Turicella otitidis gen. nov., sp. nov., a coryneform bacterium isolated from patients with otitis media. *Int. J. Syst. Bacteriol.,* 44**,** 270-3.

GODEKE, J., HEUN, M., BUBENDORFER, S., PAUL, K. & THORMANN, K. M. 2011. Roles of two Shewanella oneidensis MR-1 extracellular endonucleases. *Appl. Environ. Microbiol.,* 77**,** 5342-51.

GOK, U., BULUT, Y., KELES, E., YALCIN, S. & DOYMAZ, M. Z. 2001. Bacteriological and PCR analysis of clinical material aspirated from otitis media with effusions. *Int. J. Pediatr. Otorhinolaryngol.,* 60**,** 49-54.

GOLTERMANN, L. & TOLKER-NIELSEN, T. 2017. Importance of the Exopolysaccharide Matrix in Antimicrobial Tolerance of Pseudomonas aeruginosa Aggregates. *Antimicrob. Agents Chemother.,* 61.

GOLZ, A., ANGEL-YEGER, B. & PARUSH, S. 1998. Evaluation of balance disturbances in children with middle ear effusion. *Int. J. Pediatr. Otorhinolaryngol.,* 43**,** 21-6.

GOMEZ-GARCES, J. L., ALHAMBRA, A., ALOS, J. I., BARRERA, B. & GARCIA, G. 2004. Acute and chronic otitis media and Turicella otitidis: a controversial association. *Clin. Microbiol. Infect.,* 10**,** 854-7.

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 Immunol.,* 4**,** 625-37.

GRANDE, R., DI CAMPLI, E., DI BARTOLOMEO, S., VERGINELLI, F., DI GIULIO, M., BAFFONI, M., BESSA, L. J. & CELLINI, L. 2012. Helicobacter pylori biofilm: a protective environment for bacterial recombination. *J. Appl. Microbiol.,* 113**,** 669-676.

GRANDE, R., DI MARCANTONIO, M. C., ROBUFFO, I., POMPILIO, A., CELIA, C., DI MARZIO, L., PAOLINO, D., CODAGNONE, M., MURARO, R. & STOODLEY, P. 2015. Helicobacter pylori ATCC 43629/NCTC 11639 outer membrane vesicles (OMVs) from biofilm and planktonic phase associated with extracellular DNA (eDNA). *Front. Microbiol.,* 6**,** 1369.

GRAY, R. D., HARDISTY, G., REGAN, K. H., SMITH, M., ROBB, C. T., DUFFIN, R., MACKELLAR, A., FELTON, J. M., PAEMKA, L. & MCCULLAGH, B. N. 2018. Delayed neutrophil apoptosis enhances NET formation in cystic fibrosis. *Thorax,* 73**,** 134-144.

GU, X., KEYOUMU, Y., LONG, L. & ZHANG, H. 2014. Detection of bacterial biofilms in different types of chronic otitis media. *Eur. Arch. Otorhinolaryngol.,* 271**,** 2877-83.

GUILHEN, C., FORESTIER, C. & BALESTRINO, D. 2017. Biofilm dispersal: multiple elaborate strategies for dissemination of bacteria with unique properties. *Mol. Microbiol.,* 105**,** 188-210.

GUPTA, P., SARKAR, S., DAS, B., BHATTACHARJEE, S. & TRIBEDI, P. 2016. Biofilm, pathogenesis and prevention--a journey to break the wall: a review. *Arch. Microbiol.,* 198**,** 1-15.

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 : official journal of the European Cystic Fibrosis Society,* 12**,** 384-9.

HAAPANIEMI, J. J. 1997. Pure-tone audiometric and impedance measurements in school-aged children in Finland. *Eur. Arch. Otorhinolaryngol.,* 254**,** 269-73.

HALL-STOODLEY, L., HU, F. Z., GIESEKE, A., NISTICO, L., NGUYEN, D., HAYES, J., FORBES, M., GREENBERG, D. P., DICE, B., BURROWS, A., WACKYM, P. A., STOODLEY, P., POST, J. C., EHRLICH, G. D. & KERSCHNER, J. E. 2006. Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA,* 296**,** 202-11.

HALL-STOODLEY, L., NISTICO, L., SAMBANTHAMOORTHY, K., DICE, B., NGUYEN, D., MERSHON, W. J., JOHNSON, C., HU, F. Z., 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 Microbiol.,* 8**,** 173.

HALL-STOODLEY, L. & STOODLEY, P. 2009. Evolving concepts in biofilm infections. *Cell. Microbiol.,* 11**,** 1034-43.

HALL, A. J., MAW, A. R. & STEER, C. D. 2009. Developmental outcomes in early compared with delayed surgery for glue ear up to age 7 years: a randomised controlled trial. *Clin. Otolaryngol.,* 34**,** 12-20.

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

HAMILTON, H. L., DOMINGUEZ, N. M., SCHWARTZ, K. J., HACKETT, K. T. & DILLARD, J. P. 2005. Neisseria gonorrhoeae secretes chromosomal DNA via a novel type IV secretion system. *Mol. Microbiol.,* 55**,** 1704-21.

HARIMAYA, A., TAKADA, R., HIMI, T., YOKOTA, S. & FUJII, N. 2007. Evidence of local antibody response against Alloiococcus otitidis in the middle ear cavity of children with otitis media. *FEMS Immunol. Med. Microbiol.,* 49**,** 41-5.

HARIMAYA, A., TAKADA, R., SOMEKAWA, Y., FUJII, N. & HIMI, T. 2006. High frequency of Alloiococcus otitidis in the nasopharynx and in the middle ear cavity of otitis-prone children. *Int. J. Pediatr. Otorhinolaryngol.,* 70**,** 1009-14.

HARLOR, A. D., JR. & BOWER, C. 2009. Hearing assessment in infants and children: recommendations beyond neonatal screening. *Pediatrics,* 124**,** 1252-63.

HARMSEN, M., LAPPANN, M., KNOCHEL, S. & MOLIN, S. 2010. Role of extracellular DNA during biofilm formation by Listeria monocytogenes. *Appl. Environ. Microbiol.,* 76**,** 2271-9.

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**,** 184-90.

HAVARSTEIN, L. S., MARTIN, B., JOHNSBORG, O., GRANADEL, C. & CLAVERYS, J. P. 2006. New insights into the pneumococcal fratricide:

relationship to clumping and identification of a novel immunity factor. *Mol. Microbiol.,* 59**,** 1297-307.

HAWKE, M. 2004. *Otitis Media Guide* [Online]. Available: http://otitismedia.hawkelibrary.com/normal/1_G [Accessed 27 February 2015].

HEIJSTRA, B. D., PICHLER, F. B., LIANG, Q., BLAZA, R. G. & TURNER, S. J. 2009. Extracellular DNA and Type IV pili mediate surface attachment by Acidovorax temperans. *Antonie Van Leeuwenhoek,* 95**,** 343-9.

HERZOG, S., DACH, F., DE BUHR, N., NIEMANN, S., SCHLAGOWSKI, J., CHAVES-MORENO, D., NEUMANN, C., GORETZKO, J., DÜBBERS, A. & KÜSTER, P. 2019. High nuclease activity of long persisting Staphylococcus aureus isolates within the airways of cystic fibrosis patients protects against NET-mediated killing. *Front. Immunol.,* 10**,** 2552.

HES, H. E. S. 2019. Available: https://digital.nhs.uk/data-and-information/publications/statistical/hospital-admitted-patient-care-activity/2018-19#summary [Accessed 1 November 2019].

HEUN, M., BINNENKADE, L., KREIENBAUM, M. & THORMANN, K. M. 2012a. Functional specificity of extracellular nucleases of Shewanella oneidensis MR-1. *Appl. Environ. Microbiol.,* 78**,** 4400-4411.

HEUN, M., BINNENKADE, L., KREIENBAUM, M. & THORMANN, K. M. 2012b. Functional specificity of extracellular nucleases of Shewanella oneidensis MR-1. *Appl. Environ. Microbiol.,* 78**,** 4400-11.

HEYDORN, A., NIELSEN, A. T., HENTZER, M., STERNBERG, C., GIVSKOV, M., ERSBOLL, B. K. & MOLIN, S. 2000. Quantification of biofilm structures by the novel computer program COMSTAT. *Microbiology,* 146 (Pt 10)**,** 2395-407.

HIMI, T., KITA, H., MITSUZAWA, H., HARIMAYA, A., TARKKANEN, J., HENDOLIN, P., YLIKOSKI, J. & FUJII, N. 2000. Effect of Alloiococcus otitidis and three pathogens of otitis media in production of interleukin-12 by human monocyte cell line. *FEMS Immunol. Med. Microbiol.,* 29**,** 101-6.

HOA, M., SYAMAL, M., SACHDEVA, L., BERK, R. & COTICCHIA, J. 2009. Demonstration of nasopharyngeal and middle ear mucosal biofilms in an animal model of acute otitis media. *Ann. Otol. Rhinol. Laryngol.,* 118**,** 292-8.

HOA, M., SYAMAL, M., SCHAEFFER, M. A., SACHDEVA, L., BERK, R. & COTICCHIA, J. 2010. Biofilms and chronic otitis media: an initial exploration

into the role of biofilms in the pathogenesis of chronic otitis media. *Am. J. Otolaryngol.,* 31**,** 241-5.

HOFFMAN, H. J., DALY, K. A., BAINBRIDGE, K. E., CASSELBRANT, M. L., HOMOE, P., KVESTAD, E., KVAERNER, K. J. & VERNACCHIO, L. 2013. Panel 1: Epidemiology, natural history, and risk factors. *Otolaryngol. Head Neck Surg.,* 148**,** E1-e25.

HOIBY, N., CIOFU, O., JOHANSEN, H. K., SONG, Z. J., MOSER, C., JENSEN, P. O., MOLIN, S., GIVSKOV, M., TOLKER-NIELSEN, T. & BJARNSHOLT, T. 2011. The clinical impact of bacterial biofilms. *International journal of oral science,* 3**,** 55-65.

HOIBY, N., FLENSBORG, E. W., BECK, B., FRIIS, B., JACOBSEN, S. V. & JACOBSEN, L. 1977. Pseudomonas aeruginosa infection in cystic fibrosis. Diagnostic and prognostic significance of Pseudomonas aeruginosa precipitins determined by means of crossed immunoelectrophoresis. *Scand. J. Respir. Dis.,* 58**,** 65-79.

HOLDER, R. C., KIRSE, D. J., EVANS, A. K., WHIGHAM, A. S., PETERS, T. R., POEHLING, K. A., SWORDS, W. E. & REID, S. D. 2015. Otopathogens Detected in Middle Ear Fluid Obtained during Tympanostomy Tube Insertion: Contrasting Purulent and Non-Purulent Effusions. *PLoS One,* 10**,** e0128606.

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.

HOLZMANN, D., FUNKE, G., LINDER, T. & NADAL, D. 2002. Turicella otitidis and Corynebacterium auris do not cause otitis media with effusion in children. *Pediatr. Infect. Dis. J.,* 21**,** 1124-6.

HOMOE, P., BJARNSHOLT, T., WESSMAN, M., SORENSEN, H. C. & JOHANSEN, H. K. 2009. Morphological evidence of biofilm formation in Greenlanders with chronic suppurative otitis media. *Eur. Arch. Otorhinolaryngol.,* 266**,** 1533-8.

HORTON N.C.RICE PA, C. C. 2008. Protein-Nucleic Acid Interactions: Structural Biology. *DNA nucleases.* United Kingdom: Royal Society of Chemistry.

HUSSAIN, M., WILCOX, M. H. & WHITE, P. J. 1993. The slime of coagulase-negative staphylococci: biochemistry and relation to adherence. *FEMS Microbiol. Rev.,* 10**,** 191-207.

HUTTENHOWER, C., GEVERS, D., KNIGHT, R., ABUBUCKER, S., BADGER, J. H., CHINWALLA, A. T., CREASY, H. H., EARL, A. M., FITZGERALD, M. G., FULTON, R. S., GIGLIO, M. G., HALLSWORTH-PEPIN, K., LOBOS, E. A., MADUPU, R., MAGRINI, V., MARTIN, J. C., MITREVA, M., MUZNY, D. M., SODERGREN, E. J., VERSALOVIC, J., WOLLAM, A. M., WORLEY, K. C., WORTMAN, J. R., YOUNG, S. K., ZENG, Q., AAGAARD, K. M., ABOLUDE, O. O., ALLEN-VERCOE, E., ALM, E. J., ALVARADO, L., ANDERSEN, G. L., ANDERSON, S., APPELBAUM, E., ARACHCHI, H. M., ARMITAGE, G., ARZE, C. A., AYVAZ, T., BAKER, C. C., BEGG, L., BELACHEW, T., BHONAGIRI, V., BIHAN, M., BLASER, M. J., BLOOM, T., BONAZZI, V., PAUL BROOKS, J., BUCK, G. A., BUHAY, C. J., BUSAM, D. A., CAMPBELL, J. L., CANON, S. R., CANTAREL, B. L., CHAIN, P. S. G., CHEN, I. M. A., CHEN, L., CHHIBBA, S., CHU, K., CIULLA, D. M., CLEMENTE, J. C., CLIFTON, S. W., CONLAN, S., CRABTREE, J., CUTTING, M. A., DAVIDOVICS, N. J., DAVIS, C. C., DESANTIS, T. Z., DEAL, C., DELEHAUNTY, K. D., DEWHIRST, F. E., DEYCH, E., DING, Y., DOOLING, D. J., DUGAN, S. P., MICHAEL DUNNE, W., SCOTT DURKIN, A., EDGAR, R. C., ERLICH, R. L., FARMER, C. N., FARRELL, R. M., FAUST, K., FELDGARDEN, M., FELIX, V. M., FISHER, S., FODOR, A. A., FORNEY, L. J., FOSTER, L., DI FRANCESCO, V., FRIEDMAN, J., FRIEDRICH, D. C., FRONICK, C. C., FULTON, L. L., GAO, H., GARCIA, N., GIANNOUKOS, G., GIBLIN, C., GIOVANNI, M. Y., GOLDBERG, J. M., GOLL, J., GONZALEZ, A., GRIGGS, A., et al. 2012. Structure, function and diversity of the healthy human microbiome. *Nature,* 486**,** 207-214.

HYDEN, D., AKERLIND, B. & PEEBO, M. 2006. Inner ear and facial nerve complications of acute otitis media with focus on bacteriology and virology. *Acta Otolaryngol.,* 126**,** 460-6.

HYMES, S. R., RANDIS, T. M., SUN, T. Y. & RATNER, A. J. 2013. DNase inhibits Gardnerella vaginalis biofilms in vitro and in vivo. *J. Infect. Dis.,* 207**,** 1491-7.

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. *Front. Microbiol.,* 8**,** 1390.

IDICULA, W. K., JURCISEK, J. A., CASS, N. D., ALI, S., GOODMAN, S. D., ELMARAGHY, C. A., JATANA, K. R. & BAKALETZ, L. O. 2016. Identification of biofilms in post-tympanostomy tube otorrhea. *Laryngoscope,* 126**,** 1946-51.

INOUE, T., SHINGAKI, R., SOGAWA, N., SOGAWA, C. A., ASAUMI, J.-I., KOKEGUCHI, S. & FUKUI, K. 2003. Biofilm Formation by a Fimbriae-Deficient Mutant of Actinobacillus actinomycetemcomitans. *Microbiol. Immunol.,* 47**,** 877-881.

IWANO, T., KINOSHITA, T., HAMADA, E., DOI, T., USHIRO, K. & KUMAZAWA, T. 1993. Otitis media with effusion and eustachian tube dysfunction in adults and children. *Acta Otolaryngol. Suppl.,* 500**,** 66-9.

IZANO, E. A., AMARANTE, M. A., KHER, W. B. & KAPLAN, J. B. 2008. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms. *Appl. Environ. Microbiol.,* 74**,** 470-6.

IZANO, E. A., SHAH, S. M. & KAPLAN, J. B. 2009. Intercellular adhesion and biocide resistance in nontypeable Haemophilus influenzae biofilms. *Microb. Pathog.,* 46**,** 207-213.

JACOBY, P. A., COATES, H. L., ARUMUGASWAMY, A., ELSBURY, D., STOKES, A., MONCK, R., FINUCANE, J. M., WEEKS, S. A. & LEHMANN, D. 2008. The effect of passive smoking on the risk of otitis media in Aboriginal and non‐Aboriginal children in the Kalgoorlie‐Boulder region of Western Australia. *Med. J. Aust.,* 188**,** 599-603.

JAKUBOVICS, N. S. 2015. Intermicrobial interactions as a driver for community composition and stratification of oral biofilms. *J. Mol. Biol.,* 427**,** 3662-3675.

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

JAKUBOVICS, N. S., SHIELDS, R. C., RAJARAJAN, N. & BURGESS, J. G. 2013. Life after death: the critical role of extracellular DNA in microbial biofilms. *Lett. Appl. Microbiol.,* 57**,** 467-475.

JAMAL, M., AHMAD, W., ANDLEEB, S., JALIL, F., IMRAN, M., NAWAZ, M. A., HUSSAIN, T., ALI, M., RAFIQ, M. & KAMIL, M. A. 2018. Bacterial biofilm and associated infections. *J. Chin. Med. Assoc.,* 81**,** 7-11.

JANSSENS, Y., NIELANDT, J., BRONSELAER, A., DEBUNNE, N., VERBEKE, F., WYNENDAELE, E., VAN IMMERSEEL, F., VANDEWYNCKEL, Y.-P., DE TRÉ, G. & DE SPIEGELEER, B. 2018. Disbiome database: linking the microbiome to disease. *BMC Microbiol.,* 18**,** 50.

JENKINS, A., DIEP, B. A., MAI, T. T., VO, N. H., WARRENER, P., SUZICH, J., STOVER, C. K. & SELLMAN, B. R. 2015. Differential expression and roles of Staphylococcus aureus virulence determinants during colonization and disease. *MBio,* 6**,** e02272-14.

JENSEN, R. G., JOHANSEN, H. K., BJARNSHOLT, T., EICKHARDT-SØRENSEN, S. R. & HOMØE, P. 2017. Recurrent otorrhea in chronic suppurative otitis media: is biofilm the missing link? *Eur. Arch. Otorhinolaryngol.,* 274**,** 2741-2747.

JERVIS-BARDY, J., ROGERS, G. B., MORRIS, P. S., SMITH-VAUGHAN, H. C., NOSWORTHY, E., LEONG, L. E., SMITH, R. J., WEYRICH, L. S., DE HAAN, J., CARNEY, A. S., LEACH, A. J., O'LEARY, S. & MARSH, R. L. 2015. The microbiome of otitis media with effusion in Indigenous Australian children. *Int. J. Pediatr. Otorhinolaryngol.,* 79**,** 1548-55.

JHELUM, H., SORI, H. & SEHGAL, D. 2018. A novel extracellular vesicle-associated endodeoxyribonuclease helps Streptococcus pneumoniae evade neutrophil extracellular traps and is required for full virulence. *Sci. Rep.,* 8**,** 7985.

JOHNSON, J. S., SPAKOWICZ, D. J., HONG, B.-Y., PETERSEN, L. M., DEMKOWICZ, P., CHEN, L., LEOPOLD, S. R., HANSON, B. M., AGRESTA, H. O., GERSTEIN, M., SODERGREN, E. & WEINSTOCK, G. M. 2019. Evaluation of 16S rRNA gene sequencing for species and strain-level microbiome analysis. *Nature Communications,* 10**,** 5029.

JOHNSON, L., MULCAHY, H., KANEVETS, U., SHI, Y. & LEWENZA, S. 2012. Surface-localized spermidine protects the Pseudomonas aeruginosa outer membrane from antibiotic treatment and oxidative stress. *J. Bacteriol.,* 194**,** 813-26.

JOHNSTON, J., HOGGARD, M., BISWAS, K., ASTUDILLO-GARCIA, C., RADCLIFF, F. J., MAHADEVAN, M. & DOUGLAS, R. G. 2019. Pathogen reservoir hypothesis investigated by analyses of the adenotonsillar and middle ear microbiota. *Int. J. Pediatr. Otorhinolaryngol.,* 118**,** 103-109.

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. *J. Innate Immun.,* 5**,** 24-38.

JURCISEK, J. A. & BAKALETZ, L. O. 2007. Biofilms formed by nontypeable Haemophilus influenzae in vivo contain both double-stranded DNA and type IV pilin protein. *J. Bacteriol.,* 189**,** 3868-75.

JURCISEK, J. A., BROCKMAN, K. L., NOVOTNY, L. A., GOODMAN, S. D. & BAKALETZ, L. O. 2017. Nontypeable Haemophilus influenzae releases DNA and DNABII proteins via a T4SS-like complex and ComE of the type IV pilus machinery. *Proceedings of the National Academy of Sciences,* 114**,** E6632-E6641.

KANIA, R., VIRONNEAU, P., DANG, H., BERCOT, B., CAMBAU, E., VERILLAUD, B., CAMOUS, D., LAMERS, G., HERMAN, P., VICAUT, E., TESSIER, N. & VAN DEN ABBEELE, T. 2019. Bacterial biofilm in adenoids of children with chronic otitis media. Part I: a case control study of prevalence of biofilms in adenoids, risk factors and middle ear biofilms. *Acta Otolaryngol.,* 139**,** 345-350.

KAPLAN, J. B. 2009. Therapeutic Potential of Biofilm-Dispersing Enzymes. *The International Journal of Artificial Organs,* 32**,** 545-554.

KAPLAN, J. B., LOVETRI, K., CARDONA, S. T., MADHYASTHA, S., SADOVSKAYA, I., JABBOURI, S. & IZANO, E. A. 2012. Recombinant human DNase I decreases biofilm and increases antimicrobial susceptibility in staphylococci. *J. Antibiot. (Tokyo),* 65**,** 73-7.

KARIYA, S., OKANO, M. & NISHIZAKI, K. 2014. An association between Helicobacter pylori and upper respiratory tract disease: fact or fiction? *World Journal of Gastroenterology: WJG,* 20**,** 1470.

KAY, D. J., NELSON, M. & ROSENFELD, R. M. 2001. Meta-analysis of tympanostomy tube sequelae. *Otolaryngology-Head and Neck Surgery,* 124**,** 374-380.

KHATOON, Z., MCTIERNAN, C. D., SUURONEN, E. J., MAH, T.-F. & ALARCON, E. I. 2018. Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention. *Heliyon,* 4**,** e01067-e01067.

KIEDROWSKI, M. R., CROSBY, H. A., HERNANDEZ, F. J., MALONE, C. L., MCNAMARA, J. O., 2ND & HORSWILL, A. R. 2014. Staphylococcus aureus

Nuc2 is a functional, surface-attached extracellular nuclease. *PLoS One,* 9**,** e95574.

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.

KOBAN, I., MATTHES, R., HÜBNER, N.-O., WELK, A., SIETMANN, R., LADEMANN, J., KRAMER, A. & KOCHER, T. 2012. XTT assay of ex vivo saliva biofilms to test antimicrobial influences. *GMS Krankenhaushygiene interdisziplinär,* 7.

KOLBE, A. R., CASTRO-NALLAR, E., PRECIADO, D. & PEREZ-LOSADA, M. 2019. Altered Middle Ear Microbiome in Children With Chronic Otitis Media With Effusion and Respiratory Illnesses. *Frontiers in cellular and infection microbiology,* 9**,** 339.

KOVÁCS, Á. T. & DRAGOŠ, A. 2019. Evolved Biofilm: Review on the Experimental Evolution Studies of Bacillus subtilis Pellicles. *J. Mol. Biol.*

KREINER‐MØLLER, E., CHAWES, B., CAYE‐THOMASEN, P., BØNNELYKKE, K. & BISGAARD, H. 2012. Allergic rhinitis is associated with otitis media with effusion: a birth cohort study. *Clin. Exp. Allergy,* 42**,** 1615-1620.

KRUEGER, A., VAL, S., PÉREZ-LOSADA, M., PANCHAPAKESAN, K., DEVANEY, J., DUAH, V., DEMASON, C., POLEY, M., ROSE, M. & PRECIADO, D. 2017. Relationship of the middle ear effusion microbiome to Secretory Mucin production in pediatric patients with chronic Otitis media. *The Pediatric infectious disease journal,* 36**,** 635-640.

KUBBA, H., PEARSON, J. P. & BIRCHALL, J. P. 2000. The aetiology of otitis media with effusion: a review. *Clin. Otolaryngol. Allied Sci.,* 25**,** 181-94.

KUO, C.-L., TSAO, Y.-H., CHENG, H.-M., LIEN, C.-F., HSU, C.-H., HUANG, C.-Y. & SHIAO, A.-S. 2014. Grommets for otitis media with effusion in children with cleft palate: a systematic review. *Pediatrics,* 134**,** 983-994.

KURABI, A., SCHAERER, D., CHANG, L., PAK, K. & RYAN, A. F. 2018. Optimisation of peptides that actively cross the tympanic membrane by random amino acid extension: a phage display study. *J. Drug Target.,* 26**,** 127-134.

LACKS, S. & NEUBERGER, M. 1975. Membrane location of a deoxyribonuclease implicated in the genetic transformation of Diplococcus pneumoniae. *J. Bacteriol.,* 124**,** 1321-9.

LAGIER, J. C., ARMOUGOM, F., MILLION, M., HUGON, P., PAGNIER, I., ROBERT, C., BITTAR, F., FOURNOUS, G., GIMENEZ, G. & MARANINCHI, M. 2012. Microbial culturomics: paradigm shift in the human gut microbiome study. *Clin. Microbiol. Infect.,* 18**,** 1185-1193.

LAM, J., CHAN, R., LAM, K. & COSTERTON, J. W. 1980. Production of mucoid microcolonies by Pseudomonas aeruginosa within infected lungs in cystic fibrosis. *Infect. Immun.,* 28**,** 546-56.

LAPPAN, R., IMBROGNO, K., SIKAZWE, C., ANDERSON, D., MOK, D., COATES, H., VIJAYASEKARAN, S., BUMBAK, P., BLYTH, C. C., JAMIESON, S. E. & PEACOCK, C. S. 2018. A microbiome case-control study of recurrent acute otitis media identified potentially protective bacterial genera. *BMC Microbiol.,* 18**,** 13.

LAPPANN, M., CLAUS, H., VAN ALEN, T., HARMSEN, M., ELIAS, J., MOLIN, S. & VOGEL, U. 2010. A dual role of extracellular DNA during biofilm formation of Neisseria meningitidis. *Mol. Microbiol.,* 75**,** 1355-71.

LAUFER, A. S., METLAY, J. P., GENT, J. F., FENNIE, K. P., KONG, Y. & PETTIGREW, M. M. 2011. Microbial communities of the upper respiratory tract and otitis media in children. *MBio,* 2**,** e00245-10.

LE MAGREX-DEBAR, E., LEMOINE, J., GELLE, M. P., JACQUELIN, L. F. & CHOISY, C. 2000. Evaluation of biohazards in dehydrated biofilms on foodstuff packaging. *Int. J. Food Microbiol.,* 55**,** 239-43.

LEID, J. G., SHIRTLIFF, M. E., COSTERTON, J. W. & STOODLEY, P. 2002. Human leukocytes adhere to, penetrate, and respond to Staphylococcus aureus biofilms. *Infect. Immun.,* 70**,** 6339-45.

LEMON, K. P., KLEPAC-CERAJ, V., SCHIFFER, H. K., BRODIE, E. L., LYNCH, S. V. & KOLTER, R. 2010. Comparative analyses of the bacterial microbiota of the human nostril and oropharynx. *MBio,* 1.

LESKINEN, K., HENDOLIN, P., VIROLAINEN-JULKUNEN, A., YLIKOSKI, J. & JERO, J. 2002. The clinical role of Alloiococcus otitidis in otitis media with effusion. *Int. J. Pediatr. Otorhinolaryngol.,* 66**,** 41-48.

LETHEM, M. I., JAMES, S. L., MARRIOTT, C. & BURKE, J. F. 1990. The origin of DNA associated with mucus glycoproteins in cystic fibrosis sputum. *Eur. Respir. J.,* 3**,** 19-23.

LEWENZA, S. 2013. Extracellular DNA-induced antimicrobial peptide resistance mechanisms in Pseudomonas aeruginosa. *Front. Microbiol.,* 4**,** 21.

LI, J.-D., HERMANSSON, A., RYAN, A. F., BAKALETZ, L. O., BROWN, S. D., CHEESEMAN, M. T., JUHN, S. K., JUNG, T. T., LIM, D. J. & LIM, J. H. 2013. Panel 4: Recent advances in otitis media in molecular biology, biochemistry, genetics, and animal models. *Otolaryngology—Head and Neck Surgery,* 148**,** E52-E63.

LINDSTROM, D. R., REUBEN, B., JACOBSON, K., FLANARY, V. A. & KERSCHNER, J. E. 2004. Long‐Term Results of Armstrong Beveled Grommet Tympanostomy Tubes in Children. *The Laryngoscope,* 114**,** 490-494.

LIPFORD, G. B., HEEG, K. & WAGNER, H. 1998. Bacterial DNA as immune cell activator. *Trends Microbiol.,* 6**,** 496-500.

LIU, C. M., COSETTI, M. K., AZIZ, M., BUCHHAGEN, J. L., CONTENTE-CUOMO, T. L., PRICE, L. B., KEIM, P. S. & LALWANI, A. K. 2011. The otologic microbiome: a study of the bacterial microbiota in a pediatric patient with chronic serous otitis media using 16SrRNA gene-based pyrosequencing. *Arch. Otolaryngol. Head Neck Surg.,* 137**,** 664-8.

LIU, H., WANG, L., XIE, Z. & SHEN, P. 2012. Involvement of DNA in biofilm formation II: From bacterial adhesion to biofilm formation. *Wuhan Univ. J. Nat. Sci.,* 17**,** 162-168.

LO CANTORE, P., GIORGIO, A. & IACOBELLIS, N. S. 2015. Bioactivity of volatile organic compounds produced by Pseudomonas tolaasii. *Front. Microbiol.,* 6.

LONGAUEROVA, A. 2006. Coagulase negative staphylococci and their participation in pathogenesis of human infections. *Bratisl. Lek. Listy,* 107**,** 448-52.

LORENZ, M. G., GERJETS, D. & WACKERNAGEL, W. 1991. Release of transforming plasmid and chromosomal DNA from two cultured soil bacteria. *Arch. Microbiol.,* 156**,** 319-26.

LOUS, J., BURTON, M. J., FELDING, J. U., OVESEN, T., ROVERS, M. M. & WILLIAMSON, I. 2005. Grommets (ventilation tubes) for hearing loss

associated with otitis media with effusion in children. *Cochrane Database Syst. Rev.*, Cd001801.

LUNDGREN, K. & RUNDCRANTZ, H. 1976. Microbiology in serous otitis media. *Ann. Otol. Rhinol. Laryngol.,* 85**,** 152-5.

MAN, W. H., VAN DONGEN, T. M., VENEKAMP, R. P., PLUIMAKERS, V. G., CHU, M. L. J., VAN HOUTEN, M. A., SANDERS, E. A., SCHILDER, A. G. & BOGAERT, D. 2019. Respiratory microbiota predicts clinical disease course of acute otorrhea in children with tympanostomy tubes. *The Pediatric infectious disease journal,* 38**,** e116-e125.

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.

MANSOUR, S., MAGNAN, J., NICOLAS, K. & HAIDAR, H. 2018. Otitis Media with Effusion. *In:* MANSOUR, S., MAGNAN, J., NICOLAS, K. & HAIDAR, H. (eds.) *Middle Ear Diseases: Advances in Diagnosis and Management.* Cham: Springer International Publishing.

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.

MARCHISIO, P., CLAUT, L., ROGNONI, A., ESPOSITO, S., PASSALI, D., BELLUSSI, L., DRAGO, L., POZZI, G., MANNELLI, S., SCHITO, G. & PRINCIPI, N. 2003. Differences in nasopharyngeal bacterial flora in children with nonsevere recurrent acute otitis media and chronic otitis media with effusion: implications for management. *Pediatr. Infect. Dis. J.,* 22**,** 262-8.

MARCHISIO, P., SANTAGATI, M., SCILLATO, M., BAGGI, E., FATTIZZO, M., ROSAZZA, C., STEFANI, S., ESPOSITO, S. & PRINCIPI, N. 2015. Streptococcus salivarius 24SMB administered by nasal spray for the prevention of acute otitis media in otitis-prone children. *Eur. J. Clin. Microbiol. Infect. Dis.,* 34**,** 2377-83.

MARKS, L. R., REDDINGER, R. M. & HAKANSSON, A. P. 2012. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in Streptococcus pneumoniae. *MBio,* 3**,** e00200-12.

MARTINS, M., HENRIQUES, M., LOPEZ-RIBOT, J. L. & OLIVEIRA, R. 2012. Addition of DNase improves the in vitro activity of antifungal drugs against Candida albicans biofilms. *Mycoses,* 55**,** 80-5.

MARTINS, M., UPPULURI, P., THOMAS, D., CLEARY, I., HENRIQUES, M., LOPEZ-RIBOT, J. & OLIVEIRA, R. 2010. Presence of Extracellular DNA in the Candida albicans Biofilm Matrix and its Contribution to Biofilms. *Mycopathologia,* 169**,** 323-331.

MCDONOUGH, E., KAMP, H. & CAMILLI, A. 2016. V ibrio cholerae phosphatases required for the utilization of nucleotides and extracellular DNA as phosphate sources. *Mol. Microbiol.,* 99**,** 453-469.

MCTAGGART, L. R., COPELAND, J. K., SURENDRA, A., WANG, P. W., HUSAIN, S., COBURN, B., GUTTMAN, D. S. & KUS, J. V. 2019. Mycobiome Sequencing and Analysis Applied to Fungal Community Profiling of the Lower Respiratory Tract During Fungal Pathogenesis. *Front. Microbiol.,* 10**,** 512-512.

MEDINA, A. A. & KADOURI, D. E. 2009. Biofilm formation of Bdellovibrio bacteriovorus host-independent derivatives. *Res. Microbiol.,* 160**,** 224-231.

MEHRAJ, J., WITTE, W., AKMATOV, M. K., LAYER, F., WERNER, G. & KRAUSE, G. 2016. Epidemiology of Staphylococcus aureus Nasal Carriage Patterns in the Community. *Curr. Top. Microbiol. Immunol.,* 398**,** 55-87.

MIN, H. K., KIM, S. H., PARK, M. J., KIM, S. S., KIM, S. H. & YEO, S. G. 2019. Bacteriology and resistance patterns of otitis media with effusion. *Int. J. Pediatr. Otorhinolaryngol.,* 127**,** 109652.

MINOVI, A. & DAZERT, S. 2014. Diseases of the middle ear in childhood. *GMS Curr. Top. Otorhinolaryngol. Head Neck Surg.,* 13**,** Doc11.

MITCHELL, D. B., FORD, G. R., ALBERT, D. & WALDRON, J. 1990. Acoustic reflectometry as an aid to the diagnosis of glue ear. *Br. J. Clin. Pract.,* 44**,** 557-9.

MITTAL, R., LISI, C. V., GERRING, R., MITTAL, J., MATHEE, K., NARASIMHAN, G., AZAD, R. K., YAO, Q., GRATI, M., YAN, D., ESHRAGHI, A. A., ANGELI, S. I., TELISCHI, F. F. & LIU, X. Z. 2015. Current concepts in the pathogenesis and treatment of chronic suppurative otitis media. *J. Med. Microbiol.,* 64**,** 1103-16.

MIURA, M. S., MASCARO, M. & ROSENFELD, R. M. 2012. Association between otitis media and gastroesophageal reflux: a systematic review. *Otolaryngol. Head Neck Surg.,* 146**,** 345-52.

MOSCOSO, M. & CLAVERYS, J. P. 2004. Release of DNA into the medium by competent Streptococcus pneumoniae: kinetics, mechanism and stability of the liberated DNA. *Mol. Microbiol.,* 54**,** 783-94.

MUELLER, N. T., BAKACS, E., COMBELLICK, J., GRIGORYAN, Z. & DOMINGUEZ-BELLO, M. G. 2015. The infant microbiome development: mom matters. *Trends Mol. Med.,* 21**,** 109-117.

MULCAHY, H., CHARRON-MAZENOD, L. & LEWENZA, S. 2008. Extracellular DNA chelates cations and induces antibiotic resistance in Pseudomonas aeruginosa biofilms. *PLoS Pathog.,* 4**,** e1000213.

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. *Environ. Microbiol.,* 12**,** 1621-9.

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. *J. Antimicrob. Chemother.,* 68**,** 2550-2560.

NAVRÁTILOVÁ, L., PROCHÁZKOVÁ, P., BARDOŇ, J., NOVOTNÝ, R., ZÁPALKA, M., JAKUBEC, P., ZATLOUKAL, J., KOLEK, V., KOPŘIVA, F., FLODROVÁ, P. & RACLAVSKÝ, V. 2016. Performance of pyrosequencing versus MALDI-TOF MS in bacteria identification in chronic lung disease. *2016*.

NGO, C. C., MASSA, H. M., THORNTON, R. B. & CRIPPS, A. W. 2016. Predominant Bacteria Detected from the Middle Ear Fluid of Children Experiencing Otitis Media: A Systematic Review. *PLoS One,* 11**,** e0150949.

NICE. 2016. *Otitis media with effusion* [Online]. Available: https://cks.nice.org.uk/otitis-media-with-effusion#!topicsummary [Accessed 6 August 2018].

NICE, N. I. F. H. A. C. E. 2008. *Surgical management of otitis media with effusion.* [Online]. National Collaborating Centre for Women's and Children's Health. Available: http://www.nice.org.uk/guidance/cg60/evidence/cg60-surgical-management-of-ome-full-guideline2 [Accessed 25 February 2015].

NIJLAND, R., HALL, M. J. & BURGESS, J. G. 2010. Dispersal of biofilms by secreted, matrix degrading, bacterial DNase. *PLoS One,* 5**,** e15668.

NISTICO, L., KREFT, R., GIESEKE, A., COTICCHIA, J. M., BURROWS, A., KHAMPANG, P., LIU, Y., KERSCHNER, J. E., POST, J. C., LONERGAN, S., SAMPATH, R., HU, F. Z., EHRLICH, G. D., STOODLEY, P. & HALL-STOODLEY, L. 2011. Adenoid reservoir for pathogenic biofilm bacteria. *J. Clin. Microbiol.,* 49**,** 1411-20.

NOVOTNY, L. A., CLEMENTS, J. D., GOODMAN, S. D. & BAKALETZ, L. O. 2017. Transcutaneous Immunization with a Band-Aid Prevents Experimental Otitis Media in a Polymicrobial Model. *Clin. Vaccine Immunol.,* 24**,** e00563-16.

NOVOTNY, L. A., GOODMAN, S. D. & BAKALETZ, L. O. 2019. Redirecting the immune response towards immunoprotective domains of a DNABII protein resolves experimental otitis media. *NPJ vaccines,* 4**,** 43.

NURJADI, D., HEEG, K., WEBER, A. N. R. & ZANGER, P. 2018. Toll-like receptor 9 (TLR-9) promotor polymorphisms and gene expression are associated with persistent Staphylococcus aureus nasal carriage. *Clin. Microbiol. Infect.,* 24**,** 1210.e7-1210.e12.

NURJADI, D., LEPENDU, J., KREMSNER, P. G. & ZANGER, P. 2012. Staphylococcus aureus throat carriage is associated with ABO-/secretor status. *J. Infect.,* 65**,** 310-7.

ODER, M., ARLIČ, M., BOHINC, K. & FINK, R. 2018. Escherichia coli biofilm formation and dispersion under hydrodynamic conditions on metal surfaces. *Int. J. Environ. Health Res.,* 28**,** 55-63.

OKSHEVSKY, M., REGINA, V. R. & MEYER, R. L. 2014. Extracellular DNA as a target for biofilm control. *Curr. Opin. Biotechnol.,* 33c**,** 73-80.

OKSHEVSKY, M., REGINA, V. R. & MEYER, R. L. 2015. Extracellular DNA as a target for biofilm control. *Curr. Opin. Biotechnol.,* 33**,** 73-80.

OTTO, M. 2013. Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity. *Annu. Rev. Med.,* 64**,** 175-188.

OVESEN, T. & BORGLUM, J. D. 1998. New aspects of secretory otitis media, eustachian tube function and middle ear gas. *Ear. Nose. Throat J.,* 77**,** 770-7.

PALMER, L. J., CHAPPLE, I. L., WRIGHT, H. J., ROBERTS, A. & COOPER, P. R. 2012. Extracellular deoxyribonuclease production by periodontal bacteria. *J. Periodontal Res.,* 47**,** 439-45.

PALUCH-OLES, J., MAGRYS, A., KOZIOL-MONTEWKA, M., NIEDZIELSKI, A., NIEDZWIADEK, J., NIEDZIELSKA, G. & KOTOWSKI, M. 2011. The phenotypic and genetic biofilm formation characteristics of coagulase-negative staphylococci isolates in children with otitis media. *Int. J. Pediatr. Otorhinolaryngol.,* 75**,** 126-30.

PAPP, Z., ELGABSI, H. & TOTH, L. 2016. MALDI-TOF mass spectrometry reveals a highly complex bacterial profile of otitis media with effusion. *Int. J. Pediatr. Otorhinolaryngol.,* 86**,** 189-92.

PARADISE, J. L., BLUESTONE, C. D., COLBORN, D. K., BERNARD, B. S., SMITH, C. G., ROCKETTE, H. E. & KURS-LASKY, M. 1999. Adenoidectomy and adenotonsillectomy for recurrent acute otitis media: parallel randomized clinical trials in children not previously treated with tympanostomy tubes. *JAMA,* 282**,** 945-953.

PARK, Y. H., KIM, E. H., SEO, S. T., LEE, S. H., KIM, J. M., KOO, B. S., KIM, Y. M. & RHA, K. S. 2009. Formation of Biofilm in Patients with Chronic Otitis Media and Cholesteatoma. *Korean Journal of Otorhinolaryngology-Head and Neck Surgery,* 52**,** 124-128.

PARSEK, M. R. & SINGH, P. K. 2003. BACTERIAL BIOFILMS: An Emerging Link to Disease Pathogenesis. *Annu. Rev. Microbiol.,* 57**,** 677-701.

PARSIEGLA, G., NOGUERE, C., SANTELL, L., LAZARUS, R. A. & BOURNE, Y. 2012. The Structure of Human DNase I Bound to Magnesium and Phosphate Ions Points to a Catalytic Mechanism Common to Members of the DNase I-like Superfamily. *Biochemistry,* 51**,** 10250-10258.

PAU, B. C. & NG, D. K. 2016. Prevalence of otitis media with effusion in children with allergic rhinitis, a cross sectional study. *Int. J. Pediatr. Otorhinolaryngol.,* 84**,** 156-160.

PEETERS, E., NELIS, H. J. & COENYE, T. 2008. Comparison of multiple methods for quantification of microbial biofilms grown in microtiter plates. *J. Microbiol. Methods,* 72**,** 157-165.

PETERSEN, F. C., PECHARKI, D. & SCHEIE, A. A. 2004. Biofilm Mode of Growth of Streptococcus intermedius Favored by a Competence-Stimulating Signaling Peptide. *J. Bacteriol.,* 186**,** 6327-6331.

PETERSEN, F. C., TAO, L. & SCHEIE, A. A. 2005. DNA Binding-Uptake System: a Link between Cell-to-Cell Communication and Biofilm Formation. *J. Bacteriol.,* 187**,** 4392-4400.

PETTIGREW, M. M., LAUFER, A. S., GENT, J. F., KONG, Y., FENNIE, K. P. & METLAY, J. P. 2012a. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. *Appl. Environ. Microbiol.,* 78**,** 6262-6270.

PETTIGREW, M. M., LAUFER, A. S., GENT, J. F., KONG, Y., FENNIE, K. P. & METLAY, J. P. 2012b. Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. *Appl. Environ. Microbiol.,* 78**,** 6262-70.

PHE, P. H. E. 2014. *Investigation of Ear Infections and Associated Specimens.* [Online]. UK Standards for Microbiology Investigations. Available: http://www.hpa.org.uk/SMI/pdf [Accessed 11 February 2015].

PIETROCOLA, G., ARCIOLA, C. R., RINDI, S., DI POTO, A., MISSINEO, A., MONTANARO, L. & SPEZIALE, P. 2011. Toll-like receptors (TLRs) in innate immune defense against Staphylococcus aureus. *The International journal of artificial organs,* 34**,** 799-810.

PINCHUK, G. E., AMMONS, C., CULLEY, D. E., LI, S. M., 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. *Appl. Environ. Microbiol.,* 74**,** 1198-208.

POETKER, D. M., LINDSTROM, D. R., EDMISTON, C. E., KREPEL, C. J., LINK, T. R. & KERSCHNER, J. E. 2005. Microbiology of middle ear effusions from 292 patients undergoing tympanostomy tube placement for middle ear disease. *Int. J. Pediatr. Otorhinolaryngol.,* 69**,** 799-804.

POLLOCK, J., GLENDINNING, L., WISEDCHANWET, T. & WATSON, M. 2018. The Madness of Microbiome: Attempting To Find Consensus “Best Practice” for 16S Microbiome Studies. *Appl. Environ. Microbiol.,* 84**,** e02627-17.

PORSCHEN, R. K. & SONNTAG, S. 1974. Extracellular deoxyribonuclease production by anaerobic bacteria. *Appl. Microbiol.,* 27**,** 1031-3.

POST, J. C. 2001. Direct evidence of bacterial biofilms in otitis media. *Laryngoscope,* 111**,** 2083-94.

POST, J. C., PRESTON, R. A., AUL, J. J., LARKINS-PETTIGREW, M., RYDQUIST-WHITE, J., ANDERSON, K. W., WADOWSKY, R. M., REAGAN, D. R., WALKER, E. S., KINGSLEY, L. A., MAGIT, A. E. & EHRLICH, G. D. 1995. Molecular analysis of bacterial pathogens in otitis media with effusion. *JAMA,* 273**,** 1598-604.

PRECIADO, D., KUO, E., ASHKTORAB, S., MANES, P. & ROSE, M. 2010. Cigarette smoke activates NFkappaB-mediated Tnf-alpha release from mouse middle ear cells. *Laryngoscope,* 120**,** 2508-15.

PRESSLER, T. 2008. Review of recombinant human deoxyribonuclease (rhDNase) in the management of patients with cystic fibrosis. *Biologics : targets & therapy,* 2**,** 611-617.

PUCA, V., ERCOLINO, E., CELIA, C., BOLOGNA, G., DI MARZIO, L., MINCIONE, G., MARCHISIO, M., MISCIA, S., MURARO, R. & LANUTI, P. 2019. Detection and Quantification of eDNA-Associated Bacterial Membrane Vesicles by Flow Cytometry. *Int. J. Mol. Sci.,* 20**,** 5307.

QIN, Z., OU, Y., YANG, L., ZHU, Y., TOLKER-NIELSEN, T., MOLIN, S. & QU, D. 2007a. Role of autolysin-mediated DNA release in biofilm formation of Staphylococcus epidermidis. *Microbiology,* 153**,** 2083-92.

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

QUREISHI, A., LEE, Y., BELFIELD, K., BIRCHALL, J. P. & DANIEL, M. 2014. Update on otitis media - prevention and treatment. *Infection and drug resistance,* 7**,** 15-24.

RAJARAJAN, N., WARD, A. C., BURGESS, J. G. & GLASSEY, J. 2013. Use of physiological information and process optimisation enhances production of extracellular nuclease by a marine strain of Bacillus licheniformis. *Bioresour. Technol.,* 130**,** 552-8.

RAJENDRAN, R., WILLIAMS, C., LAPPIN, D. F., MILLINGTON, O., MARTINS, M. & RAMAGE, G. 2013. Extracellular DNA release acts as an antifungal resistance mechanism in mature Aspergillus fumigatus biofilms. *Eukaryotic cell,* 12**,** 420-9.

RANJAN, R., RANI, A., METWALLY, A., MCGEE, H. S. & PERKINS, D. L. 2016. Analysis of the microbiome: Advantages of whole genome shotgun versus 16S amplicon sequencing. *Biochem. Biophys. Res. Commun.,* 469**,** 967-977.

RAYNER, M. G., ZHANG, Y., GORRY, M. C., CHEN, Y., POST, J. C. & EHRLICH, G. D. 1998. Evidence of bacterial metabolic activity in culture-negative otitis media with effusion. *JAMA,* 279**,** 296-9.

REGEV-YOCHAY, G., TRZCIŃSKI, K., THOMPSON, C. M., MALLEY, R. & LIPSITCH, M. 2006. Interference between Streptococcus pneumoniae and Staphylococcus aureus: In Vitro Hydrogen Peroxide-Mediated Killing by Streptococcus pneumoniae. *J. Bacteriol.,* 188**,** 4996-5001.

REICHHARDT, C. & PARSEK, M. 2019. Confocal laser scanning microscopy for analysis of Pseudomonas aeruginosa biofilm architecture and matrix localization. *Front. Microbiol.,* 10**,** 677.

REN, T., GLATT, D. U., NGUYEN, T. N., ALLEN, E. K., EARLY, S. V., SALE, M., WINTHER, B. & WU, M. 2013. 16S rRNA survey revealed complex bacterial communities and evidence of bacterial interference on human adenoids. *Environ. Microbiol.,* 15**,** 535-47.

RENDUELES, O. & GHIGO, J. M. 2012. Multi-species biofilms: how to avoid unfriendly neighbors. *FEMS Microbiol. Rev.,* 36**,** 972-89.

RETTIG, E. & TUNKEL, D. E. 2014. Contemporary concepts in management of acute otitis media in children. *Otolaryngol. Clin. North Am.,* 47**,** 651-672.

RICE, K. C. & BAYLES, K. W. 2003. Death's toolbox: examining the molecular components of bacterial programmed cell death. *Mol. Microbiol.,* 50**,** 729-38.

RICE, K. C., MANN, E. E., ENDRES, J. L., WEISS, E. C., CASSAT, J. E., SMELTZER, M. S. & BAYLES, K. W. 2007. The cidA murein hydrolase regulator contributes to DNA release and biofilm development in Staphylococcus aureus. *Proceedings of the National Academy of Sciences,* 104**,** 8113-8118.

ROBB, P. J. 2006. Childhood otitis media with effusion. *Clin. Otolaryngol.,* 31**,** 535-537.

ROBB, P. J. & WILLIAMSON, I. 2012. Otitis media with effusion in children: current management. *Paediatrics and Child Health,* 22**,** 9-12.

ROGERS, K. 2016. *Human microbiome* [Online]. Encyclopædia Britannica, inc. . Available: https://www.britannica.com/science/human-microbiome [Accessed].

ROGERS, S. A., HUIGENS, R. W., 3RD, CAVANAGH, J. & MELANDER, C. 2010. Synergistic effects between conventional antibiotics and 2-aminoimidazole-derived antibiofilm agents. *Antimicrob. Agents Chemother.,* 54**,** 2112-8.

ROMLING, U. & BALSALOBRE, C. 2012. Biofilm infections, their resilience to therapy and innovative treatment strategies. *J. Intern. Med.,* 272**,** 541-61.

ROSE, S. J., BABRAK, L. M. & BERMUDEZ, L. E. 2015. Mycobacterium avium Possesses Extracellular DNA that Contributes to Biofilm Formation, Structural Integrity, and Tolerance to Antibiotics. *PLoS One,* 10**,** e0128772.

ROSENFELD, R. M. & KAY, D. 2003. Natural history of untreated otitis media. *Laryngoscope,* 113**,** 1645-57.

ROSENFELD, R. M., SCHWARTZ, S. R., PYNNONEN, M. A., TUNKEL, D. E., HUSSEY, H. M., FICHERA, J. S., GRIMES, A. M., HACKELL, J. M., HARRISON, M. F. & HASKELL, H. 2013. Clinical practice guideline: tympanostomy tubes in children. *Otolaryngology—Head and Neck Surgery,* 149**,** S1-S35.

ROSENFELD, R. M., SHIN, J. J., SCHWARTZ, S. R., COGGINS, R., GAGNON, L., HACKELL, J. M., HOELTING, D., HUNTER, L. L., KUMMER, A. W., PAYNE, S. C., POE, D. S., VELING, M., VILA, P. M., WALSH, S. A. & CORRIGAN, M. D. 2016. Clinical Practice Guideline: Otitis Media with Effusion (Update). *Otolaryngol. Head Neck Surg.,* 154**,** S1-s41.

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. *J. Dent. Res.,* 96**,** 208-216.

ROVERS, M. M., SCHILDER, A. G., ZIELHUIS, G. A. & ROSENFELD, R. M. 2004. Otitis media. *Lancet,* 363**,** 465-73.

ROWLAND, I., GIBSON, G., HEINKEN, A., SCOTT, K., SWANN, J., THIELE, I. & TUOHY, K. 2018. Gut microbiota functions: metabolism of nutrients and other food components. *Eur. J. Nutr.,* 57**,** 1-24.

RUDYARD. 2012. *Dr Rudyard Health Pictures* [Online]. Available: "http://www.rudyard.org/chronic-otitis-media/ [Accessed 24 February 2015].

SAAFAN, M. E., IBRAHIM, W. S. & TOMOUM, M. O. 2013. Role of adenoid biofilm in chronic otitis media with effusion in children. *Eur. Arch. Otorhinolaryngol.,* 270**,** 2417-25.

SADE, J. & WEISSMAN, Z. 1977. Middle ear mucosa and secretory otitis media. *Arch. Otorhinolaryngol.,* 215**,** 195-205.

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. *TheScientificWorldJournal,* 2012**,** 973436.

SALTER, S. J., COX, M. J., TUREK, E. M., CALUS, S. T., COOKSON, W. O., MOFFATT, M. F., TURNER, P., PARKHILL, J., LOMAN, N. J. & WALKER, A. W. 2014. Reagent and laboratory contamination can critically impact sequence-based microbiome analyses. *BMC Biol.,* 12**,** 87.

SANCHEZ-TORRES, V., MAEDA, T. & WOOD, T. K. 2010. Global regulator H-NS and lipoprotein NlpI influence production of extracellular DNA in Escherichia coli. *Biochem. Biophys. Res. Commun.,* 401**,** 197-202.

SASSEN, M. L., VAN AAREM, A. & GROTE, J. J. 1994. Validity of tympanometry in the diagnosis of middle ear effusion. *Clin. Otolaryngol. Allied Sci.,* 19**,** 185-9.

SAUER, K., CAMPER, A. K., EHRLICH, G. D., COSTERTON, J. W. & DAVIES, D. G. 2002. Pseudomonas aeruginosa displays multiple phenotypes during development as a biofilm. *J. Bacteriol.,* 184**,** 1140-54.

SCHILDER, A. G. M., CHONMAITREE, T., CRIPPS, A. W., ROSENFELD, R. M., CASSELBRANT, M. L., HAGGARD, M. P. & VENEKAMP, R. P. 2016. Otitis media. *Nature Reviews Disease Primers,* 2**,** 16063.

SCHOOLING, S. R. & BEVERIDGE, T. J. 2006. Membrane vesicles: an overlooked component of the matrices of biofilms. *J. Bacteriol.,* 188**,** 5945-57.

SCHOOLING, S. R., HUBLEY, A. & BEVERIDGE, T. J. 2009. Interactions of DNA with biofilm-derived membrane vesicles. *J. Bacteriol.,* 191**,** 4097-102.

SENDER, R., FUCHS, S. & MILO, R. 2016. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol.,* 14**,** e1002533.

SEPER, A., FENGLER, V. H., ROIER, S., WOLINSKI, H., KOHLWEIN, S. D., BISHOP, A. L., CAMILLI, A., REIDL, J. & SCHILD, S. 2011a. Extracellular

nucleases and extracellular DNA play important roles in Vibrio cholerae biofilm formation. *Mol. Microbiol.,* 82**,** 1015-37.

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

SEPER, A., HOSSEINZADEH, A., GORKIEWICZ, G., LICHTENEGGER, S., ROIER, S., LEITNER, D. R., ROHM, M., GRUTSCH, A., REIDL, J., URBAN, C. F. & SCHILD, S. 2013. Vibrio cholerae evades neutrophil extracellular traps by the activity of two extracellular nucleases. *PLoS Pathog.,* 9**,** e1003614.

SEVIOUR, T. W., WINNERDY, F. R., LI, W. L., XIANGYAN, S., MUGUNTHAN, S., KOHLI, G. S., SHEWAN, H. M., STOKES, J., RICE, S. A. & PHAN, A.-T. 2019. The biofilm matrix scaffold of Pseudomonas species consists of non-canonically base paired extracellular DNA and RNA. *BioRxiv***,** 527267.

SHAK, S., CAPON, D. J., HELLMISS, R., MARSTERS, S. A. & BAKER, C. L. 1990. Recombinant human DNase I reduces the viscosity of cystic fibrosis sputum. *Proceedings of the National Academy of Sciences,* 87**,** 9188-9192.

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. *Otolaryngol. Head Neck Surg.,* 147**,** 509-14.

SHEKELLE, P., TAKATA, G., CHAN, L. S., MANGIONE-SMITH, R., CORLEY, P. M., MORPHEW, T. & MORTON, S. 2002. Diagnosis, natural history, and late effects of otitis media with effusion. *Evidence report/technology assessment (Summary)***,** 1-5.

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.

SHIN, J. M., ATEIA, I., PAULUS, J. R., LIU, H., FENNO, J. C., RICKARD, A. H. & KAPILA, Y. L. 2015. Antimicrobial nisin acts against saliva derived multi-species biofilms without cytotoxicity to human oral cells. *Front. Microbiol.,* 6**,** 617.

SIGN, S. I. G. N. 2003. *Diagnosis and Management of Childhood Otitis Media in Primary Care.* [Online]. Edinburgh: Royal College of Physicians. Available: http://www.antibioticos.mscbs.gob.es/PDF/sign_Diagn_and_management_of_childhood_otitis_media_in_primary_care.pdf [Accessed 25 February 2015].

SIGURLASDOTTIR, S., WASSING, G. M., FANGLEI, Z., ARTS, M. & JONSSON, A.-B. 2019. Deletion of D-lactate dehydrogenase A in Neisseria meningitidis promotes biofilm formation through increased autolysis and extracellular DNA release. *Front. Microbiol.,* 10**,** 422.

SILLANPÄÄ, S., KRAMNA, L., OIKARINEN, S., SIPILÄ, M., RAUTIAINEN, M., AITTONIEMI, J., LARANNE, J., HYÖTY, H. & CINEK, O. 2017. Next-generation sequencing combined with specific PCR assays to determine the bacterial 16S rRNA gene profiles of middle ear fluid collected from children with acute otitis media. *mSphere,* 2**,** e00006-17.

SILVA, M. D. & SILLANKORVA, S. 2019. Otitis media pathogens–A life entrapped in biofilm communities. *Crit. Rev. Microbiol.***,** 1-18.

SIMPSON, S. A., LEWIS, R., VAN DER VOORT, J. & BUTLER, C. C. 2011a. Oral or topical nasal steroids for hearing loss associated with otitis media with effusion in children. *Cochrane Database Syst. Rev.*

SIMPSON, S. A., LEWIS, R., VAN DER VOORT, J. & BUTLER, C. C. 2011b. Oral or topical nasal steroids for hearing loss associated with otitis media with effusion in children. *Cochrane Database Syst. Rev.***,** Cd001935.

SINGH, S., SINGH, S. K., CHOWDHURY, I. & SINGH, R. 2017. Understanding the mechanism of bacterial biofilms resistance to antimicrobial agents. *The open microbiology journal,* 11**,** 53.

SMIRNOVA, M. G., KISELEV, S. L., GNUCHEV, N. V., BIRCHALL, J. P. & PEARSON, J. P. 2002. Role of the pro-inflammatory cytokines tumor necrosis factor-alpha, interleukin-1 beta, interleukin-6 and interleukin-8 in the pathogenesis of the otitis media with effusion. *Eur. Cytokine Netw.,* 13**,** 161-72.

SMITH, F., DRYBURGH, N., DONALDSON, J. & MITCHELL, M. 2011. Debridement for surgical wounds. *Cochrane Database Syst. Rev.***,** Cd006214.

STARNER, T. D., ZHANG, N., KIM, G., APICELLA, M. A. & MCCRAY, P. B., JR. 2006. Haemophilus influenzae forms biofilms on airway epithelia: implications in cystic fibrosis. *Am. J. Respir. Crit. Care Med.,* 174**,** 213-20.

STEED, D. L. 2004. Debridement. *The American Journal of Surgery,* 187**,** S71-S74.

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. *Infect. Immun.,* 79**,** 1504-1511.

STEINBERGER, R. E. & HOLDEN, P. A. 2005. Extracellular DNA in single- and multiple-species unsaturated biofilms. *Appl. Environ. Microbiol.,* 71**,** 5404-10.

STOL, K., VERHAEGH, S. J., GRAAMANS, K., ENGEL, J. A., STURM, P. D., MELCHERS, W. J., MEIS, J. F., WARRIS, A., HAYS, J. P. & HERMANS, P. W. 2013. Microbial profiling does not differentiate between childhood recurrent acute otitis media and chronic otitis media with effusion. *Int. J. Pediatr. Otorhinolaryngol.,* 77**,** 488-93.

STROMAN, D. W., ROLAND, P. S., DOHAR, J. & BURT, W. 2001. Microbiology of normal external auditory canal. *The laryngoscope,* 111**,** 2054-2059.

SUGIMOTO, S., SATO, F., MIYAKAWA, R., CHIBA, A., ONODERA, S., HORI, S. & MIZUNOE, Y. 2018. Broad impact of extracellular DNA on biofilm formation by clinically isolated Methicillin-resistant and -sensitive strains of Staphylococcus aureus. *Sci. Rep.,* 8**,** 2254.

SVENSSON, S. L., DAVIS, L. M., MACKICHAN, J. K., ALLAN, B. J., PAJANIAPPAN, M., THOMPSON, S. A. & GAYNOR, E. C. 2009. The CprS sensor kinase of the zoonotic pathogen Campylobacter jejuni influences biofilm formation and is required for optimal chick colonization. *Mol. Microbiol.,* 71**,** 253-72.

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.

SWEARINGEN, M. C., DIBARTOLA, A. C., DUSANE, D., GRANGER, J. & STOODLEY, P. 2016. 16S rRNA analysis provides evidence of biofilms on all components of three infected periprosthetic knees including permanent braided suture. *Pathogens and Disease,* 74.

SWIDSINSKI, A., GÖKTAS, Ö., BESSLER, C., LOENING‐BAUCKE, V., HALE, L. P., ANDREE, H., WEIZENEGGER, M., HÖLZL, M., SCHERER, H. & LOCHS, H. 2007. Spatial organisation of microbiota in quiescent adenoiditis and tonsillitis. *Journal of Clinical Pathology,* 60**,** 253-260.

TANG, J., KANG, M., CHEN, H., SHI, X., ZHOU, R., CHEN, J. & DU, Y. 2011. The staphylococcal nuclease prevents biofilm formation in Staphylococcus aureus and other biofilm-forming bacteria. *Science China. Life sciences,* 54**,** 863-9.

TEELE, D. W., KLEIN, J. O. & ROSNER, B. 1989. Epidemiology of otitis media during the first seven years of life in children in greater Boston: a prospective, cohort study. *J. Infect. Dis.,* 160**,** 83-94.

TEITZEL, G. M. & PARSEK, M. R. 2003. Heavy metal resistance of biofilm and planktonic Pseudomonas aeruginosa. *Appl. Environ. Microbiol.,* 69**,** 2313-20.

TETZ, G. V., ARTEMENKO, N. K. & TETZ, V. V. 2009. Effect of DNase and antibiotics on biofilm characteristics. *Antimicrob. Agents Chemother.,* 53**,** 1204-9.

TETZ, V. V. & TETZ, G. V. 2010. Effect of extracellular DNA destruction by DNase I on characteristics of forming biofilms. *DNA Cell Biol.,* 29**,** 399-405.

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. *Mol. Microbiol.,* 72**,** 1022-36.

THOMAS, V. C., THURLOW, L. R., BOYLE, D. & HANCOCK, L. E. 2008. Regulation of Autolysis-Dependent Extracellular DNA Release by Enterococcus faecalis Extracellular Proteases Influences Biofilm Development. *J. Bacteriol.,* 190**,** 5690-5698.

THORNTON, R. B., RIGBY, P. J., WIERTSEMA, S. P., FILION, P., LANGLANDS, J., COATES, H. L., VIJAYASEKARAN, S., KEIL, A. D. & RICHMOND, P. C. 2011. Multi-species bacterial biofilm and intracellular infection in otitis media. *BMC Pediatr.,* 11**,** 94.

THORNTON, R. B., WIERTSEMA, S. P., KIRKHAM, L.-A. S., RIGBY, P. J., VIJAYASEKARAN, S., COATES, H. L. & RICHMOND, P. C. 2013. Neutrophil Extracellular Traps and Bacterial Biofilms in Middle Ear Effusion of Children with Recurrent Acute Otitis Media – A Potential Treatment Target. *PLoS One,* 8**,** e53837.

THURLOW, L. R., HANKE, M. L., FRITZ, T., ANGLE, A., ALDRICH, A., WILLIAMS, S. H., ENGEBRETSEN, I. L., BAYLES, K. W., HORSWILL, A. R. & KIELIAN, T. 2011. Staphylococcus aureus biofilms prevent macrophage phagocytosis

and attenuate inflammation in vivo. *The Journal of Immunology,* 186**,** 6585-6596.

TORELLI, R., CACACI, M., PAPI, M., PARONI STERBINI, F., MARTINI, C., POSTERARO, B., PALMIERI, V., DE SPIRITO, M., SANGUINETTI, M. & BUGLI, F. 2017. Different effects of matrix degrading enzymes towards biofilms formed by E. faecalis and E. faecium clinical isolates. *Colloids and Surfaces B: Biointerfaces,* 158**,** 349-355.

TRIPATHI, R., SHARMA, P., CHAKRABORTY, P. & VARADWAJ, P. K. 2016. Next-generation sequencing revolution through big data analytics. *Frontiers in Life Science,* 9**,** 119-149.

TSUBOI, Y., KIM, Y., PAPARELLA, M. M., CHEN, N., SCHACHERN, P. A. & LIN, J. 2001. Pattern changes of mucin gene expression with pneumococcal otitis media. *Int. J. Pediatr. Otorhinolaryngol.,* 61**,** 23-30.

TURNBAUGH, P. J., LEY, R. E., HAMADY, M., FRASER-LIGGETT, C. M., KNIGHT, R. & GORDON, J. I. 2007. The human microbiome project. *Nature,* 449**,** 804-10.

VAN DEN AARDWEG, M. T., SCHILDER, A. G., HERKERT, E., BOONACKER, C. W. & ROVERS, M. M. 2010. Adenoidectomy for otitis media in children. *Cochrane Database Syst. Rev.***,** Cd007810.

VAN HOECKE, H., DE PAEPE, A. S., LAMBERT, E., VAN BELLEGHEM, J. D., COOLS, P., VAN SIMAEY, L., DESCHAGHT, P., VANEECHOUTTE, M. & DHOOGE, I. 2016. Haemophilus influenzae biofilm formation in chronic otitis media with effusion. *Eur. Arch. Otorhinolaryngol.,* 273**,** 3553-3560.

VAN ZON, A., VAN DER HEIJDEN, G. J., VAN DONGEN, T. M., BURTON, M. J. & SCHILDER, A. G. 2012. Antibiotics for otitis media with effusion in children. *Cochrane Database Syst. Rev.,* 9**,** Cd009163.

VANNESTE, P. & PAGE, C. 2019. Otitis media with effusion in children: Pathophysiology, diagnosis, and treatment. A review. *Journal of Otology,* 14**,** 33.

VAUGHAN-JONES, R. & MILLS, R. P. 1992. The Welch Allyn Audioscope and Microtymp: their accuracy and that of pneumatic otoscopy, tympanometry and pure tone audiometry as predictors of otitis media with effusion. *J. Laryngol. Otol.,* 106**,** 600-2.

VENEKAMP, R. P., BURTON, M. J., VAN DONGEN, T. M., VAN DER HEIJDEN, G. J., VAN ZON, A. & SCHILDER, A. G. 2016. Antibiotics for otitis media with effusion in children. *Cochrane Database Syst. Rev.*

VILAIN, S., PRETORIUS, J. M., THERON, J. & BROZEL, V. S. 2009. DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms. *Appl. Environ. Microbiol.,* 75**,** 2861-8.

VLAMAKIS, H., CHAI, Y., BEAUREGARD, P., LOSICK, R. & KOLTER, R. 2013. Sticking together: building a biofilm the Bacillus subtilis way. *Nat. Rev. Microbiol.,* 11**,** 157-68.

VLASTARAKOS, P. V., NIKOLOPOULOS, T. P., KORRES, S., TAVOULARI, E., TZAGAROULAKIS, A. & FEREKIDIS, E. 2007. Grommets in otitis media with effusion: the most frequent operation in children. But is it associated with significant complications? *Eur. J. Pediatr.,* 166**,** 385-91.

VON GRAEVENITZ, A. & FUNKE, G. 2014. Turicella otitidis and Corynebacterium auris: 20 years on. *Infection,* 42**,** 1-4.

VUOTTO, C., LONGO, F., PASCOLINI, C., DONELLI, G., BALICE, M. P., LIBORI, M. F., TIRACCHIA, V., SALVIA, A. & VARALDO, P. E. 2017. Biofilm formation and antibiotic resistance in Klebsiella pneumoniae urinary strains. *J. Appl. Microbiol.,* 123**,** 1003-1018.

WADE, W. 2002. Unculturable bacteria--the uncharacterized organisms that cause oral infections. *J. R. Soc. Med.,* 95**,** 81-3.

WALKER, T. S., TOMLIN, K. L., WORTHEN, G. S., POCH, K. R., LIEBER, J. G., SAAVEDRA, M. T., FESSLER, M. B., MALCOLM, K. C., VASIL, M. L. & NICK, J. A. 2005. Enhanced Pseudomonas aeruginosa biofilm development mediated by human neutrophils. *Infect. Immun.,* 73**,** 3693-3701.

WANG, J. C., HAMOOD, A. N., SAADEH, C., CUNNINGHAM, M. J., YIM, M. T. & CORDERO, J. 2014. Strategies to prevent biofilm-based tympanostomy tube infections. *Int. J. Pediatr. Otorhinolaryngol.,* 78**,** 1433-1438.

WARYAH, C. B., WELLS, K., ULLUWISHEWA, D., CHEN-TAN, N., GOGOI-TIWARI, J., RAVENSDALE, J., COSTANTINO, P., GOKCEN, 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. *Microbial drug resistance (Larchmont, N.Y.),* 23**,** 384-390.

WATTERS, G. W., JONES, J. E. & FREELAND, A. P. 1997. The predictive value of tympanometry in the diagnosis of middle ear effusion. *Clin. Otolaryngol. Allied Sci.,* 22**,** 343-5.

WEBB, J. S., THOMPSON, L. S., JAMES, S., CHARLTON, T., TOLKER-NIELSEN, T., KOCH, B., GIVSKOV, M. & KJELLEBERG, S. 2003. Cell death in Pseudomonas aeruginosa biofilm development. *J. Bacteriol.,* 185**,** 4585-92.

WHITCHURCH, C. B., TOLKER-NIELSEN, T., RAGAS, P. C. & MATTICK, J. S. 2002. Extracellular DNA required for bacterial biofilm formation. *Science,* 295**,** 1487.

WIDEN, J. E., FOLSOM, R. C., CONE-WESSON, B., CARTY, L., DUNNELL, J. J., KOEBSELL, K., LEVI, A., MANCL, L., OHLRICH, B., TROUBA, S., GORGA, M. P., SININGER, Y. S., VOHR, B. R. & NORTON, S. J. 2000. Identification of neonatal hearing impairment: hearing status at 8 to 12 months corrected age using a visual reinforcement audiometry protocol. *Ear Hear.,* 21**,** 471-87.

WILTON, M., CHARRON-MAZENOD, L., MOORE, R. & LEWENZA, S. 2016. Extracellular DNA acidifies biofilms and induces aminoglycoside resistance in Pseudomonas aeruginosa. *Antimicrob. Agents Chemother.,* 60**,** 544-553.

WINDHAM, I. H., SERVETAS, S. L., WHITMIRE, J. M., PLETZER, D., HANCOCK, R. E. W. & MERRELL, D. S. 2018. Helicobacter pylori Biofilm Formation Is Differentially Affected by Common Culture Conditions, and Proteins Play a Central Role in the Biofilm Matrix. *Appl. Environ. Microbiol.,* 84.

WINTHER, B., GROSS, B. C., HENDLEY, J. O. & EARLY, S. V. 2009. Location of bacterial biofilm in the mucus overlying the adenoid by light microscopy. *Arch. Otolaryngol. Head Neck Surg.,* 135**,** 1239-45.

WOLK, D. M. 2016. Microbiology of Middle Ear Infections: Do You Hear What I Hear? *Clin. Microbiol. Newsl.,* 38**,** 87-93.

WOLSKA, K. I., GRUDNIAK, A. M., RUDNICKA, Z. & MARKOWSKA, K. 2016. Genetic control of bacterial biofilms. *Journal of applied genetics,* 57**,** 225-238.

WU, Y., ZHANG, Q.-Z., XU, T., MA, Y., BAI, J.-N., ZHU, T., LOU, Q., GÖTZ, F., QU, D. & ZHENG, C.-Q. 2018. Nicotine enhances Staphylococcus epidermidis biofilm formation by altering the bacterial autolysis, extracellular DNA releasing, and polysaccharide intercellular adhesin production. *Front. Microbiol.,* 9**,** 2575.

YAMAN, H., YILMAZ, S., ALKAN, N., SUBASI, B., GUCLU, E. & OZTURK, O. 2010. Shepard grommet tympanostomy tube complications in children with chronic otitis media with effusion. *Eur. Arch. Otorhinolaryngol.,* 267**,** 1221-1224.

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

YANG, C. & MONTGOMERY, M. 2018. Dornase alfa for cystic fibrosis. *Cochrane Database Syst. Rev.*

YANG, L., HAAGENSEN, J. A., JELSBAK, L., JOHANSEN, H. K., STERNBERG, C., HOIBY, N. & MOLIN, S. 2008. In situ growth rates and biofilm development of Pseudomonas aeruginosa populations in chronic lung infections. *J. Bacteriol.,* 190**,** 2767-76.

YANG, L., LIU, Y., MARKUSSEN, T., HØIBY, N., TOLKER-NIELSEN, T. & MOLIN, S. 2011. Pattern differentiation in co-culture biofilms formed by Staphylococcus aureus and Pseudomonas aeruginosa. *FEMS Immunol. Med. Microbiol.,* 62**,** 339-347.

YANG, R., SABHARWAL, V., OKONKWO, O. S., SHLYKOVA, N., TONG, R., LIN, L. Y., WANG, W., GUO, S., ROSOWSKI, J. J., PELTON, S. I. & KOHANE, D. S. 2016. Treatment of otitis media by transtympanic delivery of antibiotics. *Sci. Transl. Med.,* 8**,** 356ra120-356ra120.

YOKOI, K. J., SUGAHARA, K., IGUCHI, A., NISHITANI, G., IKEDA, M., SHIMADA, T., INAGAKI, N., YAMAKAWA, A., TAKETO, A. & KODAIRA, K. 2008. Molecular properties of the putative autolysin Atl(WM) encoded by Staphylococcus warneri M: mutational and biochemical analyses of the amidase and glucosaminidase domains. *Gene,* 416**,** 66-76.

YOO, M. H., CHO, Y. S., CHOI, J., CHOUNG, Y. H., CHUNG, J. H., CHUNG, J. W., HAN, G. C., JEON, E. J., JUN, B. C., KIM, D. K., KIM, K. S., LEE, J. H., LEE, K. Y., LEE, S. H., MOON, I. S., PARK, H. J., PARK, S. N., RHEE, J., SEO, J. H. & YEO, S. G. 2018. Microbiological Results From Middle Ear Effusion in Pediatric Patients Receiving Ventilation Tube Insertion: Multicenter Registry Study on the Effectiveness of Ventilation Tube Insertion in Pediatric Patients With Chronic Otitis Media With Effusion: Part I. *Clin. Exp. Otorhinolaryngol.,* 11**,** 181-185.

ZATORSKA, B., GROGER, M., MOSER, D., DIAB-ELSCHAHAWI, M., LUSIGNANI, L. S. & PRESTERL, E. 2017. Does Extracellular DNA Production Vary in

Staphylococcal Biofilms Isolated From Infected Implants versus Controls? *Clin. Orthop. Relat. Res.,* 475**,** 2105-2113.

ZETZMANN, M., OKSHEVSKY, M., ENDRES, J., SEDLAG, A., CACCIA, N., AUCHTER, M., WAIDMANN, M. S., DESVAUX, M., MEYER, R. L. & RIEDEL, C. U. 2015a. DNase-sensitive and-resistant modes of biofilm formation by Listeria monocytogenes. *Front. Microbiol.,* 6**,** 1428.

ZETZMANN, M., OKSHEVSKY, M., ENDRES, J., SEDLAG, A., CACCIA, N., AUCHTER, M., WAIDMANN, M. S., DESVAUX, M., MEYER, R. L. & RIEDEL, C. U. 2015b. DNase-Sensitive and -Resistant Modes of Biofilm Formation by Listeria monocytogenes. *Front. Microbiol.,* 6**,** 1428.

ZHENG, Y., HE, L., ASIAMAH, T. K. & OTTO, M. 2018. Colonization of medical devices by staphylococci. *Environ. Microbiol.,* 20**,** 3141-3153.

ZHU, L., KUANG, Z., WILSON, B. A. & LAU, G. W. 2013. Correction: Competence-Independent Activity of Pneumococcal Enda Mediates Degradation of Extracellular DNA and Nets and Is Important for Virulence. *PLoS One,* 8.

ZIELHUIS, G. A., RACH, G. H. & VAN DEN BROEK, P. 1989. Screening for otitis media with effusion in preschool children. *Lancet,* 1**,** 311-4.

ZULIANI, G., CARLISLE, M., DUBERSTEIN, A., HAUPERT, M., SYAMAL, M., BERK, R., DU, W. & COTICCHIA, J. 2009. Biofilm density in the pediatric nasopharynx: recurrent acute otitis media versus obstructive sleep apnea. *Ann. Otol. Rhinol. Laryngol.,* 118**,** 519-24.

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 N eisseria gonorrhoeae. *Environ. Microbiol.,* 16**,** 1040-1052.

Appendix A: Scientific Protocol of the study

Scientific Protocol

6th June 2015, Version 5.0

Title of the study:

Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.

Name of investigator(s)

Mr Mohamed R. ElBadawey, Dr Nicholas S. Jakubovics, Prof James G. Burgess, Mr Ali M. Wayes

Summary

Chronic otitis media with effusion (COME, glue ear) is the most common childhood ear problem and it is the commonest cause of acquired hearing loss in children in the developed world. Increasing evidence implicates bacterial biofilms in the middle ear and nasopharyngeal tonsils (adenoids) as a significant cause of COME.

A bacterial biofilm is a surface associated microbial community enclosed in self-produced polymeric matrix. Resistance to both antibiotic treatment and the body's defence systems is one of the major clinical features of pathogenic bacterial biofilms.

The ability to effectively study the role of adenoid microbial community (microbiota) structure in the pathogenesis of middle ear diseases, and COME in particular, has been limited by laboratory techniques since it is often difficult to culture and identify fastidious or uncultivable microorganisms, particularly when they reside within biofilms. Advanced high throughput 16S ribosomal RNA (rRNA) gene cataloguing methods for characterising microbial community compositions have recently been developed to the point where they are accessible for general microbiology laboratories. These exciting developments are now enabling characterisation of the human-associated microbiota at a level of detail that far exceeds previous methods.

In addition, developments in our understanding of biofilms is enabling the development of new technologies that are targeted specifically to biofilm bacteria. For example, recent research in Newcastle University has discovered potent anti-biofilm molecules from marine bacteria that have been shown to work effectively in the dispersal of many non-medical biofilms and medical biofilms obtained from inflamed human paranasal sinuses.

This study aims to identify and compare microbial community components of adenoid samples from patients with COME with those of middle ear effusions, and versus adenoid microbial community components of patients without COME using combination of high throughput 16S ribosomal RNA (rRNA) gene sequencing and traditional culture assays. Furthermore, the study will also assess the effectiveness of anti-biofilm molecules, such as marine derived extracellular nuclease (NucB), in the dispersal of adenoids and middle ear biofilms outside the

body in order to establish important proof-of-concept data for the further development of anti-biofilm molecules that may be helpful in treatment of COME.

Scientific justification:

Chronic otitis media with effusion (COME) is the most common cause of chronic ear disorder and acquired hearing impairment in children in the developed world. Children between one and six years old are most likely to be affected. The prevalence of COME in children around 2 years of age is approximately 20%. It is estimated to affect about 80% of children at some stage in their development (Kubba *et al.*, 2000; Otitis media with effusion, 2011). COME is defined as the persistence of middle ear fluid beyond 12 weeks in the absence of signs and symptoms of acute inflammation (such as pain and fever). It is the leading cause of elective surgery in children (Rovers *et al.*, 2004).

In the past, middle ear effusion fluid was considered sterile. Since Senturia et al (Senturia *et al.*, 1958) first isolated bacteria from middle ear effusions in 1958, the bacterial aetiology of otitis media, including acute otitis media and chronic otitis media with effusion, has become widely accepted. Bacterial migration to the middle ear from the adenoid through the Eustachian tube is the most likely mechanism for the pathogenesis of COME (Gates *et al.*, 1988; Zuliani *et al.*, 2009; Hoa *et al.*, 2010; Nistico *et al.*, 2011). The adenoid microbiota , the collective bacterial community that lives in and on the adenoids, consists of diverse bacterial species (Brook *et al.*, 2000). The ability to effectively study the role of adenoid multi-microbial community (microbiota) structure and intermicrobial species interactions in the pathogenesis of middle ear diseases, and specifically COME, has been limited by laboratory techniques such as traditional culture-based assays and conventional polymerase chain reaction (PCR) which are known to be relatively insensitive for identifying the total population of fastidious, uncultivable microbes and bacteria residing in biofilms (Swidsinski *et al.*, 2007; Stol *et al.*, 2013).

Major advancements in microbiota research have been facilitated by molecular technologies such as a next-generation DNA sequencing platforms (such as pyrosequencing and Illumina sequencing platforms which can be used for 16S rRNA genes cataloguing), have allowed the characterization of microbial communities that may include fastidious and uncultivable organisms, which resist traditional culturing methods, and would otherwise be masked by fast-growing species in a traditional culture-based assay (Morgan and Huttenhower, 2012). Additionally, molecular methods are also more sensitive than culturing for microorganisms living in biofilms, and can provide important species level information about microbial communities. These techniques are relatively cost effective in comparison to traditional Sanger cataloguing (Swidsinski *et al.*, 2007; Kozich *et al.*, 2013; Wong *et al.*, 2013).

To the best of our knowledge, only Liu *et al.* (2011) have analysed microbial community profiles in middle ear effusion, adenoid and tonsil samples obtained from a child with COME

using open, and non-targeted *16SrRNA* gene-based cataloguing. Although there was a variation in microbial communities in the three set of samples and the results indicated that the adenoid may be a potential origin for both the middle ear and tonsil microbiota, this study was restricted to samples from a single patient and its findings cannot be generalized until reproduced in a larger study populations.

Increasing evidence supports the role of bacterial biofilms in the pathogenesis of COME (Fergie *et al.*, 2004). Biofilms were confirmed in the middle ear mucosa of children with COME and/or RAOM in 65%-92% of cases (Hall-Stoodley *et al.*, 2006b; Thornton *et al.*, 2011), on the surface of ventilation tubes removed from children with COME (Barakate *et al.*, 2007) and recurrent acute otitis media (RAOM) (Post, 2001), but not in any studied control samples, strongly supporting the role of bacterial biofilms in the pathogenesis of COME and RAOM. Previous studies using chinchilla models of COME have also confirmed biofilm formation on the middle ear mucosa through scanning electron microscopy (SEM) (Ehrlich *et al.*, 2002).

A bacterial biofilm is defined as a microbial community enclosed in a self-produced polymeric matrix that is irreversibly associated with an inert or living surface. The matrix is a complex mixture rich in polysaccharides, proteins and extracellular DNA (eDNA) (Donlan, 2002; Fergie *et al.*, 2004). Resistance to both antibiotic treatment and host defence is one of the major clinical features of bacterial biofilms (Gilbert *et al.*, 1997). This new understanding of COME aetiology also leads to potentially novel therapeutic possibilities that may improve current management options. At present, treatment of persistent symptomatic COME involves drainage of the effusion and T-tube insertion, but this does not address any persistent bacterial infection, and serves to merely remove the effusion that is the result of a middle ear inflammation. It is therefore perhaps not surprising that about a quarter of cases will require further surgical treatment within 2 years, and the average number of procedures per child is greater than two (Gates *et al.*, 1987; Daniel *et al.*, 2006).

Bacteria in biofilms are up to 1000 times more resistant to the action of antibiotics than their planktonic counterparts (Gilbert *et al.*, 1997). A number of novel approaches for treating biofilms have been proposed, including interfering with chemical communication between micro-organisms, disruption of biofilm-related genes or degrading the biofilm matrix with enzymes (Ehrlich *et al.*, 2005; Njoroge and Sperandio, 2009; Kaplan, 2010; Yang *et al.*, 2012). Different species bacterial biofilms are associated with COME and/or adenoid hypertrophy (Hall-Stoodley *et al.*, 2006a; Hoa *et al.*, 2010; Daniel *et al.*, 2012; Thornton *et al.*, 2013) with different organisms playing a role. For this reason any method to treat biofilms would need to target a component that is widely utilised by different microorganisms in the biofilm matrix.

One possible target that has received significant interest in recent years is extracellular DNA (eDNA). It has become clear that eDNA is a key structural component of many different

microbial biofilms and this molecule has been shown to serve several critical functions including stabilising the biofilm structure (Jakubovics *et al.*, 2013), enhancing initial adhesion to surfaces (Whitchurch *et al.*, 2002; Vilain *et al.*, 2009; Wessman *et al.*, 2014), promoting the exchange of genetic information (Molin and Tolker-Nielsen, 2003), and acting as a nutrient store that can be utilised during nutrient depletion (Finkel and Kolter, 2001).
Recent research in Newcastle University has discovered potent anti-biofilm molecules from marine bacteria that have been shown to work effectively in the dispersion of non-medical biofilms and some medical biofilms obtained from inflamed human paranasal sinuses by degrading eDNA (Nijland *et al.*, 2010; Shields *et al.*, 2013).

This study therefore will identify the microbes producing the biofilms in COME and adenoid enlargement and will evaluate the effect of the anti- biofilm molecules on its dispersal. If these molecules prove to be effective in the break up and removal of middle ear and adenoids biofilms, it will be a great discovery as it will change the treatment modalities of COME with a more patient friendly technique. The research is therefore justified in that it may offer improved patient care and treatment, and a reduction in costs associated with such surgery by a potentially significant amount.

Aims and Objectives of the study:

This study aims to compare microbial community components of adenoid specimens from patients with COME with those of middle ear effusions, and versus adenoid microbial community components of patients without COME using a combination of culture-independent high throughput *16S rRNA* gene cataloguing and traditional culture assays in order to develop in depth understanding of the role of adenoid microbial community structure in the pathogenesis of COME. Furthermore, the study will also assess the effect of anti-biofilm molecules (such as marine derived extracellular nuclease enzyme, NucB,) on biofilms of chronic otitis media with effusion and adenoids enlargement in vitro (outside the body). Potentially, this preliminary assessment of the effect of anti-biofilm molecules on these biofilms may set the stage to improve the treatment options for COME patients. The objectives of the study are as follows:

1. To characterise different microbial species from middle ear effusion and/or adenoid samples of patients with COME and adenoids of those with adenoid enlargement without COME using combination of high throughput 16S ribosomal RNA (rRNA) gene sequencing (cataloguing) and culturing assays.
2. To visualize biofilm community structure of adenoids and middle ear effusion samples using confocal laser scanning microscopy (CLSM).

3. To develop a laboratory model of biofilms representing the natural microbial community of individual adenoids and/or middle ear effusion biofilms.
4. To assess the role of eDNA in biofilm stability and early microbial attachment in *in vitro* biofilm models of a variety of different bacterial species from patients with COME.
5. To investigate the sensitivity of clinical isolates from middle ear effusions and adenoids in biofilm models to anti-biofilm molecules.

Patients and methods:

Inclusion criteria

The inclusion criteria are:

1. Age 16 or less.
2. Patients who are already been listed for adenoid surgery in the form of adenoidectomy or adenotonsillectomy WITHOUT any middle ear pathology at the Freeman hospital ENT department and they decide to participate in this study by signing the consent form.
3. Patients who are already been listed for short or long term ventilation tubes with or without adenoidectomy.- COME confirmed by clinical examination, hearing test and tympanometry.

Exclusion criteria

The exclusion criteria are:

1. Patients who do not provide consent.
2. Previous grommets.
3. Previous middle ear surgery.
4. Previous adenoid surgery.
5. Immunodeficiency.
6. Craniofacial malformations.
7. Underlying syndrome affecting the ear or nose.
8. Cleft palate.
9. Patients who have not had any visible middle ear effusion fluid in both ears during the operation.
10. Patients should not be involved in other medicinal trials related to COME and adenoid enlargement.
11. Patients with recurrent acute otitis media (three or more attacks of acute otitis media within 6months prior to the surgery).

Sample size of the study:

The study will need collection of two types of samples:

1. Middle ear effusion (MME) fluid samples: we will require sampling of at least 40 MMEs of patients with chronic otitis media with effusion (COME) no more than 60. It would be possible to get two MEEs samples from a single patient with bilateral COME.
2. Adenoids tissue samples, we will require recruitment of 40 patients with adenoid enlargement no more than 60 divided into two groups according to their clinical characteristics: those with COME (n=30) and those without COME (n= 30).

At least 40 samples of middle ear effusion fluid and 40 samples of adenoid tissue will give us enough material to characterize the microbial components of the biofilms and to test the anti-biofilm molecules compared to a control. We contemplate that we will never exceed 120 samples for both middle ear effusion fluid and adenoids tissue to obtain enough samples to analyse in our study.

Patients will be recruited from ENT operations theatres in Newcastle Freeman Hospital. Sampling is based on a consecutive sampling method. The patients and their parents or guardians will receive an information sheet about the study and subsequently sign a consent form to participate. Those on the list for adenoidectomy for adenoid enlargement with normal middle ear will be recruited to the study to act as controls. On the day of surgery, if the patients and the parents or guardians have agreed to participate, the project will be discussed again, also they will have an opportunity to ask questions related to the study.

The middle ear effusion fluid and/or removed adenoid specimens will be placed in a sterile collection tube containing sterile reduced tissue fluid and transported by the investigator to the Oral Biology laboratory in Newcastle University according to the Trust policy on transferring samples within the Trust. These samples are routinely removed during surgery therefore no additional intervention and sample harvest will be required during the planned surgical intervention.

These samples will be stored under a unique anonymised study identification number, so the investigator and the research team will not have an access to patient's personal information. The samples will be analysed in microbiology department in the Dental School. Samples will be divided into three parts. The first part will be examined by confocal laser scanning microscopy (CLSM) for the presence of biofilms. The second part of the sample will undergo microbial isolation using traditional culture-based methods. Then a laboratory biofilm model will be developed for the growth and analysis of isolated microorganisms. The anti-biofilm activity of potential anti-biofilm molecules such as marine derived extracellular deoxyribonuclease enzyme, NucB, against model middle ear or adenoid biofilms will be

assessed. Biofilm dispersal and breakup will be measured and quantified by confocal laser scanning microscopy, 3D imaging and quantitative image analysis, viable cell counting of planktonic bacteria and crystal violet staining methods. Finally, the third part of the samples will undergo microbial DNA extraction and purification. Subsequently, high throughput cataloguing of 16S genes from middle ear effusions and adenoids biofilms will be employed to provide information about the origin of bacteria colonising the middle ear cavity. The statistical analysis of the data will be carried out by the research team in Newcastle University. There will be no usable samples left at the end of the study and any very small amount of sample or tissue will be destroyed in accordance with Human Tissue Authority Code of Practice.

References

Barakate M, Beckenham E, Curotta J, da Cruz M (2007). Bacterial biofilm adherence to middle-ear ventilation tubes: scanning electron micrograph images and literature review. *J Laryngol Otol* 121:993-997.

Brook I, Shah K, Jackson W (2000). Microbiology of healthy and diseased adenoids. *Laryngoscope* 110:994-999.

Daniel M, Vaghela H, Philpott C, Thomas RSA, Gannon M, Spencer H, *et al.* (2006). Does the benefit of adenoidectomy in addition to ventilation tube insertion persist long-term? *Clinical Otolaryngology* 31:580-580.

Daniel M, Imtiaz-Umer S, Fergie N, Birchall JP, Bayston R (2012). Bacterial involvement in otitis media with effusion. *International Journal of Pediatric Otorhinolaryngology* 76:1416-1422.

Donlan RM (2002). Biofilms: Microbial Life on Surfaces. *Emerging Infectious Diseases* 8:881-890.

Ehrlich GD, Veeh R, Wang X, Costerton JW, Hayes JD, Hu FZ, *et al.* (2002). Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *Jama* 287:1710-1715.

Ehrlich GD, Stoodley P, Kathju S, Zhao Y, McLeod BR, Balaban N, *et al.* (2005). Engineering Approaches for the Detection and Control of Orthopaedic Biofilm Infections. *Clinical orthopaedics and related research*:59-66.

Fergie N, Bayston R, Pearson JP, Birchall JP (2004). Is otitis media with effusion a biofilm infection? *Clin Otolaryngol Allied Sci* 29:38-46.

Finkel SE, Kolter R (2001). DNA as a nutrient: novel role for bacterial competence gene homologs. *J Bacteriol* 183:6288-6293.

Gates GA, Avery CA, Prihoda TJ, Cooper JC, Jr. (1987). Effectiveness of adenoidectomy and tympanostomy tubes in the treatment of chronic otitis media with effusion. *N Engl J Med* 317:1444-1451.

Gates GA, Avery CA, Prihoda TJ (1988). Effect of adenoidectomy upon children with chronic otitis media with effusion. *Laryngoscope* 98:58-63.

Gilbert P, Das J, Foley I (1997). Biofilm susceptibility to antimicrobials. *Adv Dent Res* 11:160-167.

Hall-Stoodley L, Hu F, Gieseke A, et al. (2006a). Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 296:202-211.

Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, Hayes J, *et al.* (2006b). Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *Jama* 296:202-211.

Created on 06.06.2015 12.00 PM, Version 5.0

Hoa M, Syamal M, Schaeffer MA, Sachdeva L, Berk R, Coticchia J (2010). Biofilms and chronic otitis media: an initial exploration into the role of biofilms in the pathogenesis of chronic otitis media. *Am J Otolaryngol* 31:241-245.

Jakubovics NS, Shields RC, Rajarajan N, Burgess JG (2013). Life after death: the critical role of extracellular DNA in microbial biofilms. *Lett Appl Microbiol* 57:467-475.

Kaplan JB (2010). Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J Dent Res* 89:205-218.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013). Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl Environ Microbiol* 79:5112-5120.

Kubba H, Pearson JP, Birchall JP (2000). The aetiology of otitis media with effusion: a review. *Clin Otolaryngol Allied Sci* 25:181-194.

Liu CM, Cosetti MK, Aziz M, Buchhagen JL, Contente-Cuomo TL, Price LB, *et al.* (2011). The otologic microbiome: a study of the bacterial microbiota in a pediatric patient with chronic serous otitis media using 16SrRNA gene-based pyrosequencing. *Arch Otolaryngol Head Neck Surg* 137:664-668.

Molin S, Tolker-Nielsen T (2003). Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Curr Opin Biotechnol* 14:255-261.

Morgan XC, Huttenhower C (2012). Chapter 12: Human microbiome analysis. *PLoS Comput Biol* 8:e1002808.

Nijland R, Hall MJ, Burgess JG (2010). Dispersal of Biofilms by Secreted, Matrix Degrading, Bacterial DNase. *PLoS ONE* 5:e15668.

Nistico L, Kreft R, Gieseke A, Coticchia JM, Burrows A, Khampang P, *et al.* (2011). Adenoid reservoir for pathogenic biofilm bacteria. *J Clin Microbiol* 49:1411-1420.

Njoroge J, Sperandio V (2009). Jamming bacterial communication: new approaches for the treatment of infectious diseases. *EMBO Mol Med* 1:201-210.

Otitis media with effusion. (2011). *NICE CKS*.

Post JC (2001). Direct evidence of bacterial biofilms in otitis media. *Laryngoscope* 111:2083-2094.

Rovers MM, Schilder AG, Zielhuis GA, Rosenfeld RM (2004). Otitis media. *Lancet* 363:465-473.

Senturia BH, Gessert CF, Carr CD, Baumann ES (1958). Studies concerned with tubotympanitis. *Ann Otol Rhinol Laryngol* 67:440-467.

Shields RC, Mokhtar N, Ford M, Hall MJ, Burgess JG, ElBadawey MR, *et al.* (2013). Efficacy of a Marine Bacterial Nuclease against Biofilm Forming Microorganisms Isolated from Chronic Rhinosinusitis. *PLoS ONE* 8:e55339.

Stol K, Verhaegh SJ, Graamans K, Engel JA, Sturm PD, Melchers WJ, *et al.* (2013). Microbial profiling does not differentiate between childhood recurrent acute otitis media and chronic otitis media with effusion. *Int J Pediatr Otorhinolaryngol* 77:488-493.

Swidsinski A, Göktas Ö, Bessler C, Loening-Baucke V, Hale LP, Andree H, *et al.* (2007). Spatial organisation of microbiota in quiescent adenoiditis and tonsillitis. *Journal of Clinical Pathology* 60:253-260.

Thornton RB, Rigby PJ, Wiertsema SP, Filion P, Langlands J, Coates HL, *et al.* (2011). Multi-species bacterial biofilm and intracellular infection in otitis media. *BMC Pediatr* 11:94.

Thornton RB, Wiertsema SP, Kirkham LA, Rigby PJ, Vijayasekaran S, Coates HL, *et al.* (2013). Neutrophil extracellular traps and bacterial biofilms in middle ear effusion of children with recurrent acute otitis media--a potential treatment target. *PLoS One* 8:e53837.

Vilain S, Pretorius JM, Theron J, Brozel VS (2009). DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms. *Appl Environ Microbiol* 75:2861-2868.

Created on 06.06.2015 12.00 PM, Version 5.0

Wessman M, Bjarnsholt T, Eickhardt-Sorensen SR, Johansen HK, Homoe P (2014). Mucosal biofilm detection in chronic otitis media: a study of middle ear biopsies from Greenlandic patients. *Eur Arch Otorhinolaryngol*.

Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002). Extracellular DNA required for bacterial biofilm formation. *Science* 295:1487.

Wong KH, Jin Y, Moqtaderi Z (2013). Multiplex Illumina sequencing using DNA barcoding. *Curr Protoc Mol Biol* Chapter 7:Unit 7.11.

Yang L, Liu Y, Wu H, Song Z, Høiby N, Molin S, *et al.* (2012). Combating biofilms. *FEMS Immunology & Medical Microbiology* 65:146-157.

Zuliani G, Carlisle M, Duberstein A, Haupert M, Syamal M, Berk R, *et al.* (2009). Biofilm density in the pediatric nasopharynx: recurrent acute otitis media versus obstructive sleep apnea. *Ann Otol Rhinol Laryngol* 118:519-524.

Created on 06.06.2015 12.00 PM, Version 5.0

Appendix B: 1. Participant information sheets for parents

Participant information Sheet (Version 3.0 / 29th June 2015)

Title of the study: **Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy**

Introduction

We would like to invite your child to take part in our research study. If you feel your child would be able to understand what is involved and decide for themselves, we would like to ask for your permission to ask your child if they would like to take part. If you don't feel your child would be able to do that then we would like to ask for your permission for your child to take part. Before you decide we would like you to understand why the research is being done and what it would involve for you/your child. Please take time to read and understand the following information. **One of our team will go through the information sheet with you and answer any questions you have.**

Background information:

Chronic otitis media with effusion (COME, glue ear)

Chronic otitis media with effusion (COME) is an accumulation of fluid in the middle ear space which is located behind the eardrum, which lasts beyond 12 weeks in the absence of pain and fever. COME is the commonest cause of acquired deafness and elective surgery in children in the developed world. Children between one and six years old are most likely to be affected. Although in the majority of cases COME is transient, a significant proportion of children develop persistent symptoms that may affect hearing, education, language or behaviour. If COME persists after a three month period of watchful waiting, treatment with ventilation tubes (VTs or grommets) or hearing aids may be considered.

Adenoids (Nasopharyngeal tonsils):

The nasopharyngeal tonsils, or adenoids, are lymphoid tissue located in the upper respiratory tract at the junction of the nose and throat. As part of the immune system, the adenoids play a major role in the body's immune response to microbes that are introduced through the upper airways. Because the adenoids lie adjacent to the opening of the Eustachian tube, the passage between the back of the nose and the inside of the ear, the adenoids have long been recognized as an important factor in the causation of middle ear infection (also known as otitis media). It is believed that the adenoids serve as a potential reservoir of opportunistic pathogens. Bacteria are proposed to spread via the short Eustachian tube to the middle ear where they cause acute, recurrent or persistent infections. Significant adenoids enlargement due to recurrent infections can

also cause nasal obstruction, leading to breathing, swallowing and sleep problems. If the condition does not improve with antibiotic therapy, surgical removal of the adenoid is often recommended.

What does 'Bacterial biofilm' mean?

The term 'biofilm' is used to describe the collection of microbes in the middle ear and adenoids. The presence of the biofilms in the middle ear and adenoids is thought to be the cause of chronic otitis media with effusion and/or adenoid hypertrophy. They produce the sticky secretions that collect in the middle ear space behind the eardrum.

What are the purposes of this study?

We are studying the biology of microbes (or bugs) in the fluids collected from the middle ear space and/or in surgically removed enlarged adenoids. We would like to employ an advanced technique of microbial analysis to study the role of microbial populations in the fluids collected from middle ear space and/or removed enlarged adenoids in chronic otitis media with effusion.

Furthermore, Professor Grant Burgess, who is a member of the research team, has discovered a new compound which can dissolve and break up this sticky slime making it easier to treat and remove. This compound works with some bacteria, but we do not yet know if it works with the bacteria from the middle ear and/or adenoids. We therefore hope to see if our compound is successful at dissolving bacterial biofilms from the middle ear and adenoids.

Your child's participation in the study is entirely voluntary and will not affect the planned procedure for your/your child's ear or adenoid. A routine step of the surgery is the removal of the sticky fluid in the middle ear and/or removal of the enlarged adenoid. These sticky fluids or removed adenoids are normally discarded.

We are asking for your permission to ask your child, if you feel they would be able to understand what is involved and decide for themselves, or, if not, your permission, to use a sample for laboratory studies instead of throwing it away.

We are going to collect a sample of the middle ear fluids and/or the removed adenoids to perform our experimental research. The samples will be completely anonymous and you/your child's details will not be linked to any samples.

It is up to you/your child to decide whether or not you would like to join the study. We will describe the study to you and go through this information sheet. If you would be happy for your child to take part then what you should do depends on how old your child is and if you feel they can decide for themselves:

If you feel your child would be able to understand what is involved and decide for themselves, please sign the Consent Form yourself. Please then show your child the Information Sheet (for their age group) and ask them if they would like to take part. If

your child would like to take part then they need to also sign the Assent Form (for their age group) themselves.
There are three Information Sheets for young people of different ages, 6-11, 12-15 and 16 year olds. There are two Assent Forms, one is for 6-11 and the other for 12-15 year olds. There is a Consent Form for 16 year olds because at that age people can give Consent and are normally considered to be capable of deciding for themselves.

If you would be happy for your child to take part, but, you don't feel they would be able to understand what is involved and decide for themselves, then you can sign the Consent Form and they can take part.
If you are not happy for your child to take part then you don't need to do anything. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care that you/your child receive.

What are the benefits of taking part?

We cannot promise that the study will help you/your child but the information we get from this study will provide new information that will help us to improve the prevention and treatment of people with chronic otitis media with effusion in the future.

Will information about my child taking part in this study be kept confidential?

All information which is collected about you/your child during the course of the research will be kept strictly confidential. The only information held in our files will be your and your child's name, on the Consent form and your child's name on an Assent form (if they sign one). The consent forms will be kept in a confidential secure place.

The data collected for the study will be anonymized and will be looked at by authorized persons from the research team. They may also be looked at by authorized people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will ensure that we meet this duty.

What will happen to the results at the end of the research study?

After the study is completed, we can send you a summary of the main findings from the study. Note that this will not specifically refer to your child's samples, but will summarise the overall findings of the research. You must indicate this on the consent form and agree to retain your contact details for this purpose. If you change your mind, please contact us via email or call the contact person in the study team. Email address and phone number are provided at the end of the booklet.

Who is organizing and funding the study?

This study is sponsored the Newcastle upon Tyne Hospitals NHS Foundation Trust and funded by the Ministry of Higher Education and Scientific Research, Republic of Iraq in collaboration with Newcastle University. This work is being carried out as part of a PhD research study.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. The NHS Research Ethics Committee and Local NHS Trust R&D Team have reviewed and approved the study.

Thank you for taking the time to read this information and for considering taking part in our research study. If you would like any further information please contact:

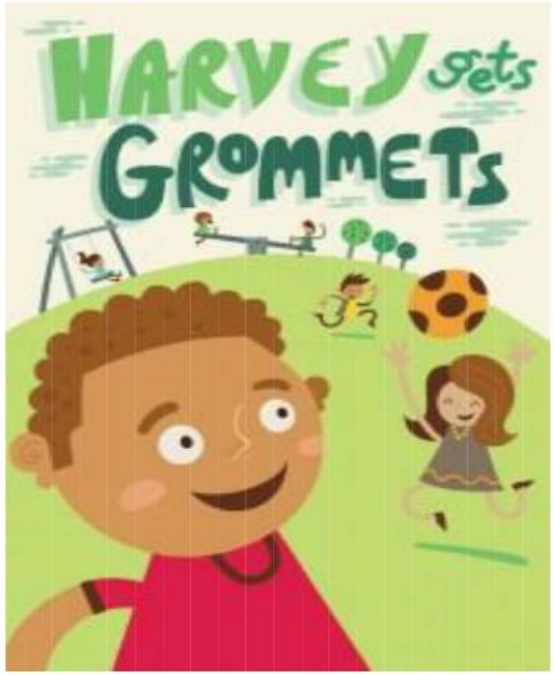
Mr Mohamed Reda ElBadawey,
ENT Consultant,
Freeman Hospital,
Newcastle upon Tyne,
NE7 7DN,
Email: Mohamed.Reda@nuth.nhs.uk
Tel: 01912448351,
Secretary Miss Dixon Tel. 0191 2231413.

2. Participant Information Sheet for Children Aged 3-5

Hello, I am Harvey.

I am feeling happy because I am here for a good reason.

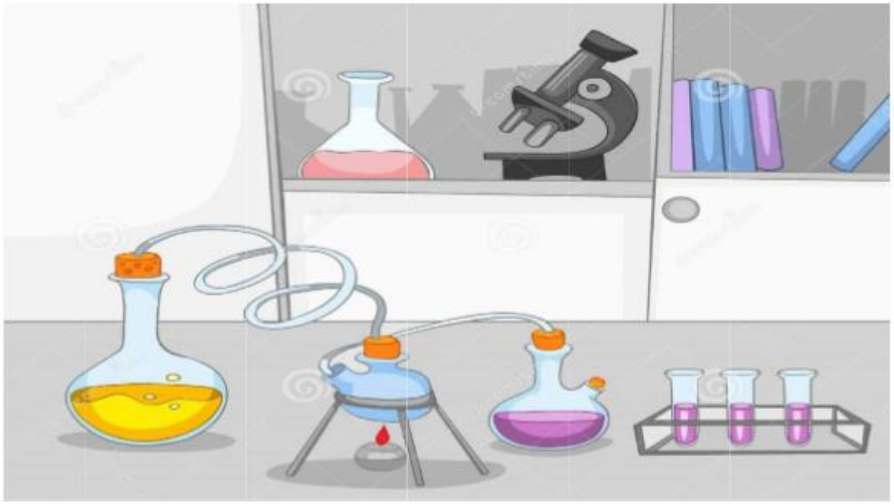
Would you like to hear my story?



Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.
Participant Information Sheet Age 3-6 years.
Created on 13.04.2015 12.00 PM, Version 2.0

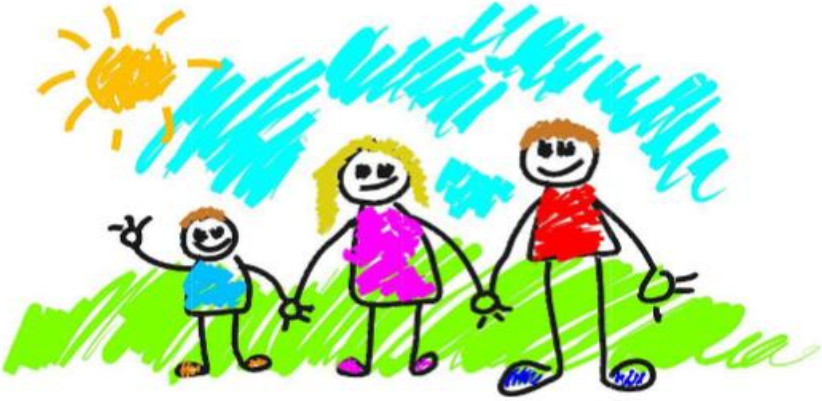
I was complaining from difficulty in hearing and block nose. When my Mam and Dad took me to the doctor, he found out that I have fluid in my ears and puffy adenoids. Therefore, the doctor removed the fluid from my ears and the puffy adenoid for my health. Then, I donated them to the researcher.

Now, my removed ear fluid and adenoid are in the laboratory. Do you know why I am happy now? I am happy because I can help the researchers to answer their questions by donating my removed ear fluid and nasal tonsils.



What will be happen to my removed ear fluid and/or adenoid next?

They will be examined by the researchers using the microscope and special machines. They want to know what kind of microbes (bugs) are in my removed ear fluid and puffy adenoid so that they can fight off the harmful microbes. This will help them to stop other people get sick.



Before I decided to give my removed ear fluid and/or adenoid, I discussed it with my Mam and Dad. Once all of us were happy to join this study, the researcher asked my Mam and Dad to sign their name on a letter.

At the end, I am very happy and my Mam and Dad are proud of me because I have given my removed ear fluid and/or adenoid for a good reason which is helping the researchers to do their work.

Bye-bye my friends. Do Well as I have done!

Chief investigator:
Mr Mohamed Reda ElBadawey,
ENT Consultant,
Freeman Hospital,
Newcastle upon Tyne,
NE7 7DN,
Tel: 01912448351,
Secretary Miss Dixon Tel. 0191 2231413.

3. Participant Information Sheet for Children Aged 6-11

Participant Information Sheet for Children Aged 6-11
To be shown and read by parent/carer if required

Title of the study: The study of bacteria associated with chronic otitis media with effusion (glue ear) and adenoids enlargement.

What is research?
Research involves careful experiments to find out the answer to an important question.

Why is this project being done?
We want to find out about bacteria in children who have glue ear and nasal tonsils swelling.

What are bacteria (microbes)?
Bacteria are tiny little creatures that are everywhere around us. We can't see them without a microscope because they are so small, but they are in the air, on our skin, in our bodies, in the ground, and all throughout nature.

Are bacteria dangerous?
Most bacteria aren't dangerous, but some are and can make us sick. These bacteria are called pathogens. Pathogens can cause diseases in human called infections. Some examples of infections are the infection of the space behind the ear drum (middle ear), also known as otitis media, and the infection of adenoids. Fortunately, we can take antibiotics which help to fight off the bad bacteria (pathogens).

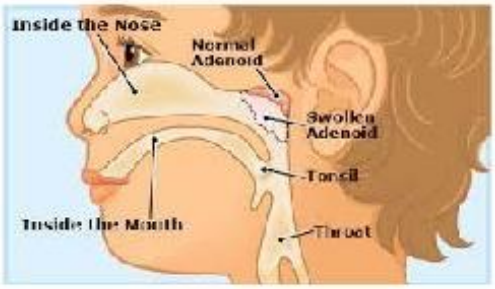
Are bacteria all bad?

Not at all. Actually, most bacteria are good, though, like the bacteria in your tummy that help you to digest food.

What are adenoids and when do they swell?

The **adenoids** are a patch of tissue that protects kids from getting sick. They sit in the back of the nasal cavity.
Adenoids do important work as infection fighters for babies and little kids by trapping harmful bacteria and viruses that you breathe in or swallow. Adenoids usually shrink after about age 5, and by the teenage years they often practically disappear.

Because adenoids trap germs that enter the body, adenoid tissue sometimes temporarily swells (gets puffier) as it tries to fight off an infection. The swelling sometimes gets better, but sometimes adenoids can get infected themselves. Swollen or infected adenoids can make it tough to breathe and cause ear problems like glue ear. Sometimes doctors recommend removing the adenoids if medicine doesn't help or if they're making a kid sick a lot. This means going into the hospital and having surgery.



What is glue ear (also known as chronic otitis media with effusion)?

Glue ear occurs when the space behind the ear drum (middle ear) is filled with fluid. The fluid can be thick and sticky which is why it's called glue ear. Glue ear can affect one or both ears. It often follows an ear infection or can occur on its own.

If both ears have fluid, child's hearing can be notably reduced. Reduced hearing for prolonged periods of time during the early years may affect speech and language development. If a child has fluid in the space behind the ear drum for more than three months, the doctor may take this fluid out and place a tiny plastic tubes called Grommet or ventilation tube into child's ear drum in a short operation.

Why is the study using ear space fluid and adenoids?

If your ears have fluid and/or you have swollen adenoids, there may be harmful bacteria in your ear fluid and swollen adenoids. So, we need to take your removed ear fluid and/or adenoids to the laboratory and grow bacteria in a special jelly-like material called Agar. Then, we use microscope and special machines to find out what kinds of bacteria are there inside your ear fluid and/or adenoids and fight off the harmful bacteria in the laboratory using a new medicine.

Why me?

You have been chosen because you have fluid in your ears and/or swollen adenoids that will be removed by an ear, nose, and throat surgeon. You can help us find some answers about the kinds of bacteria present inside your ear fluid and/or adenoids. It will help out us to understand how these bacteria can cause glue ear and find new ways to treat children with glue ear and/or swollen adenoids other than subjecting them to surgery. In some cases, new treatment could prevent children from needing to undergo further surgery. We are asking 120 children in total to take part.

We will ask for your permission to get your removed ear fluid and/or adenoids from your doctor. On your big day, when your ear fluid and/or adenoids will be removed by the doctor, we will explain the study to you and you can ask us anything about the study. If you agree to donate your removed ear fluid and/or adenoids, you and your parent will be asked to give written consent for that.

Do I have to take part?

Your mam and dad have said it is ok for us to ask you if you want to take part but that doesn't mean you have to - if you don't want to take part that's fine. You do not have to take part. It is up to you. We would like you to read this information sheet. If you are happy to take part, we would like you to write your name on two forms. We will also ask your mam or dad to write their name on the forms and give one back to us. You can still change your mind later. If you don't want to take part, just say no.

How can I be part of the project?

You need to discuss with your Mam and Dad. Once you and your Mam and Dad agree to join this study, we can go through the next step.

Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.

Participant information sheet for children Age 6-11 year.

Created on 29.06.2015 12.00 PM, Version 3.0

What is the next step?

We will take your removed ear fluid and/or adenoids with us after your doctor has removed them. We may tell you what we have found out about all the children in Newcastle, who took part in this study.

What happens when the research study stops?

We will collect all the information together and we will decide how many kinds of bacteria are there inside ear fluids and adenoids of children with glue ear and/or swollen adenoids. We can also decide whether our new medicine is good to fight off harmful bacteria obtained from their removed ear fluids and/or adenoids.

Would you like a summary of the results to be sent to your home address once it is available?

If you wish, we will tell you what we have found out from our studies in Newcastle. We will write to you at home if you and your parent allow us to keep your contact details for this purpose. These results will be a summary of the whole project, and will not contain specific information about you or your health.

What if I don't want to do the research anymore?
Just tell your Mam, Dad, carer, doctor or the investigator at any time. They will not be cross with you. You will still have the same care whilst you are at hospital.

Will anyone else know I'm doing this?
The people in our research team will know you are taking part. The doctor looking after you while you are in hospital will also know. No one else will know because we will not use your name or address. You will get a number which will be used instead.

What happens to what the researchers find out?
When we collect your information we will make sure it is stored in a safe place and only the people doing the research study can look at it.
We will use the information to tell people about kinds of good and harmful bacteria living in/on children removed ear fluid and/or adenoids, and whether our new medicine is good or bad in fighting of harmful bacteria causing glue ear and/or swollen adenoids

Did anyone else check the study is OK to do?
This study has been checked by several people, to make sure it is alright.

How can I find out more about this study?
Your mum, dad, carer or other grownup you trust may be able to answer your questions. The doctor looking after you can also help you find out more about the study.

Thank you for taking the time to read this – please ask any questions if you need to.

Chief Investigator:

Mr Mohamed Reda ElBadawey,
ENT Consultant,
Freeman Hospital,
Newcastle upon Tyne,
NE7 7DN,
Tel: 01912448351,
Secretary Miss Dixon Tel. 0191 2231413.

4. Participant Information Sheet for Young Persons Aged 12-15

Participant Information Sheet for Young Persons Aged 12-15

Title of the study: The study of bacterial biofilms in chronic otitis media with effusion (glue ear) and adenoids enlargement.

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have. So,**

What is research?
Research involves careful experiments to find out the answer to an important question.

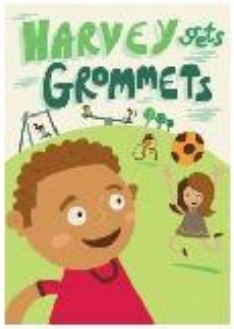
Why is this project being done?
We want to find out about bacterial biofilms in children who have glue ear and adenoids (nasal tonsils) puffiness.

What is bacterial biofilm?
The term biofilm is used to describe the collection of microbes (bugs) in the space behind the eardrum (also known as middle ear) and adenoids. The presence of the bacterial biofilms in the middle ear and adenoids are thought to be the cause of the persistence of the ear and adenoids infections. They produce the sticky fluid that collects in the space behind the eardrum leading to glue ear.

What is glue ear (also known as chronic otitis media with effusion)?

Glue ear occurs when the space behind the ear drum (middle ear) is filled with fluid. The fluid can be thick and sticky which is why it's called glue ear. Glue ear can affect one or both ears. It often follows an ear infection or can occur on its own.

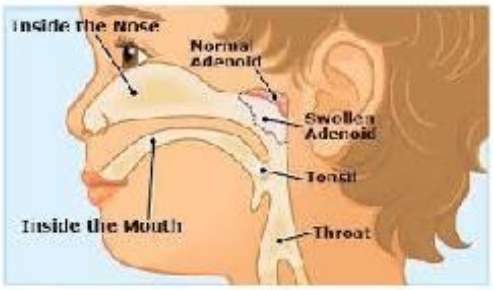
If both ears have fluid, the child's hearing can be notably reduced. Reduced hearing for prolonged periods of time during the early years may affect speech and language development. If a child has fluid in the space behind the ear drum for more than three months, the doctor may take this fluid out and place a tiny plastic tubes called a Grommet or ventilation tube into child's ear drum in a short operation.



What are adenoids and when do they swell?

The **adenoids** are a patch of tissue that protects children from getting sick. They sit in the back of the nasal cavity.
Adenoids do important work as infection fighters for babies and young children by trapping harmful bacteria and viruses that you breathe in or swallow. Adenoids usually shrink after about age 5, and by the teenage years they often practically disappear.

Because adenoids trap germs that enter the body, adenoid tissue sometimes temporarily swells (gets puffier) as it tries to fight off an infection. The swelling sometimes gets better, but sometimes adenoids can get infected themselves. Swollen or infected adenoids can make it tough to breathe and cause ear problems like glue ear. Sometimes doctors recommend removing the adenoids if medicine doesn't help or if they're making a child sick a lot. This means going into the hospital and having surgery.



Why is the study using middle ear fluid and adenoids?

We are studying the biology of microbes (or bugs) in the fluid collected from the middle ear and surgically removed swollen adenoids. If your ears have fluid and/or you have swollen adenoids, there may be microbial biofilms in your ear fluid and swollen adenoids. So, we need to take your removed ear fluid and/or adenoids to the laboratory in order to use microscope and other pieces of equipment to study bacterial biofilms inside your ear fluid and/or adenoids.

We have discovered a new compound which can dissolve and break up this sticky slime (bacterial biofilm) making it easier to treat and remove. This compound works with some bacteria, but we do not yet know if it works with the bacteria from the middle ear fluid and/or adenoids. We therefore hope to see if our compound is successful at dissolving bacterial biofilms from the middle ear and adenoids.

Why me?

You have been chosen because you have fluid in your ears and/or swollen adenoids that will be removed by an ear, nose, and throat surgeon. You can help us find some answers about the kinds of bacteria present inside your ear fluid and/or adenoids. It will help out us to understand how these bacteria can cause glue ear and find new ways to treat children with glue ear and/or swollen adenoids other than subjecting them to surgery. In some cases, new treatment could prevent children from needing to undergo further surgery. We are asking 120 children and teenagers in total to take part.
We will ask for your permission to get your removed ear fluid and/or adenoids from your doctor. On your big day, when your ear fluid and/or adenoids will be removed by the doctor, we will explain the study to you and you can ask us anything about the study. If you agree to donate your removed ear fluid and/or adenoids, you and your parent will be asked to give written consent for that.

Do I have to take part?

Your mam and dad have said it is ok for us to ask you if you want to take part but that doesn't mean you have to - if you don't want to take part that's fine. It is up to you. We would like you to read this information sheet. If you are happy to take part, we would like you to write your name on two forms. We will also ask your mam or dad to write their name on the forms and give one back to us. You can still change your mind later. If you don't want to take part, just say no.

How can I be part of the project?

You need to discuss with your Mam and Dad. Once you and your Mam and Dad agree to join this study, we can go through the next step.

What happens when the research study stops?

We will collect all the information together and we will decide how many kinds of bacteria are there inside ear fluids and adenoids of children with glue ear and/or swollen adenoids. We can also decide

whether our new compound is successful at dissolving bacterial biofilms from the middle ear and adenoids.

Would you like a summary of the results to be sent to your home address once it is available?

If you wish, we will tell you what we have found out from our studies in Newcastle. We will write to you at home if you and your parent allow us to keep your contact details for this purpose. These results will be a summary of the whole project, and will not contain specific information about you or your health.

What if I don't want to do the research anymore?
Just tell your Mam, Dad, carer, doctor or the investigator at any time. They will not be cross with you. You will still have the same care whilst you are at hospital.

Will anyone else know I'm doing this?
The people in our research team will know you are taking part. The doctor looking after you while you are in hospital will also know. No one else will know because we will not use your name or address. You will get a number which will be used instead.

What happens to what the researchers find out?
When we collect your information we will make sure it is stored in a safe place and only the people doing the research study can look at it.
We will use the information to tell people about kinds of bacterial biofilms living in/on children removed ear fluid and/or adenoids, and whether our new compound is good in dissolving and breaking up the bacterial biofilms that cause glue ear and/or swollen adenoids

Did anyone else check the study is OK to do?
This study has been checked by several people, to make sure it is alright.

How can I find out more about this study?
Your mam, dad, carer or other adult you trust may be able to answer your questions. The doctor looking after you can also help you find out more about the study.

Thank you for taking the time to read this – please ask any questions if you need to.

Chief Investigator:

Mr Mohamed Reda ElBadawey,
ENT Consultant,
Freeman Hospital,
Newcastle upon Tyne,
NE7 7DN,
Tel: 01912448351,
Secretary Miss Dixon Tel. 0191 2231413.

5. Patient information Sheet for Young Persons Aged 16

Patient information Sheet for Young Persons Aged 16

Title of the study: **Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy**

We would like to invite you to take part in our research study. Before you decide we would like you to understand why the research is being done and what it would involve for you. **One of our team will go through the information sheet with you and answer any questions you have.**

What is chronic otitis media with effusion (COME)?

Chronic otitis media with effusion (COME), also known as glue ear, is an accumulation of fluid in the middle ear space which is located behind the eardrum, which lasts beyond 12 weeks in the absence of pain and fever. Glue ear is the commonest cause of hearing loss in children in the developed world. Children between one and six years old are most likely to be affected, but occasionally it can affect older children. If glue ear persists after a three month period of watchful waiting, treatment with ventilation tubes (sometimes known as grommets) or hearing aids may be considered.

What are adenoids (Nasopharyngeal tonsils)?

The nasopharyngeal tonsils, or adenoids, are masses of lymphoid tissue situated in the upper respiratory passage at the junction of the nose and throat. As part of the body's defence system, the adenoids play a major role in our responses to microbes that enter through the upper airways. Because the adenoids lie adjacent to the opening of the Eustachian tube, the passage between the back of the nose and the inside of the ear, it is possible that ear infections may arise from bacteria that colonise the adenoids.
Significant swelling of the adenoids due to recurrent infections can also cause obstruction of the nasal passages, leading to problems with breathing, swallowing and/or sleep. If the condition does not improve with antibiotics, surgical removal of the adenoid may be recommended.

What does 'Bacterial biofilm' mean?

The term 'biofilm' is used to describe the collection of microbes in the middle ear and adenoids. The presence of the biofilms in the middle ear and adenoids is thought to be the cause of glue ear and/or adenoid enlargement. They produce the sticky secretions that collect in the middle ear space behind the eardrum leading to glue ear.

What are the purposes of this study?

We are studying the biology of microbes (or bugs) in the fluids collected from the middle ear space and/or in surgically removed enlarged adenoids. We would like to employ an advanced technique of microbial analysis to study the role of microbial populations in the fluids collected from middle ear space and/or removed enlarged adenoids in chronic otitis media with effusion.

Furthermore, our research team has discovered an exciting new compound that can break up and remove sticky bacterial biofilms from surfaces. This compound works with some bacteria, but we do not yet know if it works with the bacteria from the middle ear and/or adenoids. We therefore hope to see if our compound is successful at dissolving bacterial biofilms from the middle ear and adenoids.

Your participation in the study is entirely voluntary and will not affect the planned procedure for your ear or adenoid. A routine step of the surgery is the removal of the sticky fluid in the middle ear and/or removal of the enlarged adenoid. These sticky fluids or removed adenoids are normally discarded.
We would like your permission to use a sample for laboratory studies instead of throwing it away.
We are going to collect a sample of the middle ear fluids and/or the removed adenoids to perform our experimental research. The samples will be completely anonymous and your details will not be linked to any samples.
Your mother and father have said it is ok for us to ask you if you want to take part but that doesn't mean you have to - if you don't want to take part that's fine. We will describe the study to you and go through this information sheet. If you agree to take part, we will then ask you to sign a consent form. You are free to withdraw at any time, without giving a reason. This would not affect the standard of care you receive.

What are the benefits of taking part?

We cannot promise that the study will help you but the information we get from this study will provide new information that will help us to improve the prevention and treatment of people with chronic otitis media with effusion in the future.

Will information about my taking part in this study be kept confidential?

All information which is collected about you during the course of the research will be kept strictly confidential. The only information held in our files will be your name, on the consent form and your mother's or father's name on their own consent form. The consent forms will be kept in a confidential secure place.
The data collected for the study will be anonymized and will be looked at by authorized persons from the research team. They may also be looked at by authorized people to check that the study is being carried out correctly. All will have a duty of confidentiality to you as a research participant and we will ensure that we meet this duty.

What will happen to the results at the end of the research study?

After the study is completed, we can send you a summary of the main findings from the study. Note that this will not specifically refer to your own samples, but will summarise the overall findings of the research. You must indicate this on the consent form and agree to retain your contact details for this purpose.

Who is organizing and funding the study?

This study is sponsored the Newcastle upon Tyne Hospitals NHS Foundation Trust and funded by the Ministry of Higher Education and Scientific Research, Republic of Iraq in collaboration with Newcastle University. This work is being carried out as part of a PhD research study.

Who has reviewed the study?

All research in the NHS is looked at by an independent group of people, called a Research Ethics Committee, to protect your interests. The NHS Research Ethics Committee and Local NHS Trust R&D Team have reviewed and approved the study.

Thank you for taking the time to read this information and for considering taking part in our research study. If you would like any further information please contact:

Mr Mohamed Reda ElBadawey,
ENT Consultant,
Freeman Hospital,
Newcastle upon Tyne,
NE7 7DN,
Tel: 01912448351,
Secretary Miss Dixon Tel. 0191 2231413.

Appendix C: 1. Parent /guardian consent form

PARENT /GUARDIAN CONSENT FORM (Version 3.0/ 29th June 2015)

Title of Project: Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.

Name of Researchers: M Reda ElBadawey, N Jakubovics, JG Burgess, AM Wayes.

Please write your choice, which is 'Y' for YES and 'N' for NO at the box to confirm that:

1. I confirm that I have read and understand the information sheet dated 29th June 2015 (version 3.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	Yes [ ] No [ ]
2. I understand that my child's participation is voluntary and that I am and my child is free to withdraw at any time without giving any reason, without my child's medical care or legal rights being affected.	Yes [ ] No [ ]
3. I understand that relevant sections of my child's medical notes and data may be collected during the study, and may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my child's taking part in this research. I give permission for these individuals to have access to my child's records.	Yes [ ] No [ ]
4. Please initial the appropriate option: a) I agree for my child to take part in this study and I don't feel they are able to sign an Assent Form themselves so I have not asked them to. b) I agree that my child may be asked if they want to take part in the study and can take part if they wish to. They will complete their own Assent Form if they wish to take part. c) My child is 16 years old and will complete their own Consent Form if they wish to take part.	Yes [ ] No [ ] Yes [ ] No [ ] Yes [ ] No [ ]
5. I would like a summary of the results to be sent to my home address once it is available. I agree that my contact details will be retained for this purpose.	Yes [ ] No [ ]

Name of Child:

Name of Parent/Guardian:

Signature: Date:

Name of Person taking consent:

Signature: Date:

When completed: 1 for participant; 1 for researcher site file (original); 1 to be kept in medical notes.

2. Consent Form for Young Persons Aged 16

Consent Form for Young Persons Aged 16 (Version 1.0/ 29th June 2015)

Title of Project: Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.

Name of Researchers: M Reda ElBadawey, N Jakubovics, JG Burgess, AM Wayes.

Please write your choice, which is 'Y' for YES and 'N' for NO at the box to confirm that:

1. I confirm that I have read and understand the information sheet dated 29th June 2015 (version 1.0) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily.	Yes [ ] No [ ]
2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected.	Yes [ ] No [ ]
3. I understand that relevant sections of my medical notes and data may be collected during the study, and may be looked at by individuals from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records.	Yes [ ] No [ ]
4. I would like a summary of the results to be sent to my home address once it is available. I agree that my contact details will be retained for this purpose.	Yes [ ] No [ ]
5. I agree to take part in this study.	Yes [ ] No [ ]

Name:

Signature: Date:

Name of Person taking consent:

Signature: Date:

When completed: 1 for participant; 1 for researcher site file (original); 1 to be kept in medical notes.

Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.
Consent Form for Young Persons Aged 16
Created on 29.06.2015 12.00 PM, Version 1.0

3. Assent Form for Children Aged 6-11

Assent Form for Children Aged 6-11 (Version 3.0/ 29th June 2015)
(To be completed by the child and their parent)

Title of Project: Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.

Child (or if unable, parent on their behalf) /young person to circle all they agree with:

Have you read information (or had it read to you) about this project?	Yes/ No
Has somebody else explained this project to you?	Yes/ No
Do you understand what this project is about?	Yes/ No
Have you asked all the questions you want?	Yes/ No
Have you had your questions answered in a way you understand?	Yes/ No
Do you understand it's OK to stop taking part at any time?	Yes/ No
I would like a summary of the results to be sent to my home address	Yes/ No
Are you happy to begin this study?	☺ / ☹

If any answers are "no" or you don't want to take part, don't sign your name!
If you do want to take part, please write your name and today's date.

Your name: **Date:**

Your parent or guardian must write their name here too and sign below if they are happy for you to do the project

Name of parent/Guardian:
Signature **Date:**

The person who explained this project to you needs to sign too:

Print Name: **Date:**
Signature

Thank you for your help!

Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.
Assent Form for Children Aged 6 -11
Created on 29.06.2015 12.00 PM, Version 1.0

4. Assent Form for Young Persons Aged 12-15

Assent Form for Young Persons Aged 12-15 (Version 1.0/ 29th June 2015)
(To be completed by the child and their parent)

Title of Project: Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.

Child (or if unable, parent on their behalf) /young person to circle all they agree with:

Have you read information (or had it read to you) about this project?	Yes/ No
Has somebody else explained this project to you?	Yes/ No
Do you understand what this project is about?	Yes/ No
Have you asked all the questions you want?	Yes/ No
Have you had your questions answered in a way you understand?	Yes/ No
Do you understand it's OK to stop taking part at any time?	Yes/ No
I would like a summary of the results to be sent to my home address	Yes/ No
Are you happy to begin this study?	☺ / ☹

If any answers are "no" or you don't want to take part, don't sign your name!
If you do want to take part, please write your name and today's date.

Your name: **Date:**

Your parent or guardian must write their name here too and sign below if they are happy for you to do the project

Name of parent/Guardian:
Signature **Date:**

The person who explained this project to you needs to sign too:

Print Name: **Date:**
Signature

Thank you for your help!

Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.
Assent Form for young persons aged 12-15
Created on 29.06.2015 12.00 PM, Version 1.0

Appendix D: Research project insurance

ZURICH
MUNICIPAL

To Whom It May Concern

Our ref: CM/FEHE 30 July, 2015

Zurich Municipal Customer: University of Newcastle

This is to confirm that the above mentioned Customer has in force with this Company until the policy expiry on 31 July 2016 Insurance incorporating the following essential features:

Policy Number: NHE-08CA03-0013

Limit of Indemnity:		
Public Liability:	£ 25,000,000	any one event
Products Liability:	£ 25,000,000	for all claims in the
Pollution:		aggregate during any one period of insurance
Employers' Liability:	£ 25,000,000	any one event inclusive of costs

Excess:		
Public Liability/Products Liability/Pollution:	£2,500	any one event
Employers' Liability:	Nil	any one claim

Indemnity to Principals:
Covers include a standard Indemnity to Principals Clause in respect of contractual obligations.

Full Policy:
The policy documents should be referred to for details of full cover.

Yours faithfully

Underwriting Services
Zurich Municipal
Farnborough

Zurich Municipal
Zurich House
2 Gladiator Way
Farnborough
Hampshire
GU14 6GB

Telephone 0870 2418050
Direct Phone: 01252 387843
Direct Fax: 01252 375893
E-mail
catherine.matthews@uk.zurich.com

Communications will be monitored regularly to improve our service and for security and regulatory purposes

Zurich Municipal is a trading name of Zurich Insurance plc

A public limited company incorporated in Ireland. Registration No. 13460 Registered Office: Zurich House, Ballsbridge Park, Dublin 4, Ireland.

UK branch registered in England and Wales Registration No. BR7985. UK Branch Head Office: The Zurich Centre, 3000 Parkway, Whiteley, Fareham, Hampshire PO15 7JZ

Authorised by the Central Bank of Ireland and subject to limited regulation by the Financial Conduct Authority. Details about the extent of our regulation by the Financial Conduct Authority are available from us on request

Appendix E: Sponsorship review letter

From: Johnston, Andrew [mailto:Andrew.Johnston@nuth.nhs.uk]
Sent: 05 June 2015 16:25
To: Reda, Mohamed
Cc: Nick Jakubovics
Subject: Sponsorship review 7514

Dear Mohamad & Nick,

Project title: Biofilms study in chronic otitis media with effusion and adenoids

R&D ref: **7514**

Apologies for the delay in responding, we have had a high number of request as well as moving offices.

Thank you for submitting the above project to The Newcastle upon Tyne Hospitals NHS Foundation Trust for sponsorship. After consideration the supplied documentation has been reviewed and we are pleased to confirm that the Trust is able to act as your research sponsor in principle, subject to obtaining all relevant national and local authorisations.

Before I can electronically authorise your form as sponsor's representative, I just need to request a few changes and clarifications to your project:

IRAS form:

Filter Question 9 – It appears that this study is an educational project therefore tick 'yes' and complete sections re: academic supervisors etc.

A5-1 - please complete section with R&D ref. number and protocol version and date

A30 - please remove line stating consent forms will be destroyed.

A45 – at the end of the study the Trial Site File along with consent forms must be archived in accordance with the Joint Research Office SOP

A52 – consent forms must not be destroyed. The original consent must be stored in the Trial Site File, with a copy filed in the patient notes and a copy passed to the patient. At the end of the study all documents must be archived as stated above.

A64-1 – please add my details – address as email signature

A68-1 – please add Joanna Ho as lead NHS R&D contract – address as per email signature

A76-2 - The University may need to cover the 'design' of the study as this is a university PhD study. Who is the protocol author? Kelly Lovelock, University Insurance Officer should be able to help out with this. Email is: insurance@newcastle.ac.uk. Kelly is likely to request a copy of the protocol and REC form for review.

Protocol:

Please add version number and date to each page & ensure any of the above IRAS changes are reflected in the protocol.

Once above change(s)/clarification(s) are complete, please request sponsor authorisation via IRAS

----------//----------

Since the 01 June 2014 the process of applying to REC has changed. Guidance regarding this can be found at: http://www.hra.nhs.uk/research-community/applying-for-approvals/research-ethics-committee/ .

Please note that SPONSORSHIP is **not** the same as granting NHS Permission for the study. Once all signatures have been gained you may apply for NHS Permission, your allocated R&D Officer is Joanna Ho who can provide further advice regarding this process. Only once you receive a signed letter from a Research Governance Manager on Trust letter-headed paper can the study proceed within NUTH.

Many thanks & once again apologies for the delay.

Best wishes,

Andrew

Andrew Johnston
Research Management and Governance Manager

Newcastle upon Tyne Hospitals NHS Foundation Trust

Newcastle Joint Research Office
Regent Point
Regent Farm Road
Newcastle upon Tyne
NE3 3HD

Tel: 0191 282 5969
Fax: 0191 282 4524

Newcastle Joint Research Office

http://www.newcastlejro.org.uk/

****
****
This message may contain confidential information. If you are not the intended recipient please inform the
sender that you have received the message in error before deleting it.
Please do not disclose, copy or distribute information in this e-mail or take any action in reliance on its contents:
to do so is strictly prohibited and may be unlawful.

Thank you for your co-operation.
****
****

Appendix F: Project funder letter

Embassy of the Republic of Iraq
Cultural Attaché - London

سفارة جمهورية العراق
الدائرة الثقافية - لندن

ممثلية وزارة التعليم العالي والبحث العلمي العراقية في المملكة المتحدة وايرلندا
Representative of the Iraqi Ministry of Higher Education and Scientific Research (MOHESR) in the UK and Ireland

No: 2861/2014 – 1373
Date: 28/05/2014

Our Reference: S 1373

To: Newcastle University

Re: Mr. Ali Mostafa WAYES/ D.O.B: 25/06/1978

Dear Sir / Madam,

We are the official representative of the Iraqi Ministry of Higher Education and Scientific Research (MOHESR) in the UK and Ireland. We confirm that (MOHESR) is sponsoring the above named student. We, on behalf of MOHESR, will be responsible for payments of all fees for his full-time PhD study in (Institute of Cellular Medicine) for three years at your University (From 01/07/2014 to 30/06/2017).

Please contact us if you have any queries regarding this matter.

Yours faithfully

Prof. Mosa Almosawe
Cultural Attaché

Note: See overleaf for terms and conditions

For office use only:
نسخة منه الى:
- ملف الدائرة العامة ملف الصادر المصور.
- ملف الطالب. ملف المصور.
- المعني به.

2014/05/28 مز

V1 Jan 2014

Address: 14 - 15 Child's Place, Earls Court, London SW5 9RX Tel: 0044 (0) 20 7370 2940 **Fax:** 0044 (0) 20 7370 2941
Website: www.iraqiculturalattache.org.uk **Email:** office@iraqiculturalattach.org.uk / britain@mohesr.gov.iq

Appendix G: REC checklist

Date: 16/06/2015 | Reference: 15/NE/0225 | Online Form

APPLICATION TO RESEARCH ETHICS COMMITTEE

All studies except clinical trials of investigational medicinal products

REC Ref: 15/NE/0225
Short Title of Study: Biofilms study in chronic otitis media with effusion and adenoids
CI Name: Mr Mohamed ElBadawey
Sponsor: Newcastle upon Tyne Hospitals NHS Foundation Trust

IMPORTANT: This checklist supports an application where supporting documentation is electronically submitted, with the application form, from IRAS to the review body.

You must attach your supporting documentation to this checklist before submitting your application.

Instructions for attaching files:

1. Click on the file upload symbol in the far right hand column of the row for the document you wish to submit. This opens a pop-up window where you can select the document to upload and enter details about it.
2. Select where you want to upload the document from: *Uploading from your hard drive* - select the radio button for hard drive and then click on the button immediately below to open your computer's standard file browser window. Once you have chosen the file, simply complete the fields for document title, version number and date. Click the 'upload document' button.
   Uploading from the IRAS document storage areas called 'My documents' or 'Project Documents' - Click the appropriate radio button to open a window showing all the documents you have saved in that IRAS storage area. Select the document you want to use, enter a name for the document and click 'Add document' button.
3. When you have attached a document to the checklist the screen will show a summary of the details. Click on 'Close' to return to the checklist and select another document.

Notes about attaching files:

- The field for the type of document is pre-chosen based on the item you selected in the checklist.
- All letters must be dated. All other accompanying documents must bear version numbers and dates. These will then show in your checklist.

Notes about documents attached to this checklist:

- All documents listed below that are applicable to the application must be submitted so that the application clearly describes the study and is complete with all required documentation.
- If a document is not attached to the checklist please state why in the "reason not supplied" field. For example if not applicable, please enter "N/A". You can add/edit information in this field by clicking
- The CI must send all the relevant documents and files to each PI.
- This button allows you to add extra documents of the same type
- If any documents are revised as a result of review by any other body, the revised version must be submitted.

For information on submitting the documents and files attached to this checklist please select the "E-Submission" tab.

Document	Subtitle	Enclosed	Date	Version	Office Use	Reason not supplied
Covering letter on headed paper	Cover Letter	Yes	16/06/2015			
Research protocol or project proposal	Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy	Mandatory	06/06/2015	5.0		
Summary CV for Chief Investigator (CI)	CV- MOHAMED REDA EL BADAWEY	Mandatory	17/06/2015			

Version 5.4.0 1

Summary CV for supervisor (student research)	CV- Nicholas S. Jakubovics	Yes	16/06/2015			
Summary CV for student	CV- Ali Mostafa Wayes	Yes	15/06/2015			
Participant information sheet (PIS)	PIS for parents	Yes	29/06/2015	3		
Participant information sheet (PIS)	PIS for children Age 6-11	Yes	29/06/2015	3		
Participant information sheet (PIS)	PIS for younger children 3-6years	Yes	13/04/2015	2		
Participant information sheet (PIS)	PIS for Young Persons Aged 12-15	Yes	29/06/2015	1		
Participant information sheet (PIS)	PISfor Young Persons Aged 16	Yes	29/06/2015	1		
Letters of invitation to participant		No				N/A
GP/consultant information sheets or letters		No				N/A
Evidence of Sponsor insurance or indemnity (non-NHS Sponsors only)	Research Project Insurance	Yes	21/05/2015			
Letter from sponsor	Sponsorship review 7514	Yes	05/06/2015			
Participant consent form	Consent Form for Parents	Yes	29/06/2015	3		
Participant consent form	Consent Form for younger persons aged 16	Yes	29/06/2015	1		
Participant consent form	ASSENT FORM FOR young persons aged 12-15	Yes	29/06/2015	1		
Participant consent form	ASSENT FORM FOR CHILDREN AGED 6-11	Yes	29/06/2015	3		
Letter from statistician		No				N/A
Letter from funder	Funding letter to Newcastle University	Yes	28/05/2014			
Referee's report or other scientific critique report		No				N/A
Summary, synopsis or diagram (flowchart) of protocol in non-technical language		No				N/A
Interview schedules or topic guides for participants		No				N/A
Validated questionnaire		No				N/A
Non-validated questionnaire		No				N/A
Copies of advertisement materials for research participants		No				N/A

Instructions for use of medical device		No				N/A
Other documents						
Other (please specify)	Summary CV for supervisor (student research)	Yes	17/06/2015	1		
Other (please specify)	Cover letter response to amendments	Yes	13/07/2015	1		

Appendix H: Ethical approvals for the study

1. REC ethical approval

NHS
Health Research Authority
NRES Committee North East - Newcastle & North Tyneside 2
Room 001
Jarrow Business Centre
Rolling Mill Road
Jarrow
NE32 3DT

Telephone: 0191 428 3561

20 July 2015

Mr Mohamed ElBadawey
ENT Department
Freeman Hospital
High Heaton
Newcastle upon Tyne
NE7 7DN

Dear Mr ElBadawey

Study title:	**Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy**
REC reference:	**15/NE/0225**
IRAS project ID:	**166424**

Thank you for your letter of 15 July 2015, responding to the Proportionate Review Sub-Committee's request for changes to the documentation for the above study.

The revised documentation has been reviewed and approved by the sub-committee.

We plan to publish your research summary wording for the above study on the HRA website, together with your contact details. Publication will be no earlier than three months from the date of this favourable opinion letter. The expectation is that this information will be published for all studies that receive an ethical opinion but should you wish to provide a substitute contact point, wish to make a request to defer, or require further information, please contact the REC Assistant Miss Kerry Dunbar, nrescommittee.northeast-newcastleandnorthtyneside2@nhs.net Under very limited circumstances (e.g. for student research which has received an unfavourable opinion), it may be possible to grant an exemption to the publication of the study.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised.

Conditions of the favourable opinion

A Research Ethics Committee established by the Health Research Authority

The favourable opinion is subject to the following conditions being met prior to the start of the study.

Management permission or approval must be obtained from each host organisation prior to the start of the study at the site concerned.

Management permission ("R&D approval") should be sought from all NHS organisations involved in the study in accordance with NHS research governance arrangements.

Guidance on applying for NHS permission for research is available in the Integrated Research Application System or at http://www.rdforum.nhs.uk.

Where a NHS organisation's role in the study is limited to identifying and referring potential participants to research sites ("participant identification centre"), guidance should be sought from the R&D office on the information it requires to give permission for this activity.

For non-NHS sites, site management permission should be obtained in accordance with the procedures of the relevant host organisation.

Sponsors are not required to notify the Committee of approvals from host organisations.

Registration of Clinical Trials

All clinical trials (defined as the first four categories on the IRAS filter page) must be registered on a publically accessible database. This should be before the first participant is recruited but no later than 6 weeks after recruitment of the first participant.

There is no requirement to separately notify the REC but you should do so at the earliest opportunity e.g. when submitting an amendment. We will audit the registration details as part of the annual progress reporting process.

To ensure transparency in research, we strongly recommend that all research is registered but for non-clinical trials this is not currently mandatory.

If a sponsor wishes to request a deferral for study registration within the required timeframe, they should contact hra.studyregistration@nhs.net. The expectation is that all clinical trials will be registered, however, in exceptional circumstances non registration may be permissible with prior agreement from NRES. Guidance on where to register is provided on the HRA website.

It is the responsibility of the sponsor to ensure that all the conditions are complied with before the start of the study or its initiation at a particular site (as applicable).

Ethical review of research sites

The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" above).

Approved documents

The documents reviewed and approved by the Committee are:

Document	*Version*	*Date*
Covering letter on headed paper [Cover Letter]	1	16 June 2015
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [Research Project Insurance]	1	21 May 2015
IRAS Checklist XML [Checklist_07072015]		07 July 2015
IRAS Checklist XML [Checklist_14072015]		14 July 2015
Letter from funder [Funding letter to Newcastle University]	1	28 May 2014
Letter from sponsor [Sponsorship review 7514]	1	05 June 2015
Other [Summary CV for supervisor (student research)]	1	17 June 2015
Other [Cover letter response to amendments]	1	13 July 2015
Participant consent form [Consent Form for Parents]	3	29 June 2015
Participant consent form [Consent Form for younger persons aged 16]	1	29 June 2015
Participant consent form [ASSENT FORM FOR young persons aged 12-15]	1	29 June 2015
Participant consent form [ASSENT FORM FOR CHILDREN AGED 6-11]	3	29 June 2015
Participant information sheet (PIS) [PIS for parents]	3	29 June 2015
Participant information sheet (PIS) [PIS for children Age 6-11]	3	29 June 2015
Participant information sheet (PIS) [PIS for younger children 3-6years]	2	13 April 2015
Participant information sheet (PIS) [PIS for Young Persons Aged 12-15]	1	29 June 2015
Participant information sheet (PIS) [PISfor Young Persons Aged 16]	1	29 June 2015
REC Application Form [REC_Form_16062015]		16 June 2015
REC Application Form [IRAS NHS REC Form]		15 July 2015
Research protocol or project proposal [Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy]	5.0	06 June 2015
Summary CV for Chief Investigator (CI) [CV- MOHAMED REDA EL BADAWEY]	1	17 June 2015
Summary CV for student [CV- Ali Mostafa Wayes]	1	15 June 2015
Summary CV for supervisor (student research) [CV- Nicholas S. Jakubovics]	1	16 June 2015

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

Feedback

You are invited to give your view of the service that you have received from the National Research Ethics Service and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:
http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance

We are pleased to welcome researchers and R & D staff at our NRES committee members' training days – see details at http://www.hra.nhs.uk/hra-training/

15/NE/0225	**Please quote this number on all correspondence**

With the Committee's best wishes for the success of this project.

Yours sincerely

pp

Dr Alasdair MacSween
Chair

Email: nrescommittee.northeast-newcastleandnorthtyneside2@nhs.net

Enclosures: *"After ethical review – guidance for researchers"*
Copy to: *Dr Nicholas Jakubovics*
Mrs Joanna Ho , Newcastle upon Tyne Hospitals NHS Foundation Trust

A Research Ethics Committee established by the Health Research Authority

2. R&D ethical approval

The Newcastle upon Tyne Hospitals NHS
NHS Foundation Trust

Regent Point
Regent Farm Road
Gosforth
Newcastle upon Tyne
NE3 3HD

Tel: (0191) 233 6161

AJ/JH/MT

22 October 2015

Mr Mohammed Reda El Badawey
Consultant Otolaryngologist
Freeman Hospital
Newcastle upon Tyne
NE7 7DN

Dear Mr Mohammed Reda El Badawey,

Trust R&D Project:	**7514**
Title of Project:	***Investigation of Bioflims associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy***
Principal Investigator:	**Mr Mohammed Reda El Badawey**
Number of patients:	**120**
Funder:	**Iraqi Ministry of Higher Education and Scientific Research (MoHESR)**
Sponsor:	**The Newcastle upon Tyne Hospitals NHS Foundation Trust**
REC number:	**15/NE/0225**
IRAS Project Code:	**166424**
First participant to be recruited by:	**21 November 2015**

After completing the necessary risk and site assessments for the above research project, The Newcastle upon Tyne Hospitals NHS Foundation Trust grants NHS Management Permission for this research to take place at this Trust dependent upon:

(i) you, as Principal Investigator, agreeing to comply with the Department of Health's Research Governance Framework for Health and Social Care, and confirming your understanding of the responsibilities and duties of Principal Investigators by signing the Investigator Responsibilities Document. A copy of this document will be kept on file within the Joint Research Office.

(ii) you, as Principal Investigator, ensuring compliance of the project with all other legislation and guidelines including Caldicott Guardian approvals and compliance with the Data Protection Act 1998, Health and Safety at Work Act 1974, any requirements of the MHRA (*eg* CTA, EudraCT registration), and any other relevant UK/European guidelines or legislation (*eg* reporting of suspected adverse incidents).

(iii) where applicable, you, as Principal Investigator, should also adhere to the GMC supplementary guidance *Good practice in research* and *Consent to research* which sets out the good practice principles that doctors are expected to understand and follow if they are involved in research – see http://www.gmc-uk.org/guidance/ethical_guidance/5991.asp

The NIHR requires NHS organisations to recruit patients to CLRN Portfolio studies within 30 days from the date of this letter. The 30 day deadline for recruiting the first patient is therefore 21 November 2015.

Please note: the Department of Health 70 day bench mark requires recruitment within 70 days of a valid SSI submission. Therefore, recruiting within the NIHR 30 day target will ensure compliance with both targets.

Sponsorship

The Newcastle upon Tyne Hospitals NHS Foundation Trust will act as Sponsor for this project, under the Department of Health's guidelines for research in health and social care.

In addition, the Trust has a Research Governance Implementation Plan, agreed with the Department of Health, in order to fully comply with Research Governance and fulfil the responsibility of a Sponsor.

As the Trust is acting as Sponsor for the research and where some of the research is taking place outside of Newcastle upon Tyne, then all costs must be met for research governance audit visits to those sites. It is the responsibility of the PI to provide confirmation to the Trust of who will pay these costs. Audit is required under the Research Governance Framework for Health and Social Care. (Please note that the Trust randomly audits 10% of approved research projects annually.)

NHS Permission applies to the research described in the protocol and related documentation as listed on the favourable ethical opinion(s) from NRES Committee North East- Newcastle & North Tyneside 2, dated 20 July 2015. Specifically, the following versions of the key documents are approved:

Document	Version	Date
Research Protocol or Project Proposal	5.0	06 June 2015
Participant Consent Form [Parents]	3	29 June 2015
Participant Consent Form [Younger persons aged 16]	1	29 June 2015
Participant Consent Form [Young persons aged 12-15]	1	29 June 2015
Participant Consent Form [Children aged 6-11]	3	29 June 2015
Participant Information Sheet [Parents]	3	29 June 2015
Participant Information Sheet [Children Age 6-11]	3	29 June 2015
Participant Information Sheet [Younger children 3-6 years]	2	13 April 2015
Participant Information Sheet [Young Persons Aged 12-15]	1	29 June 2015
Participant Information Sheet [Young Persons Aged 16]	1	29 June 2015

Any changes to these documents, or any other amendments to the study must be submitted to the Research Ethics Committee and MHRA (if relevant) for review – see http://www.nres.npsa.nhs.uk/applications/after-ethical-review/amendments/ for guidance). All amendments must be submitted to the R&D office for review in parallel with ethical and regulatory review so that implications of the amendment can be assessed. You must send a copy of all amendment documents to the R&D office and if the changes or amendments to the study have implications for costs or use of resources, you must also submit details of these changes.

It is the Principal Investigator's responsibility to ensure that all staff involved in the research have Honorary Research Contracts or the necessary Letters of Access. These must be issued prior to commencing the research.

In addition, unless otherwise agreed with the Trust, the research will be covered for negligence under the CNST (Clinical Negligence Scheme for Trusts), however cover for no-fault harm is the responsibility of the Principal Investigator to arrange if required.

Please also note that for any NHS employee who generates Intellectual Property *in the normal course of their duties*, it is recognised that the Intellectual Property Rights remain with the employer and not the employee.

Yours sincerely

Andrew Johnston
Research Management & Governance (RM&G) Manager

CC: Finance Department, Level 2, Regents Point
Phillip Yates, Clinical Director, Freeman Hospital
Julia Scott, Research Team Lead, Freeman Hospital

Appendix I: R&D valid submission checklist

R&D Valid Submission Checklist

Principal Investigator	Mr Mohamed ElBadawey
Full Study Title	Investigation of Biofilms associated with Chronic Otitis Media with Effusion and Adenoids Hypertrophy.
R&D reference	7514
NIHR CSP reference (if applicable)	N/A
Clinical Directorate	ENT

Does this research project include a new device that has previously not been used in human or a new interventional procedure not carried out before in the Trust?	No
If answered yes to the above, has this research project been reviewed by the New Interventional Procedures Committee? See guidance document for further details	Yes / No

DOCUMENTS	ENCLOSED	For R&D use only
Finance		
Commercial Projects		
NIHR Industry Costing Template: * **Portfolio:** This should always be completed by Sponsor and provided to the team for review and feedback. **Non Portfolio:** If not provided by Sponsor, the local team will have to complete this	MANDATORY	
Evidence of team lead review and authorisation of Industry Costing Tool: this should be in the form of an email from the relevant Research Team Lead	MANDATORY	
Pharmacy Costing breakdown +/or confirmation of Pharmacy approval of NIHR Industry Costing Template *(for CTIMPs, provided by Trust Pharmacy)*	MANDATORY	
Non-Commercial Projects		
NuTH Costing Tool – the costing tool must have been reviewed and approved by the relevant Research Team Lead prior to submission, please ensure an email confirming this is also submitted with the costing tool	Yes	
Confirmation of Funds - one of the following must be provided		
*Grant Award Letter	YES	
Email from sponsor confirming funding arrangements	YES	
BH Reference for grants administered through Newcastle University	BH******OSR/0180/BENC/WAYE	
Written approval from relevant Clinical Director if no funding in place	NA	

Study documents		
***** NHS R&D Form fully authorised by all required signatories via IRAS (pdf & XML)	YES	
NHS SSI Form (draft)	YES	
***** Research Protocol with version and date	YES	
***** Participant Information Sheet and Consent Form with version and date	YES	
***** All other participant related documents (GP letter, questionnaires, patient diaries, posters etc)	YES	
For ctIMP projects: Confirmation from Pharmacy that they have received Pharmacy Manual from Sponsor	N/A	
2-page CV for Principal Investigator	YES	
GCP Training Certificate for Principal Investigator *(please see guidance)*	YES	
***** Evidence of Sponsorship if not confirmed by Study Agreement or authorised copy of NHS R&D Form	MANDATORY	
***** Evidence of Insurance or Indemnity (studies with non-NHS sponsors or protocol authors who hold honorary NHS contracts)	YES/CSP/NA	
***** Investigator Brochure/ Summary of Product Characteristics - *ctIMPs only*	NA	
Signed Investigator Responsibilities Form	MANDATORY	
Draft Contract/Agreements with completed financial appendix If sponsor will not provide a completed financial appendix, then R&D will require email confirmation of this from sponsor	YES/NA	
Recruitment End Date please note this is not the study end date recorded in the IRAS documents, if unsure please check with sponsor	MANDATORY	

Documents marked with * do not need to be sent to NuTH FT R&D if they are available via the NIHR Coordinated System for gaining NHS Permission (CSP) document repository.
NuTH FT must be listed as a research site on Part C of the NHS R&D Form in order for the R&D team to be able to access the study documents in the document repository.
The NIHR CSP Reference must be provided for any documents marked as available on CSP

Documents listed below are not mandatory for study submission for R&D Approvals Committee review but may be required for R&D approval	ENCLOSED	For R&D use only
Favourable Ethical opinion from NHS Research Ethics Committee (REC)	Yes	
Notice of Acceptance *letter from MHRA*	NA	
Caldicott approval (it is advisable to submit this as early as possible to prevent delays) or confirmation this has been applied for	NA	
Confirmation of any PIC sites used to aid recruitment at NuTH	NA	
Other approvals if relevant – eg CAG, IRMER, ARSAC	NA	
Has this project been submitted for adoption to the NIHR Portfolio? If so, provide the Portfolio UKCRN ID (this is not the IRAS/NIHR CSP reference)	NA UKCRN ID:	

R&D may request further information or documentation after review of the submission

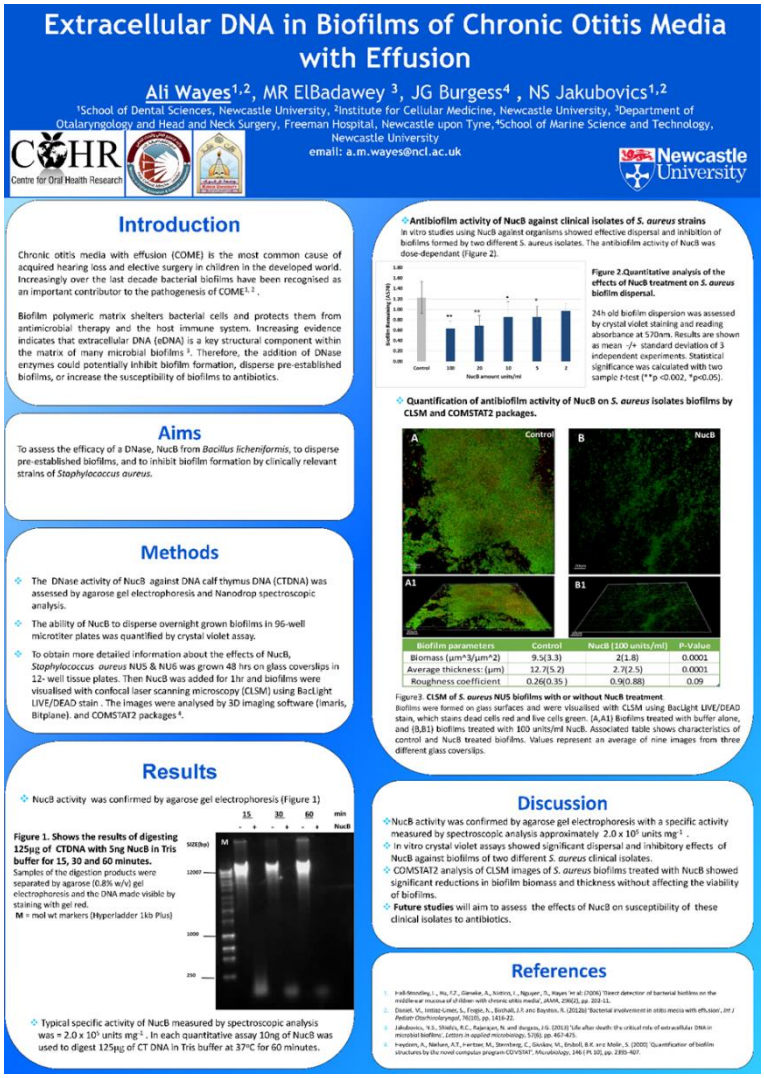
If you have any questions about the above please contact the R&D team:

The Newcastle upon Tyne Hospitals NHS Foundation Trust
Newcastle Joint Research Office, Research & Development
Level 1, Regent Point
Regent Farm Road
Gosforth
Newcastle upon Tyne
NE3 3HD

Trust.R&D@nuth.nhs.uk
0191 282 5959

Appendix J: Conferences and Meetings Attendance

1. **23-26 June 2015: EUROBIOFILMS 2015 - Fourth European Congress on Microbial Biofilms, Brno, Czech Republic (poster presentation).**



Title: Extracellular DNA in biofilms associated with chronic otitis media with effusion

Chronic otitis media with effusion (COME) is the most common cause of acquired hearing loss and elective surgery in children in the developed world. Numerous studies have implicated bacterial biofilm infections of middle ear as an important contributor to the pathogenesis of COME. Biofilm polymeric matrix shelters bacterial cells and protects them from antimicrobial therapy and the host immune system. Increasing evidence indicates that extracellular DNA (eDNA) is a key structural component within the matrix of many microbial biofilms. Therefore, the addition of DNase enzymes could potentially inhibit biofilm formation, disperse pre-established biofilms, or increase the susceptibility of biofilms to antibiotics. This study aimed to assess the efficacy of a DNase, NucB from Bacillus licheniformis, to inhibit biofilm formation by clinically relevant strains of Staphylococcus aureus. Initial experiments, using crystal violet staining, indicated that biofilm formation by two different S. aureus isolates was reduced by >50% in the presence of NucB. Structural

changes in biofilms were identified using confocal laser scanning microscopy (CLSM), IMARIS, and COMSTAT 2 computer programs. Further investigations will include testing the inhibitory effect of NucB on a variety of different bacterial species from COME patients using crystal violet assays and quantification of NucB effects on the structure of biofilms by CLSM, IMARIS, and COMSTAT2 computer programs.

2. **5-7 October 2016: ESGB & EPASG international conference "Antimicrobial resistance in microbial biofilms and options for treatment", Ghent, Belgium (oral presentation).**

Title: Antibiofilm Activitiy of the marine DNase, NucB, in biofilms associated with chronic otitis media with effusion.

Chronic otitis media with effusion (COME) is the most common disease of the ear in childhood. Numerous studies have confirmed bacterial biofilm infections as an important contributor to the pathogenesis of COME. Increasing evidence indicates that extracellular DNA (eDNA) is a key structural component within the matrix of many microbial biofilms. The aim of this study to assess the antibiofilm activity of a DNase, NucB, from Bacillus licheniformis against in vitro biofilms of COME clinical isolates. NucB treatment of some clinical isolates biofilms at concentrations range of 100-5 units/mL showed significant dose-dependent inhibition of biofilm formation and dispersion of pre-established biofilms quantified by 96-well microtiter plate crystal violet assay. Profound structural changes in biofilms were identified without affecting cells viability using confocal laser scanning microscopy (CLSM) imaging of LIVE/DEAD® stained biofilms grown on glass coverslips. There was more than 50% reduction in biomass, average thickness of the biofilms when 100 units/mL of NucB either included during biofilm formation for 24h or incubated for 1h with pre-established biofilms (48h) as calculated using COMSTAT 2. Just below 50% reduction in XTT conversion rate was observed in biofilms of these clinical biofilms which had been subjected to antimicrobial treatment at concentration 8000 – fold or greater than the minimal inhibitory concentration (MIC). Addition of NucB (100 units/mL) on these biofilms significantly increased their sensitivity to antimicrobial killing by more than 20% compared with antimicrobial treatment alone and this sensitisation was more with lower antimicrobial concentration . In summary, NucB showed efficient antibiofilm and antimicrobial –sensitizing actions against tested clinical isolates. NucB, alone or in combination with antibiotics, may potentially help in controlling biofilms associated infections including COME.

3. **18 May 2017: Annual COHR Research Afternoon, Newcastle (poster presentation).**

The same poster of EUROBIOFILMS 2015 (displayed above) was presented. Poster award winner.