Interaction of the Oral Microbiota with Respiratory Pathogens in Biofilms of Mechanically Ventilated Patients

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Abstract

Mechanically ventilated (MV) patients are at risk of ventilator-associated pneumonia. During mechanical ventilation, it has been proposed that the mouth becomes colonised by respiratory pathogens (RP) and the endotracheal tube (ETT) facilitates leakage of oropharyngeal secretions to the lower airways, whilst also supporting a biofilm. These are likely contributory risk factors for VAP. This research aimed to further establish the relationship between oral microorganisms and RP in colonisation of dental plaque and ETT biofilms. The study also investigated intervention strategies to limit RP colonisation.

The microbial composition of dental plaque, ETT biofilms, and non-directed bronchial lavages (NBLs) from MV patients was characterised using culture and molecular approaches. RPs were frequently present at all these sites, with oral microorganisms also occurring in ETTs and NBLs. Isolates from these sites in a single patient also were determined to be the same strains based on molecular typing. Additionally, NGS showed no significant difference between dental plaque and ETT biofilm microbiomes.

In vitro biofilms revealed that oral microorganisms increased RP colonisation and associated gene expression in biofilms. In *in vivo* studies, toothbrushes and foam swabs were found to be equally efficient at removing dental plaque and improving oral hygiene in MV patients. *In vitro* investigation found Chlorhexidine to be the most effective mouthwash in combatting ETT biofilms, despite high tolerance by *P. aeruginosa*. No difference between ETT biomaterials in supporting biofilms was evident.

The work highlights the importance of dental plaque as a reservoir of RP in MV patients, and these RP also colonise ETT biofilms. The synergistic effect of oral microorganisms in promoting RP colonisation reinforced the need to adequately manage oral care in MV patients. For the first time, equal effectiveness of achieving improved oral care by toothbrushes and foam swabs was demonstrated.

Abbreviations

Agr	Accessory gene regulator
AI-2	Autoinducer-2
ANOVA	Analysis of variance
APIs	Autoinducing peptides
BHI	Brain heart infusion
BLAST	Basic alignment search tool
BPE	Basic periodontal examination
C.albicans	*Candida albicans*
CAP	Community-acquired pneumonia
CFU	Colony forming units
CLSM	Confocal laser scanning microscopy
COPD	Chronic obstructive pulmonary disease
CPIS	Clinical pulmonary infection score
Crc	Catabolite repression control
CXR	Chest XR
ddNTPs	Dideoxynucleotides
DGGE	Denaturing gradient gel electrophoresis
DLVO	Derjaguin and Landau, Verwey and Overbeek
DMFT	Decayed, missing and filled teeth
DNA	Deoxyribonucleic acid

dNTPs	Deoxynucleosidetriphosphates
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
ETT	Endotracheal tube
FAA	Fastidious anaerobe agar
FAB	Fastidious anaerobe broth
FISH	Fluorescent in situ hybridisation
G	G- force
h	Hour
HAIs	Hospital-acquired infections
HAP	Hospital-acquired pneumonia
HCAP	Health care associated pneumonia
HVLP	high volume low pressure
Ica	Intercellular adhesion
ICU	Intensive care unit
IHI	Institute for health improvements
ISA	Iso-sensitest^{TM} agar
ISA	Iso-sensitive agar
M	Molar
MBEC	Minimum biofilm eradication concentration
MICs	Minimum inhibitory concentrations

Min	Minutes
MLST	Multilocus sequence typing
MRSA	Meticillin resistant *Staphylococcus aureus*
MSA	manitol salt agar
MSB	mitis salivarius bacitracin agar
MSCRAMMs	Microbial surface components that recognize adhesive matrix molecules
MSSA	Meticillin sensitive *Staphylococcus aurues*
mV	Millivolts
n/a	Not applicable
NBL	Non-directed brochial lavage
NCIB	National centre for biotechnology information
NCTC	National collection of type cultures
Ng	Nanogram
NGS	Next generation sequencing
OD	Optical Density
P. aeruginosa	*Pseudomonas aeruginosa*
P. gingivalis	*Porphyromonas gingivalis*
PBS	Phosphate buffered saline
PCR	Polimerase chain reaction
Pel	Pellicle locus

PFGE	Pulse field gel electrophoresis
pH	Power of hydrogen concentration
PIA	Polysaccharide intercellular adhesin
PNA	Peptide nucleic acid
PPFC	Parallel-plate-flow-chamber
PQS	*Pseudomonas* Quinolone Signal
PRPs	Proline-rich peptides
PsA	*Pseudomonas aeruginosa* agar
Psl	Polysaccharide synthesis locus
PVC	Polyvinyl chloride
QS	Quorum sensing
RAPD	Random amplification of polymorphic DNA
RCT	Randomised clinical trial
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
Rpm	Revolutions per minute
rRNA	Ribosomal RNA
RTF	Reduced transport fluid
S	Seconds
S. aureus	*Staphylococcus aureus*
S. mutans	*Streptococcus mutans*

SAPs	Secreted aspartyl proteinases
SD	Standard deviation
SDA	Sabouraud dextrose agar
SDB	Sabouraud dextrose broth
SEM	Scanning electron microscopy
Spl	Serine protease-like
TBE	Tris-Borate-EDTA (TBE; 0.1M Tris Base; 0.09 M Boric Acid; 0.1 mM EDTA)
U	Unit
U	Unit
V	Volt
v/v	% volume in volume
VAP	Ventilator - associated pneumonia
w/v	% weight over a 100 ml volume
w/w	% weight in weight

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1. Literature Review

1.1 Introduction

1.1.1 General commensal microflora

The human microflora is diverse and complex, with hundreds of different species constituting up to 90% of the cells that inhabit the human body (Ten Cate 2013). In a process that starts at birth, all body surfaces become colonised by permanent microbial residents and it is inevitable that microbial and host interactions will follow. In most cases, there is a mutual benefit to these interactions, however on occasions disruption to the normal balance of the microflora may lead to human disease. Every exposed body site will offer conditions that will promote colonisation by certain types of microorganisms, making each microbial community unique.

The microflora of the skin has been said to protect against colonisation by potential pathogens, as well as aiding in the processing of skin proteins, free fatty acids, and sebum (Grice *et al.* 2008). In the case of microbial residents of the gastrointestinal tract, additional help with digestion and clearance of pathogens following infection is also known to occur (Lee and Mazmanian 2010; Stecher and Hardt 2011). It has been proposed that specific microbial communities like the gut microbiome serve as additional 'organs' for the body (O'Hara and Shanahan 2006). Generally, microbial investigation has involved the study of individual species using culture-based research. When grown in liquid-based culture media, such microorganisms are said to be in a free-living or planktonic form. This growth form is not, however, representative of their natural existence where microorganisms primarily grow as part of a multispecies community within a biofilm (Johnson 2008).

1.2 Biofilms

Biofilms can be found on any moist surface including submerged substrata, pipes, medical devices, and body surfaces such as teeth and wounds. The preference for microorganisms

to grow as a biofilm is highlighted by reports that 90% of microorganisms exist naturally within biofilms (Flemming and Wingender 2010). A modern definition of a biofilm was proposed by Donlan and Costerton, describing a biofilm as a microbial-derived sessile community, characterised by cells that are irreversibly attached to a substratum or to each other, and are embedded in a matrix of extracellular polymeric substances (EPS) that they have produced, and these biofilm cells exhibit an altered phenotype with respect to growth and gene transcription (Donlan and Costerton 2002).

The above definition is greatly developed from the pioneering work of Van Leeuwenhoek in the 17th century when he first observed the scrapings of his own teeth under the microscope and described them as his 'animalcules', referring to his own dental plaque biofilm microorganisms. However, it was not until the 1940s that research into biofilms was really undertaken, this was when it became apparent that bacterial growth increased when attached to a surface (Heukelekian and Heller 1940). At the same time, it was also postulated that bacterial adherence to a surface could either be reversible or irreversible (Zobell 1943). Biofilm research suffered another relatively dormant period until the 1970s, after which, further biofilm characteristics were described, such as the apparent higher resistance of biofilm microorganisms to chlorine (Characklis 1973). In 1978, Costerton reported that in aquatic systems, with appropriate nutrient availability, bacteria adhered to surfaces and formed 'glycocalyx–enclosed biofilms' (Costerton *et al.* 1978). Costerton also postulated that chronic infections in patients with indwelling devices were also caused by bacterial biofilms residing on the device itself.

Table 1.1 shows characteristics of biofilm cells compared to 'free-living' or planktonic cells, biofilm cells are more tolerant of nutrient deprivation, and environmental changes such as pH fluctuation and exposure to oxygen free radicals (Costerton *et al.* 1995). Another feature is that the biofilm microorganisms can express genes that their free planktonic counterparts

do not (Becker *et al.* 2001; Shemesh *et al.* 2007). Interestingly, biofilm cells are described as having lower growth rates compared with planktonic cells, however, the biofilm environment would appear to provide a more sustainable form of bacterial growth perhaps better indicated by biomass and not as individual cells. A key feature of biofilms is their relatively high tolerance to antimicrobials compared with planktonic cultures, and this creates a significant challenge when treating biofilm-associated diseases (Costerton *et al.* 1999; Davey and O'Toole 2000).

Biofilms are complex heterogeneous structures, comprised and developed from multiple microcolonies. Much, like the human body, biofilms are mainly (85%) comprised of an extracellular matrix material with a relatively small percentage of cell content (15%). The biofilm cells are covered by this matrix, forming 'structures' of different sizes and shapes. Open channels occur between the microcolonies that allow fluidic flow to and from the embedded cells (Donlan and Costerton 2002).

The location of microbial cells within the biofilm also appears to have an impact on their characteristics. It has been found that since microorganisms towards the biofilm extremities have greater access to nutrients, gases, and are able to eradicate waste products more readily, they tend to more metabolically active and therefore larger in size than organisms more centrally located in the biofilm, making the former more susceptible to antibiotics (Malic 2008; Stoodley *et al.* 2002).

Table 1.1 Comparison of the characteristics of planktonic and biofilm cells.

Biofilm	**Planktonic**	**References**
Accounts for 90% of existing bacteria	10% of existing bacteria	(Flemming and Wingender 2010)
Attached to a surface	Free floating in an aqueous environment	(Costerton *et al*. 1978)
Slow growth	Comparatively rapid growth	(Brown *et al*, 1988)
Cells are dormant, smaller and not actively engaged in cell division		(Anwar *et al.* 1992)
Lower metabolic activity	Metabolic products continuously removed	(Anwar *et al*. 1992) (Costerton *et al* 1978)
Greater tolerance to antimicrobials	Greater sensitivity to antimicrobials	(Luppens *et al.* 2002)
Unique gene expression patterns		(Sauer *et al.* 2002)

1.2.1 **Biofilm formation**

There are five recognised stages involved in the development of a mature biofilm and include 1) formation of a conditioning film on the surface for microbial attachment, 2) movement of microorganisms to the conditioned surface followed by 3) reversible and irreversible attachment of microorganisms, 4) mature biofilm formation involving coaggregation/coadhesion and microbial succession, and 5) biofilm cell detachment and dispersal (Palmer and White 1997, Percival *et al.,* 2011). The stages are illustrated in Figure 1.1.

1.2.1.1 **Conditioning film formation**

Attachment of microorganisms to a surface is invariably preceded by the formation of a conditioning film that facilitates adherence of microorganisms. For biomaterials, like silicone rubber, widely used in the construction of medical devices such as catheters and endotracheal tubes (ETTs), conditioning film formation involves the adsorption of water, proteins, lipids, extracellular matrix molecules, complement, fibronectin and inorganic salts (Busscher *et al.* 1997; Garrett *et al.* 2008). This conditioning changes the physicochemical properties of the surface as well as provides a metabolically favourable environment for microbial cells with nutritional cues that trigger biofilm formation (Donlan *et al.* 2002). Multiple reports support the theory that biofilm formation occurs in response to various environmental cues (Costerton *et al*. 1995; O'Toole and Kolter 1998; Pratt and Kolter 1998). However, how these environmental signals are sensed and transduced by the biofilm-forming bacteria and the molecular mechanism(s) used to initiate the development of a biofilm are poorly understood. In the case of *Pseudomonas aeruginosa***,** these cues are integrated by the catabolite repression control (*crc*) protein, which plays a role in biofilm formation, possibly by controlling transcription of genes required for type IV pilus biogenesis. Evidence for this, is from research involving *crc* mutants which only produce a

dispersed monolayer of cells on surfaces that are devoid of microcolonies, which are typical of mature biofilms of the wild-type strain (O'Toole *et al.,* 2000).

In dental plaque, the conditioning film is called 'acquired pellicle' and starts to form immediately after a tooth has been cleaned. The acquired pellicle is primarily comprised of salivary glycoproteins, phosphoproteins, and lipids. Notable components include statherins, amylase, proline-rich peptides (PRPs) and host defence components. Bacterial-derived molecules may also contribute, and glucosyltransferases (GTFs) and glucans are important for mediating microbial attachment within dental plaque (Marsh 2004).

1.2.1.2 **Movement of microorganisms to be in proximity to the substratum surface (Mass transport)**

Several mechanism are involved in the movement of microorganisms and nutrients towards a surface of attachment and include Brownian motion, sedimentation, convective and active transport, and the Gibbs energy barrier (Palmer *et al.* 2007a).

Brownian motion is the random movement of microscopic elements in a fluid or gas caused by collisions of the molecules within the surrounding medium in thermodynamic equilibrium. The motion is named after the British botanist Robert Brown who, in 1827, first observed the movement of plant spores floating in water. This movement allows bacteria with only one flagella to translocate to find food, as otherwise, altering direction would not be possible (Li *et al.* 2008). Using total internal reflection fluorescence microscopy, Li *et al*., examined the swimming trajectories of the singly flagellated bacterium *Caulobacter crescentus* near a glass surface and observed large fluctuations over time in the distance of the cell from the solid surface. The research implied that Brownian motion, when combined with hydrodynamic interaction, had a supplementary and even greater influence between a swimming bacterium and a fluid boundary, significantly changing the direction of the microorganism closer to the surface (Li *et al.,* 2008).

Sedimentation occurs due to differences in specific gravity between bacteria and the liquid media (Palmer *et al*. 2007a). Li *et al.,* (2011), investigated the contribution of sedimentation to mass transport in parallel-plate-flow-chamber (PPFC) systems using *Staphylococcus aureus*. The research determined the height-dependent bacterial concentration, sedimentation velocity, and the sedimentation rate by microscopy. They demonstrated a five-fold difference in the initial staphylococcal deposition rate between the bottom and top plates of the PPFC, indicating that a different mass transport mechanism was operative for the bottom and top plates and that sedimentation appeared to be the predominant contributor to mass transport in the PPFC system (Li *et al.* 2011).

It has also been proposed that bacteria use the Gibbs energy barrier to aid contact with a surface. The Gibbs energy barrier is the sum of the Van der Waals interactions- commonly attractive and electrostatic interactions, usually negative due to both bacteria and the surface being negatively charged (Palmer *et al*. 2007a; Vadillo-Rodriguez *et al.* 2005).

In addition to the above, *Convective mass transport* refers to bacteria moving towards the surface by the movement of the liquid media in bulk and *active transport* explains the role of bacterial flagella and chemotaxis in bacterial attachment to a surface, although this is still poorly understood (Palmer *et al*. 2007a).

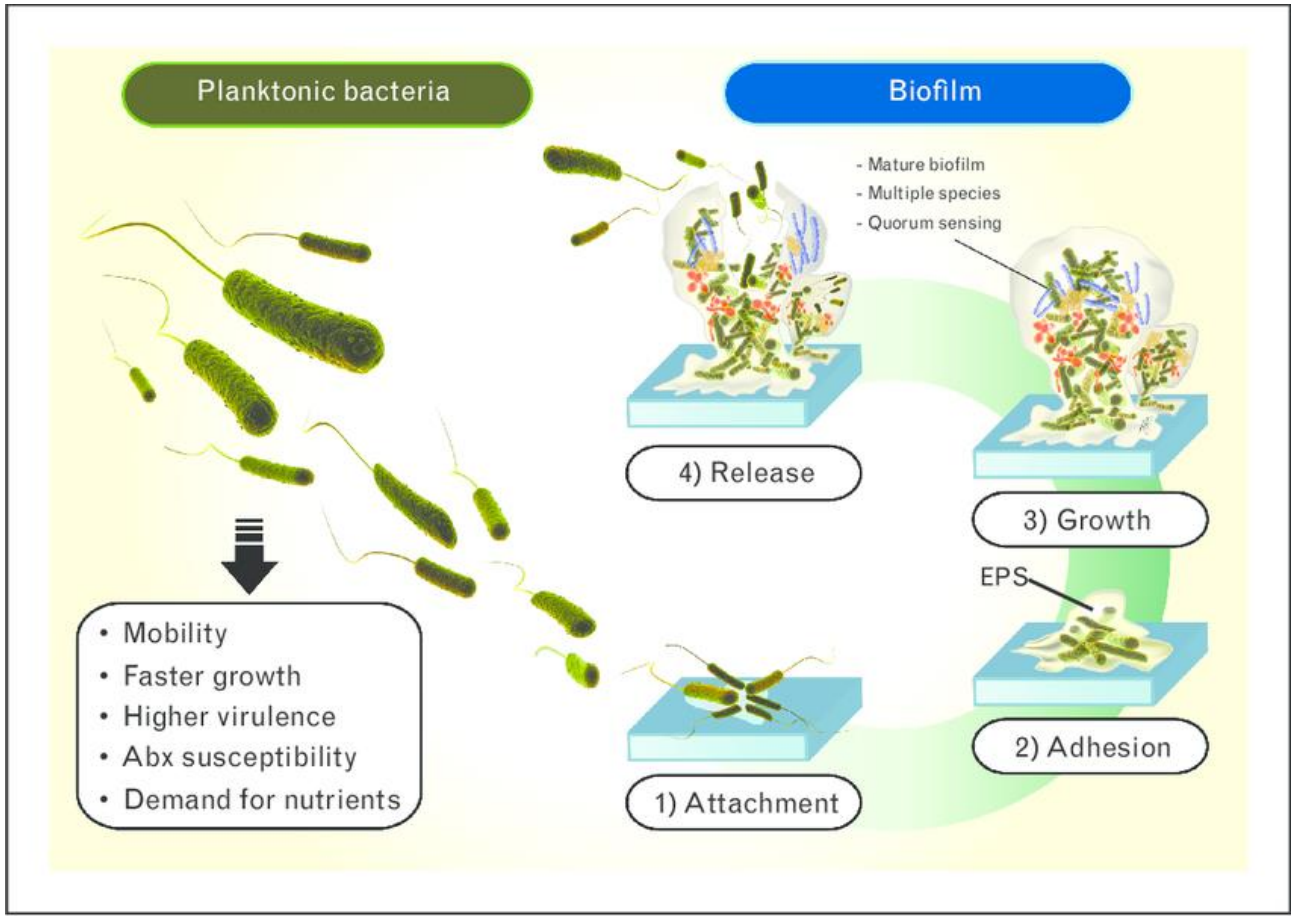

Figure 1.1 A schematic representation of biofilm formation stages.

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1.2.1.3 **Attachment of microorganisms**

The initial attachment of microorganisms to a surface is described as being weak, possibly via a single pole and is easily disrupted by fluid shear forces. It is thought that biofilms formed in low shear environments exhibit lower tensile strength and disintegrate easily. Similarly, if formed in high shear conditions biofilms exhibit much stronger mechanical properties (Donlan and Costerton 2002).

After early reversible attachment of microorganisms to the host surface, EPS substances are secreted by the microorganisms, which aid adhesion to the surface. Short-term stereochemical interactions also arise between adhesins on the microbial cells and the complementary receptors within the conditioning film. In the case of *S. aureus*, most of these adhesins are part of the Microbial Surface Components that Recognise Adhesive Matrix Molecules (MSCRAMMs) which facilitate adhesion to several host cells. Similarly, the fibronectin adhesins, FnBPA and FnBPB, participate in adhesion to surfaces and create connections with fibrin, collagen, and heparin in the host (Hall-Stoodley *et al.,* 2004). These interactions lead to the initial reversible attachment of the microorganisms to the surface to be replaced by an irreversible one. The process also involves elimination of water films between the microorganism and the host surface, allowing the surfaces to come closer together (Donlan and Costerton 2002). Importantly, transition between reversible and irreversible attachment has been documented as being as short as 5 to 10 min (Meinders *et al.* 1995; Schwab *et al.* 2005). In addition to cell-to-surface adhesion, cell-to-cell adhesion also occurs during biofilm formation, for example, *S. aureus* produces a polysaccharide intercellular adhesion (PIA) that facilitates cell-to-cell adherence (Cafiso *et al*. 2004). Some species are more adept than others at creating attachment to the surface which leads to consider them pioneer colonisers *Actinomyces* spp, *Streptococcus* spp, *Haemophilus* spp,

Capnocytophaga spp, *Veillonella* spp, and Neisseria are the main pioneer bacterial genera attaching to the tooth surface (Huang *et al*. 2011).

1.2.1.4 **Coaggregation/coadhesion and microbial succession**

As the biofilm develops, the microbiota becomes more diverse with microorganisms that were originally unable to attach to the conditioning film, adhering to the pioneer colonisers by adhesin-receptor interactions (Beachey 1981). In dental plaque, *Streptococcus* species exhibit a high degree of coaggregation, and this likely contributes to the high prevalence of these bacteria as pioneer colonisers. Indeed, the *Streptococcus* genus accounts for 47-82% of the microbiota colonising a clean tooth (Socransky and Haffajee 2002).

The metabolism of pioneer microbes in a biofilm can modulate the local environment in a way that makes it conducive for fastidious or even anaerobic bacteria to persist. For example, the anaerobic gut bacteria *Clostridium perfringens* and *Bacteriodes fragilis* has been observed to grow in *in vitro Candida albicans* biofilms , explained by a hypoxic environment within the biofilm (Fox *et al.* 2014), some species use oxygen and produce carbon dioxide and other reduced end products of metabolism, which creates a suitable environment for strictly anaerobic bacteria to survive, in an oral microbial community model, strict black pigmented anaerobes survived in an aerated system in the presence of *Fusobacterium nucleatum* but failed to do it in its absence. (Bradshaw *et al.* 1998). The effect of local metabolic activity by pioneer colonisers also generates nutrients and fermentation products that other microorganisms can use. It is such environmental changes that ultimately drive microbial succession within the biofilm (Costerton 1999).

1.2.1.5 **Mature biofilm formation**

The biofilm growth rate decreases during maturation to its final 3-dimensional structure. Bacteria produce extracellular polymers that contribute to the EPS, including soluble and insoluble glucans, fructans and heteropolymers. Glucans contribute to further acquired pellicle and microbial adhesion, whereas fructans act as extracellular nutrient storage compounds (Matsumi *et al.* 2015; Rozen *et al.* 2001). Mature biofilms contain channels within their structure, which enable flow of nutrition and gases into the biofilm and also facilitate removal of waste products (Stoodley *et al.* 1999).

In a mature biofilm, differing microenvironments form at distinct locations, microelectrodes have shown that oxygen does not always reach the 'deep' layers of a biofilm, and the result is the creation of anaerobic niches (de Beer *et al.* 1994).

The EPS of biofilms not only provides structural support, but also protects embedded cells from environmental threats such as desiccation, and exposure to antimicrobials and host immune molecules. The EPS also acts a source of nutrients and enzymes as well as containing extracellular DNA (eDNA), which can be used in genetic exchange processes (Flemming and Wingender 2010; Mann *et al.* 2009; Mulcahy *et al.* 2008).

1.2.1.6 **Dispersal of biofilm cells**

External forces such as the physical effects of fluid shear can cause shedding or sloughing of biofilm cells (Stoodley *et al.* 2002). Internal biofilm processes, such as endogenous enzymatic activity leading to the release of EPS and surface-binding proteins can also trigger cell dispersal (Boyd and Chakrabarty 1994; Lee *et al.* 1996) creating a risk of distal site infection. Three types of dispersal (see below) have been described, swarming, clumping and surface dispersal (Hall-Stoodley *et al.* 2004).

In *S. aureus* biofilms, the accessory gene regulator (*agr)* quorum-sensing system is required to form biofilms and its reactivation in established biofilms through autoinducing peptides (AIPs) addition or glucose depletion triggers cells detachment. Importantly, detachment also restored sensitivity of the dispersed cells to the antibiotic rifampicin (Boles and Horswill 2008).

1.2.1.6.1 Swarming dispersal

Also known as seeding dispersal, swarming dispersal refers to bacterial cells being locally released. In some species, gliding or twitching motility allows cells to move individually along a surface (Bartova *et al.* 2014). A study using flow cell microscopy showed that in 9-day old *P. aeruginosa* biofilms, the inner microcolonies of the biofilm liquefy leading to free movement of cells and generating empty spaces surrounded by walls of non-motile cell clusters (Sauer *et al*. 2002). *Aggregatibacter actinomycetemcomitans*, *Haemophilus aphrophilus* and *Streptococcus mitis* (Bellissimo-Rodrigues *et al.* 2009). In the case of non-motile species, it was proposed that bacterial translocation was dependant on convection currents and temperature gradients in the bulk media (Kaplan and Fine 2002).

1.2.1.6.2 Clumping dispersal

Instead of single cells, aggregates of cells encased in EPS can disperse from biofilms (Stoodley 2001). Biofilm clumps may contain thousands of microorganisms that likely exhibit a similar phenotype to those retained within the biofilm, including properties such as higher antibiotic resistance. Such cell clusters can act as 'metastatic foci' of infection at distant sites. This type of dispersal has been reported for non-motile species including *S. aureus* (Hall-Stoodley *et al.* 2004).

1.2.1.6.3 Surface dispersal

As mentioned previously, environmental conditions such as those facilitated by increased shear can mediate transport of whole biofilm sections. For example, *in vitro* studies have shown that aggregates of endotracheal tube biofilms can travel up to 50 cm away from the end of the endotracheal tube when exposed to the forces of mechanical ventilation (Inglis 1993). This effect is particularly important in the clinical scenario where developed biofilm aggregates from the endotracheal tube, potentially with higher tolerance to host immune mechanisms and antimicrobials, would contaminate the lung. Surface dispersal has also been reported *in vitro* for *S. aureus* biofilms using a glass model of a central venous catheter model where biofilm was seen to detach, 'roll' and then re-attach to a distal point (Hall-Stoodley *et al.* 2004).

1.2.2 Oral Biofilms: Dental Plaque

Dental plaque was the first discovered 'biofilm', first described by Anthony van Leeuwenhoek in 1684 as "The number of these animalcules in the scurf of a man's teeth are so many that I believe they exceed the number of men in a kingdom". Dental plaque is the most studied biofilm, it covers the oral cavity, particularly the teeth and tongue. Dental plaque is known to harbour over 600 bacterial species (Paster *et al.* 2001), of which about 350 have been cultivated. The remaining species have been identified by sequencing of 16S rDNA (Dewhirst *et al.* 2010; Socransky and Haffajee 2002). Culture analyses estimate that up to 200 species can be found in one host (Paster *et al.* 2006), however, next generation sequencing estimates this number could be close to 500 species per person (Nelson *et al.* 2010; Zaura *et al.* 2009).

1.2.2.1 **Mature dental plaque**

In early stages of biofilm development, a condensed layer of only a few bacteria is seen, but later a thicker layer demonstrating less orientation and higher morphological diversity occurs. In mature plaque, due to enzymatic activity in the acquired pellicle, plaque microorganisms can be seen in direct contact with enamel and food particles (Marsh 2004). Dental plaque deposition continues until it reaches a critical size where surrounding structures (*i.e.* lips, tongue, cheeks), mastication and salivary clearance limit its expansion. Dental plaque is, however, dynamic in both structure and composition and continuously reorganises (Rosan and Lamont 2000).

1.2.2.2 **Microbial composition of dental plaque**

Dental plaque microbial communities differ at distinct sites in the mouth. Sites such as the teeth, tongue and gingival pockets provide different oxygen and nutrient levels as well as mechanical and temperature challenges to plaque. In addition, there will be different levels of contact with the host immune system and secretions like saliva and gingival crevicular fluid (Kolenbrander *et al.* 2010).

Dental plaque is described as supragingival when above the gingival margin or subgingival when below. Supragingival plaque is mainly composed of Gram-positive bacteria, including *Streptococcus sanguinis, Streptococcus mutans, Streptococcus mitis, Streptococcus salivarius* and lactobacilli. Less prevalent but are encountered, are facultative anaerobic Gram-negative species such as *Neisseria* species and *Haemophilus parainfluenza*, and occasionally obligate anaerobes including species of *Veillonella* and *Propionibacterium* (Kroes *et al.* 1999; Nyvad and Kilian 1987). The differing morphology of the anatomical structures of the mouth offer distinct microenvironments that will be reflected by the respective microbial communities. For example, the base of the teeth fissures are commonly colonised by *Streptococcus mutans* and *Lactobacillus,* whereas interproximal plaque, is

predominantly comprised of Gram-positive bacilli, in particular *Actinomyces* species (Kuramitsu *et al.* 2007; Huang *et al.* 2011).

Subgingival plaque occurs below the gum line, in a space between the tooth surface and the gingival epithelium called the gingival crevice or sulcus. In health, subgingival plaque is composed primarily of Gram-positive facultative anaerobic cocci (40%; *e.g.* streptococci) and facultative anaerobic bacilli (35% *e.g. Actinomyces*). Anaerobic bacteria constitute the remaining 25% of the subgingival plaque microflora, and spirochetes and anaerobic streptococci are considered almost exclusive to subgingival plaque (Kroes *et al*. 1999; Moore and Moore 1994).

In disease, the gingival crevice can enlarge into a periodontal pocket and the flow of gingival crevicular fluid increases. In periodontal pockets, plaque presents as a thin compact layer on the root surface of the tooth with a looser structure towards the epithelial lining of the pocket. The periodontal pocket is anaerobic, and the most frequent inhabitants are Gram-negative anaerobic bacteria such as *Prevotella intermedia*, *Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Tannerella forsythia* and spirochetes such as *Treponema denticola* (Kuramitzu *et al.* 2007).

Dental appliances may also harbour dental plaque. In the case of the fitting surface of a denture, plaque tends to be more acidogenic, and the most commonly found bacteria are streptococci along with yeast of the genus *Candida* species. Obligate anaerobes like *Actinomyces israelii* have been cultured from dentures, whilst molecular techniques have detected periodontal pathogens such as *P. gingivalis, Tannerella forsythia* and *A. actinomycetemcomitans* (Socransky and Haffajee 2002).

1.2.2.3 **Interspecies interactions in dental plaque**

Interestingly, many species encountered in the oral cavity *e.g. S. mutans* and *P. gingivalis*, are not usually found anywhere else in the body. Oral microorganisms need to attach to oral surfaces (teeth or mucosa) or to existing microorganisms and biofilms, as otherwise they will be transported to the digestive tract during salivary clearance and swallowing (Kolenbrander *et al*. 2010). With such an abundance of microbial species, intercellular interactions play a key role in dental plaque formation and maintenance. Previous research shows that in the oral environment, each microbial species has an affinity to another genetically different microorganism. *Fusobacterium nucleatum* has partnerships with multiple species including early and late colonisers as well as aerobic and anaerobic species. For this reason *F. nucleatum* has often been described as a 'bridging' bacterium (Huang *et al.* 2011; Kolenbrander *et al*. 2010). A well-documented example of multispecies affinity occurs between *Porphyromonas gingivalis* and *Treponema denticola*. *P. gingivalis* produces isobutyric acid, and this in turn enhances growth of *T. denticola.* Meanwhile*, T. denticola* produces succinic acid which can be incorporated into the *P. gingivalis* cell wall (Huang *et al.* 2011). *F. nucleatum* and *P. intermedia* are tolerant of pH levels as low as 5.0 and produce ammonia and organic acids through fermentation of glutamate and aspartate. These metabolites increase alkalinity and will make the environment more tolerable for species like *P. gingivalis*, which optimally grows at neutral pH (Huang *et al*. 2011). Similarly, aerobic species consume oxygen in the environment creating localised anaerobic conditions that allow the growth of strictly anaerobic bacteria (Kuramitsu *et al.* 2007).

1.2.3 **Gene expression in biofilms**

Microorganisms can live as 'free floating' (planktonic) cells or as part of a biofilm. Clearly, different challenges and requirements present themselves when microorganisms grow within a biofilm. These include the need to adhere to a surface, generate EPS, grow in an

environment where nutrient availability or oxygen is limited, and the requirement to both communicate with other cells and to disperse from the biofilm when needed (O'Toole *et al.* 2000). In order to meet these lifestyle challenges, differential expression of specific biofilm genes is required (Jefferson, 2004). The first indication that changes in gene expression occurred in biofilms originated from a gene-fusion study where up to 38% of the *Escherichia coli* genome was differentially expressed during biofilm formation (Prigent-Combaret *et al.* 1999). Later, a study using DNA microarray analysis estimated that only a 1% difference in terms of gene expression occurred between planktonic and biofilm growth of *P. aeruginosa*, with a 50% split between up-regulation and down-regulation of genes. However, these apparently subtle differences were significant in terms of biofilm formation and also antibiotic resistance (Whiteley *et al.* 2001). Furthermore, microarray analyses of *E. coli* and *Bacillus subtilis* growth showed that a change of up to 15% in biofilm gene expression occurred compared with planktonic cells (Beloin *et al.* 2004; Ren *et al.* 2004; Schembri *et al.* 2003; Stanley *et al.* 2003). In a study of gene expression patterns in *S. aureus* biofilms, up-regulation of over 160 genes for biofilm cells compared with planktonic cells was noted. The up-regulated genes included those involved in the synthesis of binding factors, peptidoglycan and PIA, as well as those associated with the detoxification of formate, urea, and reactive oxygen species (Resch *et al.* 2005).

Use of Affymetrix® GeneChip microarrays has facilitated analyses of almost the entire *P. aeruginosa* and *E. coli* genomes under defined growth conditions. In such studies, differences of 4-14% in *E. coli* gene expression has been reported between planktonic and biofilm cells (Schembri *et al.* 2003), and for *P. aeruginosa* the difference in total gene expression was determined to be approximately 3% when planktonic cells were compared with those in developing biofilms. Interestingly, this figure increased to 14.3% in more established biofilms (Waite *et al.* 2005). More recently, a study using next generation

sequencing (RNAseq) examined global gene expression of *P. aeruginosa* biofilms in 96-well plate microtitre plates and compared these to stationary phase planktonic growth (Dotsch *et al.* 2012). In this study, it was reported that some genes were similarly expressed in both the stationary phase of planktonic cultures and in biofilms, but there was also a group of genes exclusively expressed in biofilms, most of which were related to adaptation to microaerophilic growth conditions, repression of type three secretion proteins and production of extracellular matrix components (Dotsch *et al*. 2012).

Table 1.2 shows changes in gene expression have also been reported during different stages of biofilm development, indicating that it is not only biofilm establishment, but also survival of cells within the biofilm that involves differentially regulated gene expression.

During the early stages of biofilm development, flagella motility may be required for initial attachment of motile bacteria to a surface. However, once attached, these genes are subsequently down-regulated (Prigent-Combaret *et al*. 1999; Sauer and Camper 2001; Stanley *et al*. 2003). In the case of *P. aeruginosa*, type IV pili and *cupA* fimbriae have been shown to be involved in the initiation of biofilm formation (D'Argenio *et al.* 2002; O'Toole and Kolter 1998; Sauer and Camper 2001; Vallet *et al.* 2001). Once attached to a surface, production of EPS is required to provide biofilm support and is also important in self-aggregation of cells. In *S. aureus* and *S. epidermidis* biofilms, polysaccharide production is mediated by the *icaABCD* operon which encodes for enzymes needed to synthesise the polymer PIA/PNAG (polysaccharide intercellular adhesion/poly-N-acetylglucosamine) (Heilmann *et al.* 1996). In addition to the intercellular adhesion (*ica)* operon and PIA production, the AtLE and dltA surface proteins are thought to facilitate adhesion to polystyrene surfaces (Hall-Stoodley, *et al.* 2004).

Table 1.2 Genes required for biofilm formation, adapted from (Jefferson 2004).

Genes	Function	Species
Adhesion		
gbpA	Polysaccharide formation	*Streptococcus mutans*
tarC	Regulator of glucosyltransferase S and glucan binding protein	*Streptococcus mutans*
ica ADBC	Intercellular adhesin synthesis	*Staphylococcus aureus*
clf A	Clumping factor A, fibrinogen binding protein	*Staphylococcus aureus*
bopABCD	Biofilm on plastic surface operon	*Enterococcus faecalis*
Quorum sensing		
com X	Competence	*Streptococcus gordonii*
com ACDE	Competence	*Streptococcus mutans*
LasI	Synthesis of 3OC12-HSL quorum sensing signal	*Pseudomonas aeruginosa*
Cell Wall		
brpA	Possible regulator of autolysis	*Streptococcus mutans*
glmM	Peptidoglycan synthesis	*Streptococcus gordonii*
bacA	Peptidoglycan synthesis	*Streptococcus gordonii*
Metabolism		
ccpA	Carbon catabolite control protein	*Streptococcus mutans*
Crc	Global carbon metabolism regulator	*Pseudomonas aeruginosa*
Stress response		
Dgk	Stress response regulator	*Streptococcus mutans*
pur R	Regulator of purine synthesis, metabolism	*Staphylococcus epidermis*
mut T	DNA mismatch repair	*Streptococcus gordonii*

For *P. aeruginosa*, the *pel* locus (referring to pellicle, a biofilm formed at the air-medium interface) containing the genes *pelA-G*, is responsible for synthesis of a glucose-rich component of the matrix, whereas *psl* (polysaccharide synthesis locus), containing the *pslA-O* genes, is responsible for a mannose and galactose rich EPS (Friedman and Kolter 2004; Khan *et al.* 2010), and although alginate is often considered a major component of *P. aeruginosa* extracellular matrix, this may only be true for mucoid strains commonly isolated from cystic fibrosis patients. Mucoid strains are proficient alginate producers and non-mucoid strains do not produce significant quantities of alginate, but can still form biofilms (Friedman and Kolter 2004).

In mature biofilms, embedded cells need to adapt to a new microenvironment and altered gene expression allows the cells to compete in lower nutrients levels, reduced oxygen tension and pH changes. For example, anaerobic growth was found to induce expression of the *ica* operon and associated PIA production in both *S. epidermidis* and *S. aureus* biofilms (Cramton *et al.* 2001), whilst in *P. aeruginosa* biofilms, the stationary phase sigma factor, *rpoS*, has been shown to be either repressed by 2-3-fold or slightly activated (Whiteley *et al.* 2001; Xu *et al.* 2001).

1.2.3.1 **Differential gene expression as a stress response**

Environmental changes may occur in biofilms and are frequently inhibitory to healthy microbial cell growth and function. Such changes can include nutrient depletion, oxygen limitation, extreme pH, osmolarity imbalances and temperature shifts, and in turn will induce stress in the cells. Examples of these environmental changes, include the stress-inducing changes that occur in dental plaque biofilms following the presence of sucrose immediately after eating and drinking. Fermentation of sucrose by certain bacteria leads to lactic acid production with a subsequent reduction of local pH in the biofilm from pH 7.5 to as low as pH 3.5 (Welin-Neilands and Svensäter 2007). Extremes of temperature in *S. aureus*

biofilms occur when host core temperature increases as evident in patients with a septic status. In order to survive these environmental fluctuations, microorganisms need to rapidly modulate expression of certain genes (de Nadal *et al.* 2011).

Modulation of gene expression and subsequent differential protein production first requires the cells to sense the environmental change. In yeast, osmostress is mainly sensed by two upstream mechanisms that converge on the high osmolarity glycerol (HOG) signal transduction pathway, which is the central pathway of the yeast osmostress response (de Nadal *et al*. 2011). Once the stress is detected, the cell needs to rapidly respond by regulating expression of proteins that counter the effects of the experienced stress (de Nadal 2004). For instance, in the example of lowered pH in dental plaque, *S. mutans* increases specific activity of the membrane F1 H+/ATPase involved in proton efflux during pH homeostasis (Belli & Marquis, 1991; Hamilton & Buckley, 1991). In heat shock, in the yeast *Saccharomyces cerevisiae*, the main defensive response is protein unfolding as well as the induction of genes involved in respiration and the use of alternative carbon sources (Causton 2001).

1.2.3.2 **Quorum sensing in biofilms**

It is widely recognised that microorganisms generate signalling molecules that effectively allows communication between the cells. The process is termed Quorum Sensing (QS) and can lead to coordinated gene expression (Socransky and Haffajee 2002). Cells detect the signalling molecule, which if in sufficient concentration allows the entire microbial population to harmonically respond to changes in cell density (Binkley *et al.* 2004).

QS in Gram-positive and Gram negative bacteria can regulate a number of physiological activities, including competence development, sporulation, antibiotic biosynthesis, and induction of virulence factors (Miller and Bassler 2001). Additionally, biofilm growth has been reported to promote cell-cell signalling systems in order to activate genetic competence and facilitate genetic exchange (Cvitkovitch *et al.* 2003).

Several chemical classes of microbially-derived signalling molecule have now been identified. Broadly, these can be split into two main categories: oligopeptides commonly utilised by Gram-positive bacteria (Binkley *et al*. 2004) and fatty acid derivatives like acyl-homoserine lactones (AHLs) frequently utilised by Gram-negative bacteria (Sadikot *et al.* 2005).

In Gram-positive bacteria, QS systems generally consist of three components, a signal peptide and a two-component regulatory system (TCRS) or two-component signal transduction system (TCSTS) that has a membrane-bound histidine kinase sensor and an intracellular response regulator (Kleerebezem *et al.* 1997).

In *S. aureus*, the QS system is encoded by the *agr* operon, and the communication molecules are the autoinducing peptides (AIPs). It has been reported that AIPs bind to a surface histidine kinase receptor, initiating a regulatory cascade that modulates expression of a multiple genes including virulence factors, such as proteases, haemolysins and toxins (Boles and Horswill 2008; Novick 2003).

In *Pseudomonas aeruginosa,* two AHL-based QS systems have been described, namely the *Las* and *Rhl* systems. The *Las* system comprises of the transcriptional regulator *LasR* and its cognate *AHL* signal, *N*-(3-oxododecanoyl)-l-homoserine lactone (3-oxo-C12- HSL), which is synthesised by the *AHL* synthase *LasI*. The *Rhl* system is comprised of *RhlR* together with its cognate *AHL*, *N*-butyryl-l-homoserine lactone (C4-HSL), synthesised by the *RhlI AHL* synthase (Özçaka *et al.* 2012). Additionally, another signalling molecule called *Pseudomonas* Quinolone Signal (PQS) has been reported (Pesci *et al.* 1999). Structural genes for PQS production have been identified (*pqsABCDH*) together with a transcriptional regulator (*pqsR*) and the response effector (*pqsE*) (Özçaka *et al*. 2012).

1.2.4 **Antibiotic resistance in biofilms**

Biofilm cells exhibit higher resistance to antimicrobial agents compared to their planktonic counterparts, and this has been documented as being up to 1000-fold higher (Luppens *et al.* 2002). Multiple factors contribute to this biofilm resistance, including the reduced metabolism and growth rate of certain biofilm cells, which is thought to be accentuated in the central regions of the biofilm (Evans *et al.* 1991; Mah and O'Toole 2001). Furthermore, the biofilm matrix may provide mechanical protection by impairing diffusion of the antibiotic molecules into the deeper cell layers, and its concentration decreased below a therapeutic level due to degradation by enzymes entrapped in the matrix. (Normark and Normark 2002), in *P. aeruginosa* sequestration of the antibiotic tobramycin by glucose polymers in the periplasm has been observed (Mah *et al.* 2003). Additionally, ionic interactions occur between antibiotics and the biofilm matrix, recently it was reported that the positively charged antibiotic tobramycin was sequestered to the biofilm periphery, while the neutral antibiotic ciprofloxacin readily penetrated (Tseng *et al.* 2013).

The ability of ciprofloxacin to pass through a *P. aeruginosa* biofilm revealed that the antibiotic was able to penetrate some, but not all, of the biofilm, leaving remnant bacteria (Suci *et al.* 1994). Furthermore, the trait of the microorganism creates an additional challenge to the penetration of the antibiotic. Biofilms formed by a β-lactamase negative *Klebsiella pneumonia* mutant were readily penetrated by ampicillin, whereas biofilms of the wild type (β-lactamase positive *K. pneumonia*) were not (Anwar *et al*. 1992). Interestingly, β-lactamase negative *K. pneumonia* mutants were resistant to ampicillin, and this suggested that impedance of biofilm penetration was not the mechanisms of resistance (Vrany *et al.* 1997). Penetration of the biofilm also depends on the antibiotic itself, in a study determining antibiotic penetration of *S. aureus* and *S. epidermidis* biofilms, it was found that the movement of β-lactams (oxacillin and cefotaxime) and glycopeptide (vancomycin)

antibiotics was significantly impeded, this contrasted with movement of the aminoglycoside amikacin and the fluoroquinolone ciprofloxacin, which was unaffected (Singh *et al.* 2010).

In the case of therapy for infection, if a significant number of planktonic and biofilm bacteria are killed, then improvement of the clinical condition would be expected. However, as bacteria in protected biofilm regions often survive, these cells can rapidly re-establish the biofilm. As a result, mechanical or surgical removal of the biofilm may often be the only effective treatment options (Davies 2003; Marrie *et al.* 1982).

Biofilm cell density also contributes to antimicrobial resistance. Interestingly, in experiments comparing antibiotic sensitivities of planktonic *P. gingivalis* at similar cell densities to those encountered in biofilms, higher minimum inhibitory concentrations (MICs) to a range of antibiotics were observed compared to conventionally determined MICs. However, these MICs were still 2 to 8-fold lower than those of biofilm populations (Larsen, 2002).

As well as serving as a barrier to antibiotics, biofilms contain microenvironments that exhibit different gradients in oxygen levels, pH and osmolarity. These can affect the relative effectiveness of the antibiotic (Costerton and Stewart, 2001). In addition, as biofilm cells may exhibit reduced metabolic activities and growth rates, antibiotics such as penicillin that target active cell wall synthesis will have no effect on non-dividing cells (Davies 2003; Tuomanen *et al.* 1986).

As described previously, biofilm cells exhibit differential gene expression compared with planktonic cells and this may enhance resistance against the antimicrobials. For example, increased expression of efflux pumps in the cell membrane that remove antibiotics from the cell, has been reported for *P. aeruginosa* biofilm cells. In this species, the MexAB-OprM pump has been shown to be able to transport multiple drugs (Davies 2003; Donlan and Costerton 2002; Sauer and Camper 2001).

As well as having higher resistance to antimicrobials, biofilms are also more resistant to the host immune responses. *P. aeruginosa* has been shown to be resistant to antibodies produced during cystic fibrosis infection in a rat model (Meluleni *et al.* 1995). Phagocytosis may be impaired and the host tissues in vicinity to the biofilm may be affected by neutrophil and complex system response (Stewart and Costerton, 2002).It has also been reported that extracellular DNA, a biofilm matrix component, induces antibiotic resistance due to its ability to bind and sequester cations, including magnesium, from the surrounding environment. This environmental cue was then detected by *P. aeruginosa* leading to induction of genes involved in modification of the cell surface component, lipopolysaccharide, resulting in physical alterations in the bacterial outer membrane (Mulcahy *et al.* 2008).

1.2.5 **Biofilms in human disease**

Biofilms account for at least 80% of infectious diseases in humans and include infection such as otitis and periodontitis. In addition, biofilms can also cause infection when they colonise medical devices including contact lenses, venous catheters and endotracheal tubes (Table 1.3). Furthermore, medical equipment including dental unit waterlines and ventilators may be colonised by biofilms and serve as a sources of infection within hospital environments (Donlan and Costerton, 2002).

1.2.5.1 **Biofilms on medical devices**

Microorganisms frequently live in biofilms attached to inert surfaces in the environment, therefore it is hardly a surprise that biomaterials are not exempt form colonisation. Implanted medical devices are especially prone to biofilm development, leading to both infection and/or failure of the device (Høiby *et al.* 2011; Reid 1999).

The high incidence of infections due to medical devices has led to the term ‘chronic polymer associated infection’ with involved species often being those not previously thought of as

pathogens *e.g. S. epidermis* which is a normal habitant of the skin (Hall-Stoodley *et al.* 2004). The primary function of the device may also be compromised by biofilm formation, for example, biofilm aggregates can block catheter lumens rendering the device unusable and necessitating replacement (Lindsay and von Holy 2006).

Colonisers of medical devices can be Gram-positive and Gram-negative bacteria as well as yeast, and the biofilms involved can be single or multispecies (Donlan 2001). These organisms frequently originate from the patient's own body (endogenous) or may be of exogenous origin *e.g*. from carers or the local environment (Table 1.4) (Hall-Stoodley *et al.* 2004).

Colonisation of devices can occur within hours of implantation, and the duration the device is in place influences the risk of biofilm development and patient morbidity (Donlan and Costerton 2002).

Biofilm prevention strategies include flushing with antimicrobials in venous catheters as well as impregnation of the biomaterial with antibiotics and antimicrobials such as chlorhexidine and silver sulfadiazine antiseptics. Unfortunately, such methods often do not reliably eradicate or prevent biofilm formation. Similarly, a range of materials including silicone, polyurethane, composites and hydrogel-coated materials have failed to avoid biofilm formation (Donlan, 2001).

Table 1.3 Biofilm related infections.

Infection	Description	Reference
Native valve endocarditis	*Streptococcus spp*, are the most prevalent bacteria in this infection, but *Staphylococcus spp* and *Candida* have also been identified. Biofilms can cause valve dysfunction and heart disease as well as the production of emboli leading to thromboembolic disease, fungal biofilms have been recognized to be larger in size, and larger size biofilms are reported to cause more emboli.	(Donlan and Costerton 2002).
Cystic fibrosis	Cystic fibrosis is a heterogeneous genetic disease that affects the lower respiratory tract, where the mucocilliary system impaired and the epithelium of the lung becomes covered in viscous mucus that has been linked to bacterial lung infections that account for 90% of early deaths. *Pseudomonas aeruginosa* is the most common pathogen	(Valenza *et al.* 2008)
Chronic bacterial prostatitis	Scanning electron microscopy has shown biofilm growth in the prostate ducts. It has been reported that bacteria recovered from prostatitis samples have shown the characteristic slow growth and antibiotic resistance of biofilm organisms.	(Donlan and Costerton 2002).
Otitis media	Otitis media is an inflammation of the mucoperiosteal lining of the middle ear. Commonly associated with *Streptococcus pneumoniae*, *Haemophilus influenzae, Moraxella catarrhalis*, group A beta- haemolytic Streptococci, enteric bacteria, *Staphylococcus aureus, Staphylococcus epidermis* and *Pseudomonas aeruginosa*. A collection of highly viscous fluid occurs and to drain this fluid and thus relieve symptoms and prevent hearing loss, tympanostomy tubes are fitted. These tubes accumulate biofilms in their inner surfaces and to complicate treatment, the middle ear fluid absorbs significantly less antibiotics than plasma therefore middle ear infection are thus difficult to treat.	(Donlan and Costerton, 2002)

Table 1.4 Common pathogens in medical devices infection.

Infection	Microorganisms associated	Reference
Central venous catheter	*Staphylococcus epidermis, Staphylococcus aureus, Candida albicans, Pseudomonas aeruginosa, Klebsiella peumoniae* and *Enterococcus faecalis*	(Elliott *et al.* 1997; Raad *et al.* 1992)
Prosthetic valves	*Streptococcus epidermis, Staphylococcus aureus, Streptococcus spp., Gram negative bacilli, diphtheroids, enterococci* and *Candida species.*	(Donlan 2001)
Urinary catheters	*Staphylococcus epidermis, Enterococcus faecalis, Proteus mirabilis, Pseudomonas aeruginosa and Klebsiella pneumonia.*	(Donlan 2001)
Prosthetic Joints	*Staphylococcus aureus and Staphylococcus epidermidis, other Coagulase Negative Staphylococci, Pseudomonas aeruginosa and Enterococcus faecalis.*	(Campoccia *et al.* 2006)

1.3 Ventilator associated pneumonia

Ventilator-associated pneumonia (VAP) is a respiratory infection that develops in patients after 48 hours of mechanical ventilation (MV). Depending on the number of days of mechanical ventilation, VAP can be classified as early or late onset (Ibrahim *et al.* 2000; Kollef *et al.* 2012). VAP within the first 4 days after initiation of MV is considered 'early onset' and thought to be caused by possibly endogenous pathogens associated with the community, namely, *Haemophilus influenzae*, *Escherichia coli* or meticillin sensitive *Staphylococcus aureus* (MSSA) (Ibrahim *et al*. 2000; Wiener-Kronish and Dorr 2008). After 5 days mechanical ventilation, VAP is consider to be 'late onset' and is more associated with antibiotic resistant bacteria typically considered endogenous to healthcare facilities such as *Pseudomonas aeruginosa* and meticillin resistant *Staphylococcus aureus* (MRSA) (Masterton *et al.* 2008).

1.3.1 Diagnosis of VAP

Diagnosis of VAP is complicated by the absence of a 'classic sign' or a gold standard guideline for diagnosis, and this frequently creates discrepancies in clinical opinion. The most widely used diagnostic tool is the Clinical Pulmonary Infection Score (CPIS) a clinical score of 0-12 with a score >6 considered indicative of VAP, it is based on the following 6 variables: body temperature, leukocyte count, volume and character of tracheal secretions, arterial oxygenation, chest radiograph findings, Gram stain results, and results of culture of tracheal aspirate specimens (Table 1.5) (Pugin 2002; Pugin *et al.* 1991). However the CPIS originates as a secondary outcome from a relatively small sample of 40 blind bronchial lavages from 28 patients and has no evidence of its poor specificity (Zilberberg and Shorr 2010). In view that potentially hospital acquired pneumonia could be "underdiagnosed" if all of the CPIS criteria had to be met, in 2008, the British Society of Antimicrobial Chemotherapy (BSAC) recommended that pneumonia should be considered for patients presenting with the

presence of purulent tracheal secretions, and new and/or a persistent infiltrate on chest X-ray (CXR), which was otherwise unexplained, increased oxygen requirement, core temperature above 38.3°C, Blood leucocytosis (>10000/mm³) or leukopenia (<4000 mm³) (Masterton *et al.,* 2008). The most accurate method for VAP diagnosis would be a lung biopsy, however such a procedure is clearly highly invasive and therefore a recommendation against its routine use was made by the BSAC (Masterton *et al.,* 2008).VAP diagnosis can be facilitated by quantitative microbial culture from bronchoscopic specimens and subsequent identification of pathogenic microorganisms. Such an approach has been recommended by the American Thoracic Society (American Thoracic and Infectious Diseases Society of 2005). There are currently two bronchoscopic methods including use of a protected specimen brush (PSB) or a bronchoalveolar lavage (BAL). PSB employs a double lumen catheter with a telescopic cannula and a distal plug, whilst BAL (involving guidance from the bronchoscope), involves irrigation and subsequent aspiration of the bronchus using approximately 120 ml of sterile saline (Chastre *et al.* 2010). A bacterial load of $>10^3$ colony forming units (CFU)/ml from a PSB or 10^4 CFU/ml from a BAL have been found to positively correlate with histology reports of VAP (Bonten 1999).An alternative to bronchoscopic techniques is the non-directed bronchial lavage (NBL). In this technique, a catheter is inserted into the endotracheal tube (ETT) to the point where the operator can feel resistance, and then approximately 20 ml of sterile saline is introduced and immediately aspirated. As with BAL, the diagnostic threshold is microbial growth of up to 10^4 CFU/ml. This technique has been found to be of similar sensitivity to BALs for VAP diagnosis, but is preferred by some clinicians as it is deemed to be less invasive, less technically demanding and more cost effective (Felton *et al.* 2010; Flanagan *et al.* 2000).

1 Table 1.5 Clinical pulmonary infection score (CPIS).

Parameter	Points
Temperature,° C	
36.5 - 38.4	0
38.5 – 38.9	1
≥39.0 and ≤36.	2
Blood leukocyte level, leukocytes/mm^{-3}	
4000 -11,000	0
<4000 or 11,000	1
Plus bands forms ≥ 500	2
Tracheal secretions	
<14+	0
≥14+	1
Pus purulence	2
Oxygenation, Pa:FiO_2, mm HgO_2 **	
>240 or ARDS*	0
≤240 and no ARDS	2
Pulmonary radiograph finding	
No infiltrate	0
Diffuse or patchy infiltrate	1
Localised infiltrate	2
Culture of tracheal aspirate specimen (semi- quantitative: 0-1, -2, or 3+)	
Pathogenic bacteria cultured ≤1 or no grown	0
Pathogenic bacteria cultured	1
Plus same pathogenic bacteria on Gram stain >1+	2

2 *ARDS: acute respiratory distress syndrome.

3 **PaO2:FiO2, radio of partial pressure of arterial oxygen to the fraction of inspired oxygen

4 Adapted from Zilberberg and Shorr (2010)

1.3.2 **Aetiology of VAP**

In a healthy individual, the respiratory tract has protective mechanisms against pathogen colonisation. These mechanisms include the normal function of the glottis, larynx and cough reflexes, salivary flow over the mucosa, and the action of tracheobronchial secretions and mucocilliary structures which entrap microorganisms which are later expelled through the oropharynx or swallowed into the stomach. In the case of patients receiving MV a predisposition of their respiratory tract to being colonised by pathogenic bacteria arises because the intubation process can not only lead to mucosal damage, but also impairs the physiological mucocilliary clearance and cough reflexes (Koeman *et al.* 2001).

An accumulation of subglottic secretions pools above the ETT cuff in MV patients and despite nursing efforts to regularly remove these secretions by suction, leakage around the cuff occurs. Such leakage is thought to arrive through pressure changes occurring in the cuff creating spaces between the cuff and the trachea. The ETT cuff does not provide an efficient seal to the lower airways as microchannels form from folding of the cuff biomaterial (Blot *et al.* 2014). Unsurprisingly, the most important risk factor for VAP appears to be aspiration of microorganisms from the oropharynx (Brennan *et al.* 2004; Rodrigues *et al.* 2009; Rumbak 2005). Microorganisms originating from the gastrointestinal tract have also been associated with the infection, although further investigation as to precise involvement is required (Garrouste-Orgeas *et al.* 1997).

It has been suggested that microbial laden secretions from the oropharynx leak around the cuff of the ETT entering the lower respiratory tract. In addition to the lower respiratory tract becoming colonised, a biofilm also forms within the lumen of the ETT, which is protected from the normal host defence mechanisms or the action of administered antimicrobials (Inglis *et al.* 1989).

Other factors that may contribute to microbial aspiration include sedation, decreased level of consciousness, and use of a nasogastric tube and contamination of equipment (Rumbak 2005). Patient dependent factors include age, the presence of chronic lung disease or acute respiratory distress syndrome, and admission for other medical or neurological reasons (Vincent 2004). Additional factors are related to the patient's hospital stay, such as increased duration of intubation, manipulation of airway, re-intubation, frequent ventilation circuit changes, low intra-cuff pressure of the ETT, failed subglottic suction, patient transport between hospitals, supine body position, pH altering agents, enteral feeding and multiple central venous line insertions (Brennan *et al*. 2004). Previous use of antibiotics has been found increase the risk for VAP (Rodrigues *et al*. 2009).

1.3.3 **Epidemiology of VAP**

VAP is the most frequent hospital-acquired infection in Intensive Care Units (ICUs) and defined as a pneumonia that occurs 48 h after commencement of mechanical ventilation (Ibrahim *et al*. 2000). A recent prospective surveillance study found that VAP prevalence was 15.6% globally (13.5% in the United States, 19.4% in Europe, 13.8% in Latin America, and 16.0% in Asia Pacific) (Kollef *et al.* 2014). Previous reports had estimated an incidence between 8 and 28% (Amin 2009; Chastre and Fagon 2002), with an estimated 17 cases occurring per 1000 ventilator days (Le Berre *et al.* 2008). Reports on actual mortality rates are variable, with a recent meta-analysis estimating an overall attributable mortality of 13% (Melsen *et al.* 2013). However, mortality rates will vary with severity of underlying disease, with studies showing that surgical patients exhibit high VAP mortality (69%), whilst lower mortality (38%) is reported in patients with moderate grade of illness as determined by an APACHE (Acute Physiology, Age, Chronic Health Evaluation) score of 20-29 (Melsen *et al*. 2013; Shen *et al.* 2011). In contrast, trauma patients have lower mortality compared with non-trauma patients (odds ratio [OR] = 0.37, 95%CI = 0.21-0.65), (Bonez *et al.* 2013). For

patients who are severely ill, it is however difficult to conclusively establish VAP as the ultimate cause of death. A prolonged ICU stay 5 to 7 days; (Safdar *et al.* 2005) also increases patient risk of further morbidities. Additionally, costs attributable to VAP are in the range of £6000 to £22000 per patient and represent a significant burden to the health care system (Kollef *et al*. 2012).

1.3.4 **VAP causative microorganisms**

In 2002, a review of the microbiology of VAP was undertaken by Chastre and Fagon (2002). This review examined the findings of 24 previous studies and concluded that that 58% of VAP causative pathogens were Gram-negative bacteria (GNB) including *P. aeruginosa* (24%), Enterobacteriaceae (14%), *Haemophilus* (10%) and *Acinetobacter* species (8%). Gram-positive bacteria that were also prevalent included *S. aureus* (20%) and *Streptococcus* species (8%). Over half of the pneumonias caused by *S. aureus* involved strains that were meticillin resistant (*i.e.* MRSA). In a recent Brazilian study, *A. baumannii* was the most commonly isolated microorganism from VAP (28% of cases). It was also noted in this study that 48% of patients had received inappropriate antibiotic therapy (Rodrigues *et al*. 2009). In a review comparing Asian and western VAP data, it was found that MRSA occurred more frequently in western countries (Chawla 2008). It seems apparent that the environment has an effect on the predominant pathogens for VAP; therefore, antibiotic therapy needs to be adjusted to local data to avoid inappropriate antibiotic prescribing.

1.3.5 **The endotracheal tube (ETT)**

The ETT (Figure 1.2) plays an important role in the development of VAP as its presence will impair the natural patient defence mechanisms of mucocilliary clearance and cough reflexes (Levine and Niederman 1991) leading to accumulation of tracheobronchial secretions (Pneumatikos *et al.* 2009). Furthermore, the insertion of the ETT can lead to traumatic damage to the tracheal mucosa. The ETT itself can also deliver exogenous microorganisms

(Rello *et al.* 1996) to the airway and this could contribute to the view that re-intubation represents an independent risk factor for development of VAP (de Lassence *et al.* 2002; Torres *et al.* 1995).

1.3.5.1 **Leakage around the endotracheal tube cuff**

The ETT cuff (Figure 1.3) functions to seal the trachea and prevent oropharyngeal secretions reaching the lower airways. The cuffs of first generation ETTs were made of rubber and had a design for low volume and high pressure, and were considered to provide an adequate seal (Blot *et al.* 2014). However, this design promoted ischemia and damage to the tracheal mucosa, and was subsequently modified to one of high volume and low pressure (HVLP), with the cuff material being made from polyvinyl chloride (PVC) (Blot *et al*. 2014; Haas *et al.* 2014).

To achieve an effective seal, the ETT cuff is inflated to a cuff pressure of approximately 25-30 cm of H_2O. Since an ETT has to fit a range of different sized tracheas, the inflated cuff can be 1.5 to 2 times the diameter of an average adult trachea (Pneumatikos *et al.* 2009). The result of such differences in the diameter of the inflated cuff and the trachea leads to folding of the cuff material, and these folds provide microchannels allowing microaspiration of microbial laden secretions that have pooled above the cuff (Hamilton and Grap 2012; Young *et al.* 2006).

Despite different types of ETT cuff are available with distinct designs (including tapered and cylindrical shapes), and these are often constructed from PVC or polyurethane. There would however appear to be little impact of these designs on VAP incidence, although two clinical trials favoured the tapered shape polyurethane cuff to deliver better seals (Dave *et al.* 2010). The most recent commercially available ETT is the PneuX ETT (formerly known as LoTrach™; Venner Medical, Singapore), which is a straight, wire-reinforced silicone tube that aims to address multiple factors in VAP prevention, including clearing subglottic secretions and

minimising tracheal damage thanks to an atraumatic tip. The main purpose of this ETT is to reduce or even eliminate leakage of tracheal secretions and the tube has recently been used with a tracheal seal monitor, which in a pilot study produced promising results in VAP prevention with incidence rates of 1.8% (Doyle *et al.* 2011).

Recently, Hwang *et al*., (2013), designed an ETT with two cuffs separated by a 5 mm gap. Between the cuffs is an outlet port of a line through which water soluble gel can be injected. The design was tested *in vitro* using artificial tracheas and compared with 4 commercially available tubes and the authors found no leakage after a 48 h period only with the double cuff prototype (Hwang *et al.* 2013). The design has yet to be clinically evaluated.

In addition to cuff design and materials, strategies to prevent microbial leakage past the cuff have employed cuff inflators to maintain a constant pressure. Whilst it has been reported that inflators unreliably estimate cuff pressure (Blanch 2004), a recent randomised controlled study evaluating a pneumatic device (Nosten®) in 64 patients found this approach to be effective in controlling cuff pressure. Nevertheless, the impact of this device on microaspiration was not significant (Jaillette *et al.* 2013).

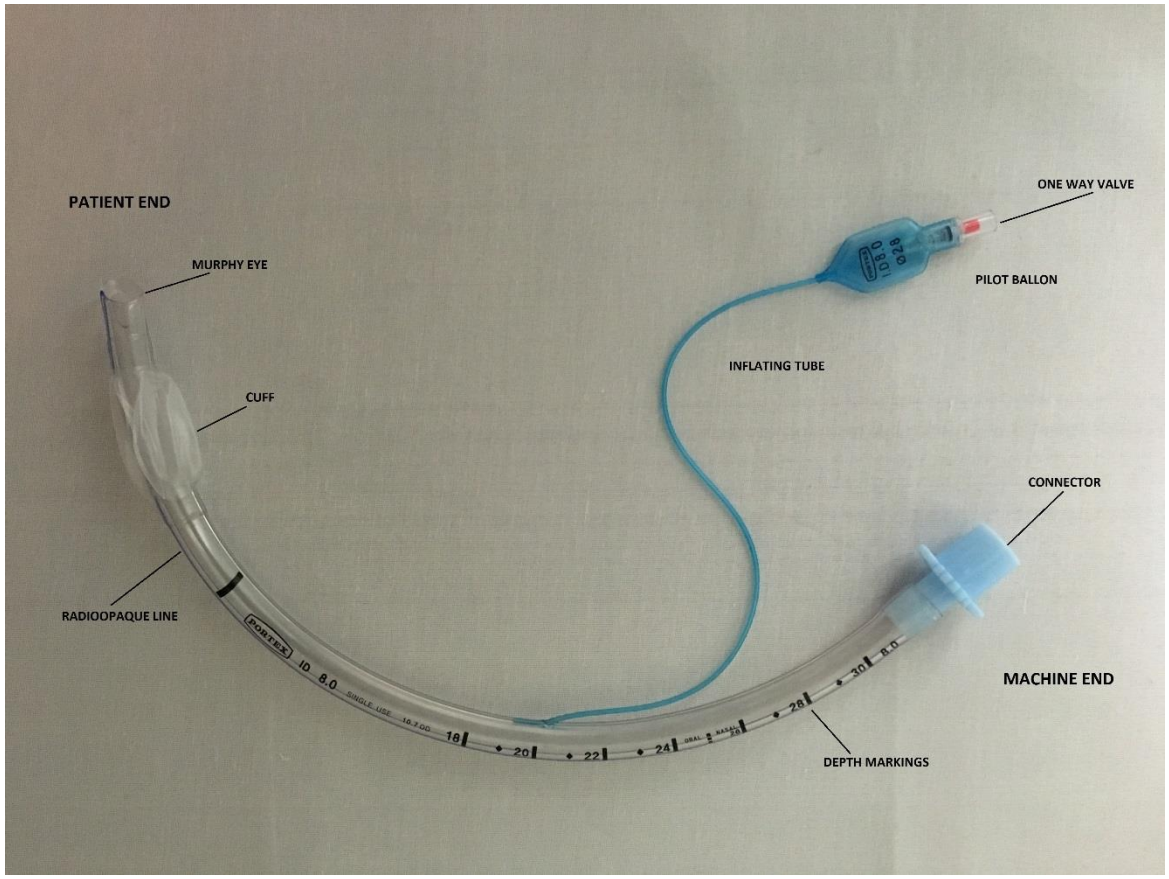

Figure 1.2 Picture of a standard polyvinyl chloride endotracheal tube.

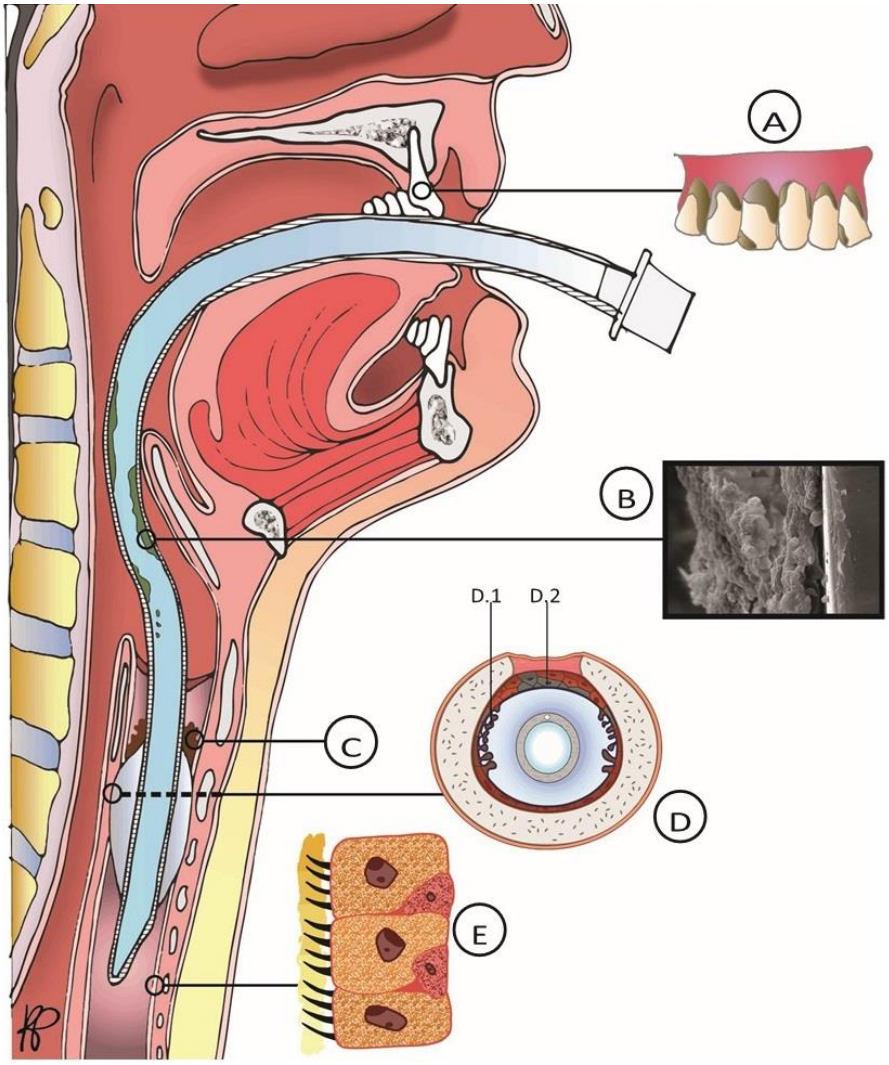

Figure 1.3 VAP pathophysiology: the endotracheal tube -related injury.

(a) Dental plaque becomes colonised by respirator pathogens. (b) A microbial biofilm with potential pathogens forms in the endotracheal tube (c) oropharyngeal secretions accumulate in the subglottic space, just above the cuff (d1) These contaminated secretions leak into the lungs trough the inflated micro-channels that form around the cuff (d2).The inflated cuff causes mucosal damage because of tracheal wall ischemia, damaging the integrity of the mucosa and impairing host-defence mechanisms. (e) The functional tracheal mucociliary apparatus is impaired. Reproduced with permission (Pirrone *et al*.2016).

The use of lubricant gel seals has also been proposed to reduce formation or impact of microchannels. In a clinical trial of lubricating ETT cuffs with a water based lubricating gel (KY® Jelly), leakage was significantly reduced (11% compared to 83%; $p<0.001$). However the effect was relatively limited, as cuff leakage occurred after 48 h and was not therefore deemed a viable solution for patients requiring longer periods of ventilation (Blunt *et al.* 2001). There was also no evidence of the effect of these prevention strategies on reducing incidence of VAP, mortality rates or hospital stay.

1.3.6 **VAP prevention**

Prevention of VAP has evolved significantly in the recent years as the medical community realised that a multifactorial approach was required and several recommendations have been proposed; the lack of structured prevention strategies to implement the recommendations led to publishing of so-called 'VAP bundles'. The bundles are an approach to take evidence-based guidelines to clinical practice, they take a limited number of prevention strategies (usually 3 to 5) that have independently demonstrated to be effective and combine them into one guidance that should be applied to every patient providing a cohesive effect in the improvement of outcomes (Rello *et al.* 2010b; Wip and Napolitano 2009).

The current components of the Institute for health improvements (IHI) ventilator bundle are elevation of the head of the bed, daily 'sedation vacations' and assessment of readiness to extubate, peptic ulcer disease prophylaxis, deep venous thrombosis prophylaxis and daily oral care with chlorhexidine, interestingly, although the bundle was created originally in 2003, oral care was only included in 2010 (Cambridge 2012; Klompas *et al.* 2014a; Zilberberg *et al.* 2009).

1.3.6.1 **Elevation of the head of the bed**

The evidence for head elevation is limited but a recent meta-analysis found that a semi-recumbent position with the head of the bed elevated at 45° as opposed to a supine position with elevation between 15° and 30° significantly reduced the risk for VAP (Alexiou *et al.* 2009).

1.3.6.2 **Sedation vacations and extubation assessment**

Kress *et al*., (2000) conducted a randomized controlled trial in 128 adult mechanically ventilated patients. Patients were randomized to receive daily interruption of sedation until awake versus management at the clinician's discretion. Daily interruption resulted in a highly significant reduction in time spent on mechanical ventilation. The duration of mechanical ventilation decreased from 7.3 days to 4.9 days (p=0.004) (Kress *et al.* 2000). Similar results had been reported previously with daily assessment of spontaneous breathing has also proved to shorten the intubation period for 1 or 2 days (Ely *et al.* 1996; Esteban *et al.* 1995). Importantly, whenever possible, the sedation vacation and trial for spontaneous breathing should be simultaneous to increase the chances of patients successfully breathing independently (Strøm *et al.* 2010).

1.3.6.3 **Peptic ulcer disease prophylaxis**

Mechanical ventilation creates a significant risk to the onset of stress-related mucosal disease including gastric bleeding, which in turn dramatically increases mortality in ICU patients by 5-fold (Cook *et al.* 1994). This strategy involves the prescription of H_2 receptor inhibitors like ranitidine or proton pump inhibitors which increases the pH of the gastric contents (Steinberg 2002), this however creates the potential risk of facilitating bacterial growth in a less hostile environment and the concern lays in that oesophageal reflux and aspiration of gastric contents along the endotracheal tube may lead to endobronchial

colonization and pneumonia. Recently a slight risk of VAP has been reported with proton pump inhibitors compared to a H_2 receptor inhibitor (Bateman *et al.* 2013).

1.3.6.4 **Daily oral care**

Oral care in conjunction with use of chlorhexidine has been studied in several randomised controlled clinical trials, which have subsequently been analysed in a minimum of 9 meta-analysis studies including a Cochrane review. Results from these suggest that the use of chlorhexidine reduces the incidence of VAP from 25% to 19%, with cardiac patients having the most benefit but there is, however, no evidence that the use of chlorhexidine has an impact on mortality or length of ICU stay (Chan *et al.* 2007; Hua *et al.* 2016; Klompas *et al.* 2014b; Shi *et al.* 2013). Unlike use of chlorhexidine, evidence for the effect of toothbrushing in VAP is limited by the lack of randomised controlled trials and to date impact on VAP outcomes has not been documented (Alhazzani *et al.* 2013; Hua *et al*. 2016; Pobo *et al.* 2009). Similarly, research including use of other antimicrobial agents is scarce (Shi *et al.,* 2013). Importantly, oral health has been demonstrated to decline in ICU patients (Fourrier *et al.* 1998; Munro *et al.* 2006; Sachdev *et al.* 2013), but there remain only limited studies that evaluated the efficacy of oral hygiene in mechanically ventilated patients (Needleman *et al.* 2011; Oliveira *et al.* 2014). There is no clear guidelines on the method and frequency of the delivery of oral care for mechanically ventilated patients, and wide variation in practices has been reported (Feider *et al.* 2010; Rello *et al.* 2007).

1.3.6.5 **Other recommended strategies**

***Selective gastric decontamination (SDD)*:** This a practice is based on the theory that the normal anaerobic intestinal flora prevents secondary colonisation with Gram-negative bacteria like *Pseudomonas aeruginosa*, which poses a risk for the immunocompromised patient. The aim of SDD is to eradicate the Gram-negative bacteria and fungi from the digestive tract whilst maintaining the anaerobic microflora. SDD consists of four steps that

include (i) oral cavity and gastrointestinal tract decontamination with topical non-absorbable antibiotics (polymyxin E, tobramycin and amphotericin), (ii) systemic prophylaxis, usually with cefotaxime, (iii) regular monitoring of microbial composition through culture of throat and faecal specimens, and (iv) good cross-infection practice (Bonten *et al.* 2000; de Smet *et al.* 2009). Selective oropharyngeal decontamination (SOD) is a variation of SDD which refers to application of topical antibiotics in the oropharynx only (de Smet *et al.* 2009). Despite some evidence of that both SOD and SDD reduce ICUs mortality rates (Roquilly *et al.* 2015), this practice has seldom been adopted (Bastin and Ryanna 2009; Oostdijk *et al.* 2012) and remains controversial due concerns that the use of antibiotics may promote emergence of resistant bacteria, although no reports have been made during clinical trials, the long term effect promotion of antibiotic resistant microorganisms has not been discarded (Price and Cuthbertson 2016).

Subglottic secretion drainage: The reduction of pooling of secretions above the endotracheal cuff with the use of subglottic secretion drainage endotracheal tubes was previously reported to reduce VAP incidence, the duration of mechanical ventilation and reduce antibiotic needs (Bouza *et al.* 2008; Muscedere *et al.* 2011). However two recent meta-analysis that include 17 and 20 randomised clinical trials report that the use of subglottic drainage reduce VAP incidence but no impact on mortality, length of ICU stay or use of antibiotics (Caroff *et al.* 2016; Mao *et al.* 2016).

Physical activity: Early stimulation of physical activity has reported to speed Increase extubation and reduces cost. (Klompas *et al*. 2014a).

Probiotics: preparations of non-pathogenic microorganisms like *Lactobacillus rhamnosus* may improve microbial balance, particularly in competing against pathogenic bacteria that colonises the oropharynx and stomach, lower incidence of VAP has been reported in recent meta-analysis (Manzanares *et al.* 2016; Siempos *et al.* 2010).

1.3.7 **Oral biofilms and Ventilator-Associated Pneumonia**

Dental plaque provides a wide variety of microorganisms that may be aspirated into the respiratory tract. It is thought that oral hygiene can deteriorate in the medically compromised patient leading to higher plaque levels (Fourrier *et al*. 1998; Sachdev *et al*. 2013).

In mechanically ventilated patients, the placement of an endotracheal tube (ETT) is essential to facilitate gaseous exchange to the lower airway. However, the ETT also impairs the patient's ability to clear oral secretions through coughing and mucocilliary activity. There is also reduced salivary flow in such patients, that combined with broad-spectrum antibiotic therapy creates a high-risk environment for resistant bacteria to colonise the oropharynx (Chastre and Fagon 2002; Munro *et al*. 2006). Hence, an increase in bacterial load in dental plaque has been shown during intubation (Munro *et al.* 2006) and multidrug resistant bacteria like MRSA and *P. aeruginosa* are commonly associated with VAP (Chastre and Fagon, 2002).

Lower salivary flow may cause a reduction in the adherence of oral commensal streptococci to the oral mucosa because of the decreased levels of fibronectin, facilitating overgrowth of pathogenic bacteria. *In vitro* studies reported that salivary fibronectin inhibited adherence of the Gram negative *Escherichia coli* to saliva-treated buccal cells (Hasty and Simpson 1987), whereas others have reported that salivary components like mucin and secretory immunoglobulin A facilitate the adherence of *Streptococcus gordonii* and *Streptococcus mutans* (Ito *et al.* 2012; Ligtenberg *et al.* 1992). In critical care patients, it has been demonstrated that the oropharynx becomes colonised within 48 h post intubation with Gram negative bacteria and this was a predictor factor on the onset of pneumonia (Ewig *et al.* 1999). Indeed, respiratory pathogens including *P. aeruginosa*, *Acinetobacter* and *Staphylococcus* species have repeatedly been found in plaque and saliva of hospitalised

patients (Didilescu *et al.* 2005; Fourrier *et al.* 1998; Zuanazzi *et al.* 2010). A link between periodontal disease and oral colonisation by respiratory pathogens has also been suggested by different authors who also propose that the inflammatory products of periodontitis may promote colonisation (Paju and Sannapieco 2007). More research is however needed to fully understand this relationship.

Poor oral care and difficulties in swallowing were associated with pneumonia in a study involving 613 elderly patients in a nursing home (Terpenning *et al.* 2001), whilst dentate patients have also shown higher incidence of aspiration pneumonia compared with edentulous patients (Mojon 1997).

Bahrani-Mougeot *et al.,* (2007) took tongue swabs and bronchial lavage fluids (BALs) from 40 patients diagnosed with VAP and analysed these by 16S rRNA gene amplification, cloning and sequencing. This revealed several novel species at both sites. At least one of the associated species (*H. Influenzae, Escherichia sp., Streptococcus pneumoniae, S. aureus, Pseudomonas* sp., and *Proteus mirabilis*) were found in BAL and tongue samples of 14 of the 16 patients. Additionally, members of the normal oral microflora were detected in BAL samples, including *Streptococcus, Lactobacillus* and *Porphyromonas* species. These results support the hypothesis that the oral cavity serves as a reservoir for VAP related microorganisms (Bahrani-Mougeot *et al.* 2007).

In collaboration with the critical care unit of the University Hospital of Wales, researchers at Cardiff Dental School collected 24 ETTs from 20 patients and analysed these using PCR and molecular profiling. Results revealed the presence of the oral bacteria *S. mutans* (n=5) and *P. gingivalis* (n=5) as well as the yeast *Candida albicans* (n=6) and supported the hypothesis that oral microbes participated in ETT biofilms (Cairns *et al.* 2011).

There are no studies that have simultaneously analysed ETT biofilms, bronchial lavages and plaque from the oral cavity. Such data could aid understanding of the pathogenesis of VAP and promote prevention strategies.

1.4 Methods for the detection microbial species in clinical specimens

1.4.1 Traditional microbiology techniques

Traditional microbiology methods for identification of microbial species in environmental samples are based on microbial metabolism, for example oxidation-reduction potential, and gaseous requirements (aerobic, anaerobic, CO_2 dependant).

It is imperative that microorganisms are viable and culturable on the selected culture medium. Bacteria grow as colonies on agar plates, which allows assessment of morphological characteristics including shape, size, consistency, and opacity, and subsequent antibiotic resistance testing. Culture media can be one for general purposes such as blood agar, which supports the growth of many microorganisms, or be tailored for selective recovery of certain species and suppression of others. Such selective media are often supplemented with additives such as antibiotics. Differential media are designed to distinguish between different groups of microorganisms through biochemical reactions that result in a colour change in the medium to indicate the presence of a particular species.

The culture of bacteria allows a comprehensive characterisation of individual species and strains' phenotypic traits, however, for a significant proportion of species this is not yet possible. In environmental samples it is considered that over 90% of bacteria are unculturable (Wade 2002). Even though dental plaque is one of the most studied biofilms, it is estimated that half of the oral microbial community are not yet culturable (Paster *et al*. 2001) and to screen for a wide range of microorganisms by species specific PCR or cloning would be formidable expensive and time consuming. Therefore, microbial composition of

communities such as those in the oral cavity, lower airway and ETT cannot be fully determined without use of culture-independent molecular techniques.

1.4.2 **Polymerase chain reaction (PCR)**

PCR relies on the ability of *Taq* polymerase to repeatedly synthesise new strands of DNA complementary to template targets following repeated thermal cycles. An oligonucleotide primer is required for the incorporation of the first nucleotide. On completion of PCR, the specific target sequence will typically have been amplified 10^9-fold and these products are often called amplicons (Van Pelt-Verkuil *et al.* 2008).

There are multiple applications of PCR in the study of bacteria and yeast, including genotyping and gene sequencing, both of which will be used in the research presented in this thesis.

To identify cultured bacteria isolates, amplification and sequencing of the bacterial 16S ribosomal RNA (rRNA) gene can be undertaken. Sequences are compared to those in a database and identification made based on similarity. Amplification of 16S rRNA genes (rDNA) can also identify bacteria in mixed bacterial populations and without prior culture. Historically, in order to separate different amplicons prior to sequencing from mixed communities, a cloning step was required, which was both costly and labour intensive. However, with the advent of next generation sequencing approaches, the need for cloning has been circumvented. Species-specific PCR can also be used to detect individual species directly from mixed populations and such methods have successfully been used in the direct analysis of clinical samples. For example species-specific PCR has been successfully used to detect bifidobacteria from the gut and respiratory pathogens in children with parapneumonic empyema (Blaschke *et al.* 2013; Matsuki *et al.* 2003).

1.4.3 **DNA sequencing**

DNA sequencing is the process of finding the exact order of nucleotides (adenine, guanine, cytosine and thymine) in a DNA molecule, the technology to achieve this has advanced significantly in the last decade allowing the study of whole microbiome populations of an environment like the gut, oral cavity (Dewhirst *et al*. 2010; Shreiner *et al.* 2015).

The characterisation of complex bacterial communities derives from the discovery of the 16S rRNA gene as a bacterial phylogenic marker (Woese 1987). The 16S rRNA gene is a housekeeping gene that is considered a "molecular chronometer" and as such a measure representative of evolution change. (Woese 1987). The 16S rRNA gene includes regions that are highly conserved and others which are species specific. (Hanage *et al.* 2006).

1.4.3.1 **The Sanger method**

The start of genomic sequencing happened in the late 1970s with the introduction of the Sanger method, which allowed DNA to be sequenced in a reliable and reproducible way (Sanger *et al.* 1977). This method uses a single stranded DNA template, a DNA primer that is complementary to the known sequence, DNA polymerase, deoxynucleosidetriphosphates (dNTPs) and importantly dideoxynucleotide-triphospate (ddNTPs), this are nucleotide analogues that lack the 3'-hydroxyl group essential in phosphodiester bond formation thus, terminating the DNA elongation process. All four ddNTPs are were labelled initially with a radioactive probe which was later replaced with a fluorescent dye, the label on each ddNTP corresponds to the nucleotide identity. In the early years products were visualised by gel based electrophoresis, this later changed to capillary based polymer gel and where products were separated by size. When a fluorescent dye is used a laser identifies the ddNTP labels and assigned one of four colours, this provides a chromatogram from which a software translates the reads into a DNA sequence and generates error probabilities for each base (Shendure and Ji 2008).

A variation of the Sanger method is the called shot gun *de novo* sequencing where DNA is randomly fragmented and cloned into a high copy number plasmid which is the incorporated into *Escherichia coli*, as the bacterium reproduces creates clones of the plasmid inserted that can be cultured and picked as a single colony from an agar plate, to then be sequenced as above (Shendure and Ji 2008).

The first automated capillary electrophoresis platform (AB370; Applied Biosystems) was introduced in 1987. This technology which was used in the Human Genome Project in 2001 increased the output from a maximum of 200 nucleotides per day to approximately 1 megabase.

1.4.3.2 **Pyrosequencing**

Pyrosequencing was the first generation of Next generation sequencing, introduced in 2004 by Roche with the 454 platform. (Mardis 2008). In pyrosequencing DNA fragments are fixed on DNA-capture beads in a water-oil emulsion and then amplified by PCR. The beads are loaded with DNA polymerase on a PicoTiterPlate. The pyrosequencing reaction results in the release of pyrophosphate, which initiates a series of downstream reactions that produce light by the firefly enzyme luciferase. The amount of light produced is proportional to the number of nucleotides and converted into a sequence (Mardis 2008; Oulas *et al.* 2015). This technology was widely used in the early years but it has become less popular due to higher costs and more difficult data analysis (Werner *et al.* 2012).

1.4.3.3 **Next Generation Sequencing (NGS)**

NGS, also known as massive parallel sequencing was first commercially introduced in 2005 with the Genome Analyser, where in a single sequencing run produced one gigabase of data and by 2014 the output increased to 1.8 terabases with the latest platform HiSeqX Ten (Illumina). The output increase has had an inverse proportional effect on the cost of

sequencing making it much more accessible for small research projects. The high efficiency of this technology has changed genomic sequencing as well as opening new opportunities in other areas like characterisation of ecological diversity (Mardis 2008).

NGS differs from the Sager sequencing in that it is based on spatially separated, clonally amplified DNA templates or single DNA molecules on a flow cell where the process is extended across millions of fragments. Similarly to the previous technologies a DNA polymerase (*i.e*. *Bst*) catalyses fluorescently labelled dNTPs into a DNA template strand during a series of PCR cycles, at each cycle the nucleotides are identified by fluorophore excitation (Nakazato *et al.* 2013). NGS incorporates adapter sequences that allow for selective amplification by PCR, this eliminates the need for bacterial cloning to achieve amplification of genomic fragments, for some platforms (Helicos and Pacific Biosystems) the amplification of DNA fragments is not required before sequencing (Mardis 2008).

1.4.3.4 **Metagenomics**

One of the fields of study that has benefited greatly by NGS is microbial ecology, which has led to the rise of metagenomics which has been defined as" the direct genetic analysis of genomes contained within an environmental sample without the prior need for cultivating clonal cultures" (Oulas *et al*. 2015). This aspect of NGS was used initially to characterise the microbial genomes in an environmental sample, for which is also known as "full shotgun metagenomics" (Xia *et al.* 2011). However, metagenomics can also be used for PCR amplification of selected genes called "meta-genetics" (Handelsman 2009).

Full shotgun metagenomics, uses inventories of 16S rRNA genes to provide a snapshot of the bacterial diversity and relative abundance within a sample creating a community biodiversity profile (Gee *et al.* 2004; Ziesemer *et al.* 2015). Currently this technology can identify the species present in a sample as well as what their function and some interspecies interactions that maintain homeostasis in their ecosystem (Oulas *et al*. 2015).

Metagenomics has been used to study a wide range of environmental samples including those from difficult conditions like extreme temperature, hypoxic environments and volcanic zones (Benson *et al.* 2011; Kilias *et al.* 2013; Stevens and Ulloa 2008).

The study of the human microbiomes has progressed remarkably in the last decade with the use of metagenomics. In 2007 the National Health institutes launched the Human Microbiome Project with the intention of achieving a comprehensive characterisation of the human microbiota and its role in human health and disease, to date there are 564 publications from this project (NHI 2007). From the human body, the gut microbiome has been the most studied (Qin *et al.* 2010; Shreiner *et al*. 2015). Recently, Browne *et al*. developed a workflow that combines whole-genome and metagenomic sequencing, with computational and phenotypic analysis, with this new approach the researchers were able to culture 90% of the gut bacterial microbiome (Browne *et al.* 2016) despite the fact it had previously been considered as predominantly unculturable (Walker *et al.* 2014).

1.4.4 **Molecular fingerprinting of microbial species**

1.4.4.1 **Random Amplification of Polymorphic DNA (RAPD)**

RAPD is a form of PCR used for genotyping of microorganisms and can differentiate between genetically distinct strains of the same species. The key to this approach is to employ 'random' primer sequences whose targets are widely distributed through the bacterial DNA genome. Multiple amplicons are generated for a given bacterial strain, which when separated by gel electrophoresis resembles a 'barcode' of different sized bands. The number of bands and their sizes are dependent on the positions of the targets for the original primers. In RAPD, the primers used are typically arbitrary and short (8 to 10) nucleotide sequences and importantly it is not necessary to have prior knowledge of the genetic material for RAPD (Bart *et al.* 1998).

1.4.4.2 **Pulsed-field gel electrophoresis (PFGE)**

PFGE is a genotyping technique that until the development of multilocus sequence typing was considered the gold standard in epidemiological investigations (Prevost *et al.* 1991). PFGE still remains a valuable method as it is relatively inexpensive and is highly discriminatory with proven benefits in the investigation of nosocomial infections (David *et al.* 2013).

PFGE separates chromosome-sized DNA molecules through application of an alternating electric field between spatially distinct pairs of electrodes. Varying electrical pulses promote separation of DNA molecules sized between 30 to 2000 Kb. During PFGE, DNA fragments have to re-orientate themselves within the electrophoresis gel matrix when the direction of the electric field changes, and the time to do this is dependent on fragment size (Prevost *et al*. 1991).

Clamped Homogeneous Electric Field (CHEF)-PFGE is a variation of PFGE. CHEF-PFGE was developed to improve high resolution, create sharper bands and straight lanes of DNA profiles. The system uses 24 electrodes along the perimeter of a hexagonal electrophoresis tray. The electrodes generate two different alternating electric field vectors where each electrode ‘clamps’ the voltage of its individual region of space as necessary to maintain field homogeneity (O'Brien *et al.* 2007).

1.5 Target species

To study interaction between typical oral microorganisms and respiratory pathogens, this current research focussed on five microbial species namely, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Candida albicans*, *Streptococcus mutans* and *Porphyromonas gingivalis*.

1.5.1 ***Pseudomonas aeruginosa***

Pseudomonas aeruginosa is a Gram-negative bacillus-shaped bacterium ubiquitously found in the environment, inhabiting waters, soils and plants (Driscoll *et al.* 2007). In the hospital setting, *P. aeruginosa* has been isolated from the floors, bed rails, sinks of hospitals, as well as from hands of nurses (Chitkara and Feierabend 1981). *P. aeruginosa* causes opportunistic infections in plants, insects and mammals (Battle 2009). In humans, *P. aeruginosa* is a known opportunistic pathogen of immunocompromised patients and is the one of most prevalent microorganisms in nosocomial pneumonia with approximately 24% of cases attributed to it (Chastre and Fagon 2002; Pearson 1996; Rello *et al.* 2002).

Pseudomonas aeruginosa is not a frequent member of the normal microbiota of humans, with colonisation rates ranging between 0 and 3.3% for skin and nasal mucosa, 0 to 6.6% for the oropharynx and 2.6 to 24% for the gut (Lister *et al.,* 2009, Morrison and Wenzel, 1984). In contrast, colonisation of up to 50% of hospitalised patients have been reported. Patients who have skin injuries, catheters, or in receipt of mechanical ventilation or surgery, are most at risk (Ohara and Itoh 2003; Ozkurt *et al.* 2005; Thuong *et al.* 2003; Vallés *et al.* 2004). In a 3-year prospective study of mechanically ventilated patients, it was found that a 54.2% of patients were colonised with *P. aeruginosa* either before or after intubation, and tracheal colonisation was 30.5% (Vallés *et al.,* 2004), indeed several studies report *P. aeruginosa* to

be frequently found in the dental plaque of mechanically ventilated patients (El-Solh *et al.* 2004; Heo *et al.* 2008; Sands *et al.* 2016 (a); Zuanazzi *et al*. 2010).

Pseudomonas aeruginosa is associated with multiple infections in the body, including those of the skin, eyes and ears. Chronic lung infection, nosocomial pneumonia, septicaemia, bacterial keratitis and urinary tract infections are also caused by *P. aeruginosa*, and these infections are frequently difficult to treat (Boyle *et al.,* 2013, Valenza *et al.,* 2008). Additionally, in mechanically ventilated patients, respiratory infections associated with *P. aeruginosa* are considered to result in higher mortality than those caused by other microorganisms (Chastre and Fagon, 2002).

Pseudomonas aeruginosa can maximise available nutrients, produces and releases a wide range of exoproteins, the majority of which are toxins and hydrolytic enzymes which have significant impact in its pathogenicity. For example, in environments of low iron concentrations commonly found in the host, *P. aeruginosa* secretes the protein HasAp, a haemophore that generates haem release from haemoglobin, a characteristic considered important in the onset of infection (Bleves *et al.* 2010; Wandersman and Delepelaire 2004).

Pseudomonas aeruginosa is naturally resistant to multiple antibiotics and also rapidly adapts to generate resistance to new drug therapies, a feature which is possible due to its ability to acquire resistance genes from plasmids or through changes in gene expression (Lister *et al.* 2009). In patients initially colonised with sensitive *P. aeruginosa* strains, between 27 to 72% of those were found to change to exhibit multidrug resistance (Obritsch *et al.* 2005), which clearly creates difficulty in eradicating infection.

1.5.2 ***Staphylococcus aureus***

Staphylococcus aureus is a Gram-positive coccus-shaped bacterium frequently found in the respiratory tract and skin of humans, it is a permanent coloniser in around 30% of the population, or a transient coloniser in 30 to 50% (Chambers 2001; Kluytmans *et al.* 1997). Although *S. aureus* is not considered part of the normal oral microbiota, oral carriage occurs in between 3% to 46% of healthy individuals and a higher occurrence in saliva compared to dental plaque is evident (Eick *et al.* 2016; Ohara-Nemoto *et al.* 2008) (Kluytmans *et al.,* 1997 Chambers, 2001).

Within the *Staphylococcus* genus, *S. aureus* is generally regarded the most pathogenic species and is commonly associated with multiple infections including those of the skin, respiratory tract and this species is also a cause of food poisoning. On occasions, infections can be life threatening, as is the case of pneumonia, meningitis, osteomyelitis, endocarditis and toxic shock syndrome (Lowy 1998). Staphylococci are recognised as the most common causes of nosocomial infection in intensive care units (Otto 2008) and *S. aureus* accounts for approximately 20% of VAP cases (Chastre and Fagon 2002).

Natural defence mechanisms against *S. aureus* include the relatively low temperature of the skin surface and its acidic pH, which impeded *S. aureus* growth as well as its colonising ability by inhibiting clumping factor B and fibronectin binding protein A (Arciola *et al.* 2005). Furthermore, other commensal microorganisms may prevent *S. aureus* colonisation. For example, some strains of *S. epidermidis* produce *Esp*, a serine protease that prevents *S. aureus* biofilm formation and destroys pre-existing biofilms (Iwase *et al*, 2010). Skin commensals also promote the innate immune response by inducing expression of antimicrobial peptides and activation of signalling pathways in keratinocytes, which facilitate killing of pathogenic bacteria such as *S. aureus* (Wanke *et al.* 2011).

As mentioned earlier, to facilitate adhesion, *S. aureus* uses microbial surface components recognising adhesive matrix molecules (MSCRAMMs), which include fibronectin-binding protein A (Fnbp A) and Fnbp B (Arciola *et al*. 2005; Patti *et al.* 1994). Additionally *S. aureus* expresses fibrinogen-binding proteins (ClfA and ClfB), iron-regulated surface determinant A (IsdA) and wall teichoic acid (Weidenmaier *et al.* 2004). Strains of *S. aureus* may produce the toxin, Panton-Valentine Leucocidin (PVL), which enhances virulence by causing leukocyte lysis or apoptosis via pore formation (Kaneko and Kamio 2004). *S. aureus* can also demonstrate resistance to multiple antibiotics including meticillin and vancomycin (Diekema *et al.* 2001).

1.5.3 ***Candida albicans***

There are more than 200 species of *Candida*, several of which are opportunistic pathogens of humans. Generally, *C. albicans* is regarded as the most important clinical species and is commonly found in the oral cavity, eyes, and genitourinary and gastrointestinal tracts of humans (Spampinato and Leonardi 2013). Other important *Candida* species found in healthy individuals but also regarded as opportunistic pathogens include *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, and *Candida krusei* (Maccallum 2012). Oral carriage of *Candida* has been estimated at 50-75% in healthy individuals depending on the population group studied (Ariyawardana *et al.* 2007; Javed *et al.* 2013).

Candida infection (candidosis) usually occurs in debilitated or immunocompromised patients, after surgery, during prolonged stay in an intensive care unit, or extended use of broad-spectrum antibiotic therapy (Azoulay *et al.* 2006). Infections may be superficial (oral and vaginal thrush) or systemic and in the case of the latter, are frequently life threatening (Wisplinghoff *et al.* 2004).

Candida albicans has several putative virulence factors, the secretion of aspartyl proteinases (Sap1 to Sap10) facilitates colonization and invasion of host tissues through the disruption

of host mucosal membranes (Silva *et al.* 2011) and phospholipases are thought to contribute to host cell-membrane damage, which could promote cell damage and/or expose receptors to facilitate adherence of Candida (Ghannoum 2000). *Candida albicans* can express multiple adhesins (agglutinin-like sequence, hypha-associated GPI-linked protein) to enable colonisation of host cells or abiotic surfaces (Mayer *et al.* 2013; Murciano *et al.* 2012). The organism also exhibits polymorphism with regards to its growth and can change from spherical yeast forms (ovoid-shaped budding) to hyphal or pseudohyphal forms (filamentous) (Berman and Sudbery 2002). The hyphal form of *C. albicans* is linked to tissue invasion, whereas the yeast from is associated with dissemination to distant sites (Cleary *et al.* 2011; Malic *et al.* 2007).

Another virulence factor of *C. albicans* is its ability to form biofilms on mucosal and biomaterial surfaces including those of dentures, catheters and endotracheal tubes (Vandecandelaere *et al.,* 2012, Mayer *et al.,* 2013). Importantly, *C. albicans* biofilms, are more resistant than their planktonic counterparts to antifungal therapy due to similar mechanisms to those previously described for bacterial biofilms. These mechanisms include increased expression of efflux pumps, higher cell densities, extracellular matrix production and modified gene expression (Al-Fattani and Douglas 2006; Mukherjee *et al.* 2003; Taff *et al.* 2013).

Airway colonisation by *C. albicans* in the intensive care setting has been identified as a risk factor for colonisation by the multidrug resistant bacterium *P. aeruginosa* pneumonia, as well as a risk for systemic candidiasis (Azoulay *et al.* 2006). Additionally, poorer clinical outcomes have been reported in patients with suspected VAP who have *Candida* colonisation of the respiratory tract (Delisle *et al.* 2011; Williamson *et al.* 2011). Hamet *et al*., (2012) conducted a prospective observational study including 323 suspected VAP patients a higher mortality rate (44.2% vs 31.5%; p: 0.02) in patients with Candida

colonisation of the airway, this same study also found Candida airway colonization was one independent risk factor for multidrug resistant bacteria isolation [odds ratio (OR) = 1.79, 95 % confidence interval 1.05-3.05; p= 0.03 (Hamet *et al.* 2012).

1.5.4 ***Streptococcus mutans***

The *Streptococcus* genus currently consists of more than 100 species, which are often classified into 6 phylogenetic clusters based on their 16S rRNA gene sequences (Nobbs *et al.* 2009).

Streptococcus mutans is a Gram-positive coccus-shaped bacterium frequently found in dental plaque. This species was first associated with causing dental caries in 1924 when Clarke isolated it from a carious lesion. As it is not a microorganism readily present in the environment, several studies have suggested that the most likely route of colonisation of an individual is vertical transmission from the mother/carer to the infant (Tanzer *et al.* 2001). *S. mutans* requires a solid, non-shedding surface for colonisation, and as a consequence, it is not normally found in infants until the first tooth appears. However, insertion of prosthetic appliances during treatment of edentulous cleft palate in new-borns can lead to earlier colonisation with *S. mutans* (de Soet *et al.* 1998).

S. mutans ferments mannitol and sorbitol to produce organic acids that demineralise tooth enamel during caries (Decker *et al.* 2014). The species also produces extracellular polysaccharides in the form of glucans, which allow it to firmly adhere to the smooth tooth surface (Kuramitsu 1993; Loesche 1986). The production of glucan-binding proteins (GbpA, -B, -C and -D) is thought to play an important role in subsequent cell-cell aggregation and biofilm development (Shah and Russell 2004; Smith and Taubman 1996). Additionally, antigen I/II surface protein in *S. mutans* is involved in binding to salivary pellicle, collagen type I and fibronectin (Petersen *et al.* 2002). These characteristics promote *S. mutans* as a pioneer coloniser of dental plaque and also facilitates co-adherence of other

microorganisms to the existing biofilm. Indeed it has been suggested that *S. mutans* presence will promote lactobacilli colonisation within dental caries lesions (Tanzer *et al*. 2001). In a similar manner, *S. mutans* could aid colonisation of teeth by other bacteria including potential respiratory pathogens, and this clearly has implications with regards to VAP. *S. mutans* also has sucrose-independent mechanisms to promote cell to cell aggregation, in sucrose depleted conditions it produces the wall-associated protein A (WapA) (Zhu *et al.* 2006) which could be relevant in the mechanically ventilated patient as there is no oral intake, is produced

1.5.5 ***Porphyromonas gingivalis***

Porphyromonas gingivalis is a Gram-negative obligate anaerobic bacterium (Benedyk *et al.* 2016). This bacterium is as non-motile, asaccharolytic, coccobacillus in shape and grows as smooth, raised colonies on blood supplemented agar media, that are initially white to cream coloured but within 4 to 8 days start to darken and turn to a deep red to black colour. This colony colouration correlates with the concentration of protoheme, which derives from the erythrocytes (Holt *et al.* 1999; Nakayama 2015). *Porphyromonas gingivalis* is frequent member of the oral microbiota and a recognised periodontal pathogen. The prevalence of *P. gingivalis* in patients with gingivitis has been reported at 79% compared to 25% in healthy individuals (Griffen *et al.* 1998). *Porphyromonas gingivalis* is able to produce biofilms independently, but this function is best seen in the presence of other bacterial species like *Tannerella forsythia* and *Treponema denticola* where synergistic relationships have previously been described (Bao *et al.* 2014).

The main virulence factors of *P. gingivalis* are its gingipains, which comprise of three related cysteine proteases (Bao *et al*. 2014). Gingipains are thought to contribute to several functions including biofilm formation through fimbriae assembly, nutrition by digestion of

host proteins, proteolysis, and alteration of the host immune response (Olsen and Potempa 2014).

Periodontal disease has been proposed to have association with respiratory disease (Paju and Scannapieco 2007). Lower airway colonisation by anaerobic bacteria in mechanically ventilated patients has been reported to happen in >50% of mechanically ventilated patients (Agvald-Ohman *et al.* 2003; Robert *et al.* 2003). Species isolated from subglottic and tracheal secretions include *Peptostreptococc*i and *Prevotella* spp. (Agvald-Ohman *et al*. 2003). Furthermore, *P. gingivalis* has been detected by molecular methods in endotracheal tube biofilms (Cairns *et al.* 2011).

The impact of anaerobic species in respiratory disease may have important clinical implications, in an experimental mouse model, where a mixed culture of *P. gingivalis* and *T. denticola* was inoculated into the mouse trachea; the resulting infection induced inflammatory cytokine production and caused pneumonia (Kimizuka *et al.* 2003).

1.6 Hypothesis

The underlying hypothesis of this present body of work is that oral microorganisms play a key role in biofilm development and colonisation of respiratory pathogens in the inner lumen of the ETT and thus the promotion of ventilator-associated pneumonia in intubated patients.

1.7 Aims

The principle aim of this study was to use a combination of cultural and molecular based methods to characterise *in vitro* and *in vivo* biofilms on ETT surfaces and establish involvement of oral microorganisms in the development of respiratory pathogen biofilms.

Specific aims:

I. Establish the presence of oral microflora and respiratory pathogens in dental plaque, ETTs, and the lower airways of mechanically ventilated patients and elucidate genetic similarities between detected species at each site.

II. Characterisation of the bacterial microbiota from dental plaque, ETTs and lower airways of mechanically ventilated patients using metataxonomics.

III. Characterise the relationship between oral microorganisms and respiratory pathogens, by investigating potential synergistic effects in gene expression and growth of respiratory pathogens.

IV. Evaluate oral intervention methods for potential VAP patients. In this research two oral hygiene methods (brushes vs. swabs) will be compared in a clinical trial and *in vitro* experiments will compare different surfaces and designs of ETTs and susceptibility of respiratory pathogens to antimicrobial mouthwashes.

2. Analysis of dental plaque, endotracheal tube biofilms and non-directed bronchoalveolar lavages from mechanically-ventilated patients

2.1 Introduction

Mechanical ventilation is required in the majority of critically-ill patients to facilitate management of respiratory failure and impaired consciousness. However, this patient group is at high risk (15.6%; Kollef *et al*. 2014) of developing ventilator-associated pneumonia (VAP). The overall attributable mortality is estimated at 13%, but can be higher depending of the patient group (Melsen *et al*. 2013). VAP also results in an extended hospital stay of 5 to 7 days (Safdar *et al.* 2005) and up to £22000 additional economic cost per patient (Kollef *et al.* 2012; Wagh and Acharya 2009). The pathogenesis of VAP is not yet fully understood and multiple variables contribute to its occurrence (Kollef 1999). The endotracheal tube (ETT) impairs the natural mucocilliary clearance that occurs in the upper airway and keeps the epiglottis open exposing the lower airway to the contents of the pharynx (Bauer *et al*. 2002). One important factor in the development of VAP is the aspiration of oropharyngeal secretions that accumulate above the inflated cuff; it has been reported that the cuff material folds onto itself creating microchannels that allow leakage of the secretions into the lower airway (Dave *et al.* 2010; Haas *et al.* 2014). If these secretions are loaded with potential pathogens an obvious risk for infection is present (Figure 2.1).

The work outlined in this Chapter focuses on two factors which have been identified as contributors of VAP, namely biofilms within the endotracheal tube and the composition of the oral microflora.

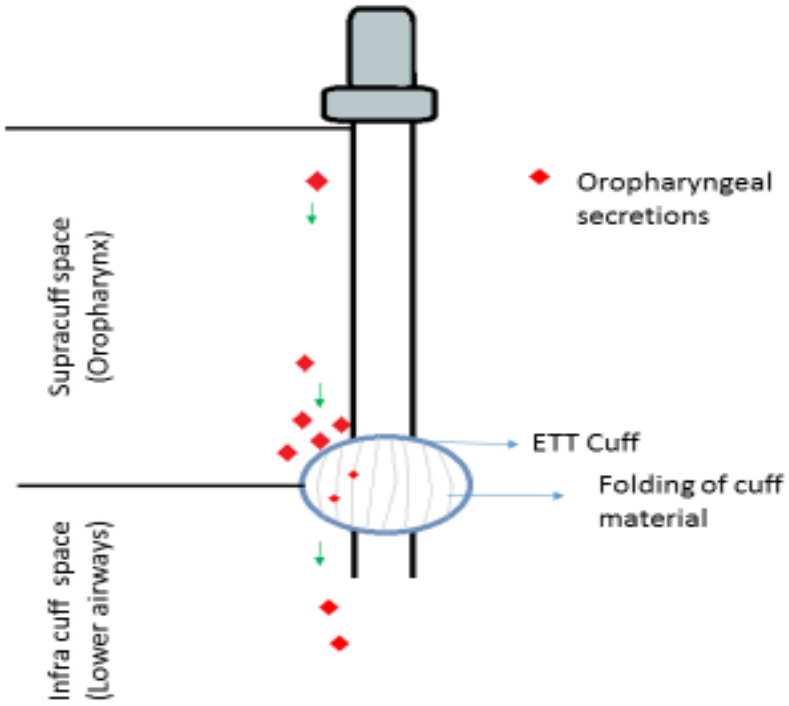

Figure 2.1 Schematic diagram showing the leakage of oropharyngeal secretions around the endotracheal tube cuff

2.1.1 Biofilm development in endotracheal tubes

Biofilms are communities of microorganisms that are often attached to a surface and embedded in extracellular polymeric substances (Hall-Stoodley *et al.* 2004). It is now thought that over 65% of infectious diseases are caused by biofilms and importantly, medical devices are prone to colonisation by biofilms (Donlan and Costerton 2002). Biofilms are much more than the 'sum of their parts', primarily due to altered gene expression. As discussed previously, biofilms also exhibit increased antimicrobial resistance (Chapter 1, Section 1.2.4).

The lumen of the ETT creates an environment that is protected from the patient's defence mechanisms. Unsurprisingly, ETT biofilms have been identified as risk factors for VAP (Adair *et al.* 1999; Danin *et al.* 2015; De Souza *et al.* 2014).

The first study employing imaging techniques for describing ETT biofilms was published in the late 1980s and used scanning electron microscopy (SEM) to image biofilms in 25 ETTs (Sottile *et al.* 1986). Results showed that 86% of ETTs were completely covered by biofilm and 16% were partially covered (Sottile *et al.* 1986). In a recent study using atomic force microscopy (AFM), full biofilm coverage of the lumen of 6 ETTs was noted (Danin *et al*. 2015). Inglis *et al.,* (1989) analysed ETT biofilm presence and the effect that ventilator gas flow had on the biofilm. Using SEM, 30 out of 40 ETTs examined had at least 50 mg (dry weight) of biofilm and once connected to a ventilator, fragments of the biofilm detached in 50% of the ETTs, and were projected up to 45 cm away from the ETT tip (Inglis *et al.* 1989). ETT biofilms can be detected after only 12 h of intubation and the biofilm will generally accumulate with increased duration of ventilation (Inglis *et al.* 1989; Perkins *et al.* 2010), but this is not always the case (Wilson *et al.* 2012).

The importance of ETT biofilms in VAP occurrence has been demonstrated numerous times (De Souza *et al.* 2014; Inglis *et al.* 1989; Perkins *et al.* 2010). Importantly, Adair *et al.,* (1999)

compared the microbiology of tracheal samples with ETT biofilms and found that 70% of patients with VAP had identical pathogens at both sample sites.

The origin of ETT biofilm microorganisms is an important question, and Feldman *et al.,* (1999) were amongst the first researchers to investigate this. This group proposed that colonisation progressed from the stomach, the oropharynx, then the lower respiratory tract and finally the ETT. At the same time, it was suggested that proteins and other adhesion materials interacted with the colonising bacteria facilitating biofilm formation (Feldman *et al.* 1999).

Perkins *et al.,* (2010) analysed the bacterial content of 8 ETTs from a medical and trauma intensive care unit using PCR sequencing of bacterial 16S rDNA. It was evident that over 70% of sequences were from typical members of the normal oral microflora. The most prevalent species belonged to the *Streptococcus* genus (7 of the 8 tubes), and many of these were normal components of the oropharynx microflora and adept biofilm producers. Species of *Prevotella* and *Neisseria* were also frequently detected, and 20% of the sequences were similar to known VAP pathogens, with only 6% of sequences typical of gastrointestinal microflora (Perkins *et al.* 2010).

Cairns *et al.,* (2011) assessed the microbial diversity of 20 ETTs using PCR and denaturing gel electrophoresis (DGGE). This study showed a significant microbial diversity in ETT biofilm samples, with between 3 and 22 bands per sample detected by DGGE. Since each distinct DGGE band can represent more than one species (Li *et al.* 2007), it is clear that the ETT biofilms were very complex and diverse in terms of microbial composition. This same study used species-specific PCR to detect target key oral microorganisms and respiratory pathogens. Interestingly, in these studies *Streptococcus mutans* (N=5) and *Porphyromonas gingivalis* (N=5) were detected along with *Staphylococcus aureus* (N=6) and *Pseudomonas*

aeruginosa (N=4) which are primary VAP pathogens. Additionally *Candida albicans* was cultured from 6 of the analysed ETTs (Cairns *et al.* 2011).

The findings of Cairns *et al.,* (2011) were later corroborated by Vandecandelaere *et al.,* (2012) in a study involving pyrosequencing, sequencing of 16S rRNA gene clone libraries and traditional culture techniques targeting ETT biofilms. Once more, a high level of diversity of ETT biofilms was evident and potential VAP pathogens were identified using culture and pyrosequencing. The study only analysed 4 samples by pyrosequencing, and in all of these, the predominant bacteria were members of the oral microflora, namely *Prevotella* species, *Peptostreptococcus* species and lactic acid bacteria (Vandecandelaere *et al.* 2012). In addition to pathogenic bacteria, it has also recently been demonstrated that the ETT biofilm is firmly attached to the surface and rinsing with saline does not effectively remove it (Danin *et al.* 2015).

Other sources of contamination of the ETT and lower airways could be the ventilator and suction equipment, but evidence for this is limited (Sole *et al.,* 2002). Furthermore, the presence of a nasogastric tube may facilitate gastroesophageal reflux. Therefore, gastric fluid may be aspirated into the lungs, carrying bacteria and provoking local inflammation, however colonisation originating from the gastric contents remains a matter of debate (Bassis *et al.* 2015; Bonten and Gaillard 1995).

2.1.2 The oral microflora

The oral mucosa and dental plaque has been identified as potential reservoirs of pathogenic microorganisms, particularly in hospitalised and critically ill patients (Gilbert *et al.* 2002; Pollitt *et al.* 2014; Scannapieco *et al.* 1992). Traditional culture based methods have implicated oral microbiota as contributors to infectious respiratory disease (Fourrier *et al.* 1998; Johanson *et al.* 1969; Scannapieco 1999). However, as valuable as culture based methods are, there are a number of important limitations. In particular, non culturable or

difficult to grow organisms may play an important role in the pathogenesis of disease and these may be missed by culture methods. Furthermore, comparing genetic relationships between micoorganisms is often preferable to phenotypic approaches when assessing similarity of isolates from different origins. Such information may be key in understanding the origin of infection, pathogenesis and formulating prevention strategies.

A pioneering study on the molecular analysis of the diversity and genetic relationship between the microbes from the oral cavity and lungs provided the first culture-free evidence on the subject. In this study, Bahrani-Mougeot *et al.,* (2007) collected bronchoalveolar lavage (BAL) fluid and dorsal tongue swabs from 39 patients. After total bacterial DNA extraction and PCR amplification of 16S rRNA gene sequences, the amplicons were cloned into *Escherichia coli* and sequenced. This study found a diversity of bacterial species in both samples and confirmed the presence of respiratory pathogens colonising the oral cavity and lungs (Bahrani-Mougeot *et al.* 2007). Later, a separate study compared genetic relationships between respiratory pathogens from dental plaque and bronchoalveolar lavages (BALs) using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing and found that microorganisms from dental plaque and BALs, including *S. aureus* and *P. aeruginosa* were identical (Heo *et al.* 2008).

2.2 Objectives

Current evidence suggests that oral microorganisms contribute to biofilm formation in ETT lumens. These ETT biofilms are also known to contain pathogenic bacteria able to cause VAP. However, studies demonstrating a microbiological continum between the oral cavity, the ETT and the lung are limited. The principle aims of this Chapter were therefore to:

- Determine simultaneous presence of representative oral microorganisms (*i.e. Streptococcus mutans*, *Porphyromonas gingivalis* and *Candida albicans*) along with

potential respiratory pathogens (*i.e. Staphylococcus aureus* and *Pseudomonas aeruginosa*) in dental plaque, ETT biofilms and NBLs of MV patients.

- Determine whether strains of isolated and targeted species from different sites in individual patients were genetically identical. In cases where identical strains were detected, evidence would thus be generated that was supportive of a microbiological link between the sampled sites. The methods used to assess strain similarities were PCR and PFGE-based genotyping approaches.

2.3 Materials and Methods

2.3.1 Ethical approval for patient recruitment

Ethical approval was obtained from the Research Ethics Committee for Wales (trial registration: Clinical Trials.Gov NCT01154257 14th June 2010) for recruitment of 50 patients. Informed and written consent was obtained from patients or relatives complying with the Mental Capacity Act 2005.

2.3.2 Patient recruitment

Patients were recruited soon after admission to the adult intensive care unit at the University Hospital of Wales (UHW), Cardiff, UK. The UHW is a major 1000-bed reference hospital, with an adult intensive care unit consisting of 33 beds. The unit is involved in the treatment of all adult patients with the exception of burns and cardiothoracic cases.

Patient inclusion criteria were the requirement that patients were aged >18 years, were mechanically ventilated with an ETT placed via the oral route, and had >20 teeth of broadly symmetric (left and right) distribution. Patients that did not meet the inclusion criteria or had thrombocytopenia (platelet count <30) or uncontrolled coagulopathy were not included, as risk of bleeding from tooth brushing, facial or oral trauma would be a consideration.

A total of 28 patients were recruited, of whom 7 withdrew because of death or hospital transfer, leaving 21 patients to complete investigations. These 21 patients included 10 males and 11 females with a mean age of 48.8 years.

2.3.3 Collection of clinical samples

Dental plaque samples were obtained for up to 7 consequitive days. Plaque was recovered from the upper and lower first molars, first bicuspid and central incisors on each side of the mouth. For patients with missing teeth, the remaining teeth in closest proximity were sampled. Plaque was collected using sterile endodontic paper points (size ISO 45; QED, UK), with one paper point used per tooth. Sampling commenced at the distal part of the buccal aspect of the tooth with 1 mm of the paper point placed into the gingival sulcus, then, with a slow and continuous motion the paper point was drawn towards operator to recover the plaque.

In addition to dental plaque, non-directed bronchial lavages (NBLs) were obtained up to twice a week (Figure 2.2). The ETT was collected when extubation was clinically indicated for biofilm assessment using microbial culture and molecular analysis.

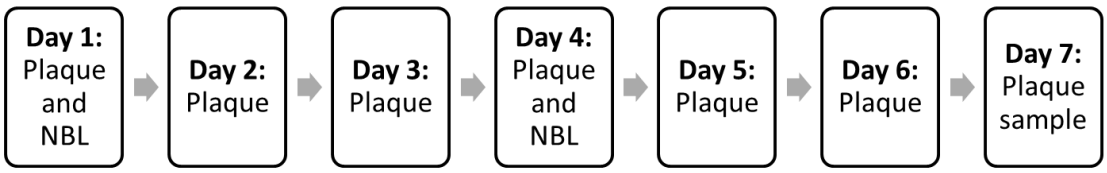

Figure 2.2 Chronology of collection of dental plaque and non-directed bronchial lavages (NBL).

2.3.4 Processing of clinical specimens

Paper points were immediately immersed in 1 ml of microbiological transport medium Reduced Transport Fluid (0.045% (w/v) K_2HPO_4, 0.045% (w/v) KH_2PO_4, 0.09% (w/v) NaCl, 0.09% (w/v) $(NH_4)_2SO_4$, 0.018% (w/v) $MgSO_4$, 0.038% (w/v) EDTA, 0.04% (w/v) $NaCO_3$, 0.02% (w/v) dithiothreitol) to protect sample integrity prior to microbial culture (Syed and Loesche 1972).

Immediately after extubation, ETTs were wrapped in sterile paper towels previously dampened with sterile saline and placed in a sterile bag. ETTs were immediately transferred to the microbiology laboratory and stored at 4°C for a maximum of 6 h until processed. A 1 cm section of from the middle of the ETT was cut and the biofilm in the lumen recovered by scraping with a sterile surgical blade. This biofilm was suspended in 1 ml of phosphate saline buffer (PBS) and subjected to microbiology and molecular analysis.

The reduced transport fluid containing plaque, the resuspended biofilm from the ETT and the NBL samples, were vortex mixed for 30 s and serially diluted in PBS, prior to a 50-µl volume being inoculated on to appropriate agar media using a spiral plating system (Don Whitley Scientific, Shipley, UK). All culture media were obtained from Lab M (Heywood, UK) unless otherwise stated. The following media were used to culture microorganisms: Blood Agar (BA) for aerobic bacteria, Fastidious Anaerobe Agar (FAA) for anaerobic bacteria, Sabouraud's Dextrose Agar (SDA) and CHROMagar® Candida (Chromagar; Paris, France) for *Candida* and yeast species, Mannitol Salt Agar (MSA) for detection of *Staphylococcus* species, Mitis Salivarius Sucrose Bacitracin agar (MSB; Difco, BD; Oxford, UK) (Schaeken *et al.* 1986) for *S. mutans* and finally, a selective agar for *Pseudomonas aeruginosa* (PsA). The inoculated media were incubated under appropriate gaseous environments at 37°C for 48 h with the exception of MSB (5 d) and FAB (7 d). After incubation, colony-forming units (CFU) on agars were enumerated. Suspected target species were provisionally identified based on

colony appearance and selected and distinct colonies stored at -80°C using microbeads (Pro-Lab Microbank®, Bromborough, UK).

2.3.5 Phenotypic analysis

Isolate identification was provisionally based on the conditions of growth, colony morphology and colour and a variety of phenotypic tests including catalase and coagulase tests. For suspected *S. aureus* isolates, meticillin resistance was determined.

2.3.5.1 Catalase test

The catalase test differentiates staphylococci (catalase positive) from streptococci. A single colony was submerged in 2 ml of 3% (v/v) hydrogen peroxide (Fisher Scientific, Loughborough, UK) and effervescence was indicative of a positive catalase reaction.

2.3.5.2 Coagulase test

The coagulase test is the gold standard to identify *S. aureus* based on coagulase (clumping factor) production. A staphylase test kit (Oxoid, Altrincham, UK) was used according to the manufacturer's instructions. Briefly, two to three colonies were mixed in a drop of distilled water and deposited in two demarked regions of the provided test card. Test and control reagents were mixed and observed for agglutination, which indicated a positive result for *S. aureus* (Appendix II).

2.3.5.3 Oxidase test

The oxidase test reveals the presence of the enzyme cytochrome oxidase, which is characteristic of *Pseudomonas* and *Neisseria* species (Gaby and Hadley 1957). Oxidase strips (Mast group Ltd, Bootle, UK) impregnated with oxidase reagent (N,N-Dimethyl-p-Phenylenediamine) and ascorbic acid were used. Isolates were cultured overnight on PsA agar and the strip placed over the grown colonies; a deep blue colour change within 10 s of was considered a positive reaction (Appendix II).

2.3.5.4 Testing for meticillin susceptibility

Meticillin resistance was tested using oxacillin strips and cefoxitin discs (Brown *et al.* 2005). For oxacillin strips, bacterial cultures were grown overnight on BA. Two colonies were subsequently streaked onto iso-sensitest™ agar (ISA; Oxoid) in parallel horizontal lines on the same plate. Positive MRSA (NCTC 12493) and negative meticillin sensitive *S. aureus* (MSSA; NCIB 9518) control strains were also tested. An oxacillin strip was aseptically placed perpendicular to the culture streaks and incubated at 30°C for 24 h (Appendix II). For the cefoxitin discs assay, cultures were grown as previously described, and colonies resuspended in sterile water to a 0.5 McFarland standard and an iso-sensitive agar (ISA) plate inoculated. Agars were dried at room temperature and a cefoxitin disc (Mast group Ltd) was placed in the centre of the plate. Up to four isolates were tested simultaneously. Agar containing a positive and negative control were concurrently tested. All agars were incubated at 30°C for 24 h (Appendix II).

2.3.6 Molecular Analysis

2.3.6.1 DNA Extraction

DNA extraction was undertaken for all isolates,ETT and NBL samples, however tipically one dental plaque sample (trial day 3 or closest) was subject to molecular analysis. The the Gentra Puregene® Yeast/Bacteria kit (Qiagen Manchester, UK) was used. Bacteria and yeast isolates were subcultured overnight in Fastidious Anaerobe Broth (FAB) and DNA extracted following either the Gram-positive or yeast protocols, as appropriate, and as detailed by the manufacturer. Where optional extended incubation times were possible, they were used at the maximum recommended time. Clinical samples previously stored at -80°C, were brought to room temperature and centrifuged at 10000 *g* for 10 min and resuspended in PBS prior the start of the DNA extraction protocol.

For NBLs and ETT biofilms that presented with high viscosity, pre-treatment with Sputasol® (Oxoid) was employed to liquidise the specimen. The sample volume was doubled using Sputasol® and incubated at 37°C with rotation at 100 rev/min for 2 h (Stuart orbital incubator SI500). In addition, portions of samples that remained viscous following Sputasol® treatment, were placed in 1.5-ml microcentrifuge tubes with 50 µl of sterile glass beads (425–600 µm in diameter, Sigma) and mixed using 30 s pulses in a mini bead beater (Stratech Scientific Ltd., Soham, UK).

2.3.6.2 Species-specific PCR

All DNA extracts were subjected to PCR using species-specific primer pairs to detect the target microorganisms (Table 2.1). All reactions were repeated on two separate occasions.

The PCR mix (25-µl final reaction volumes) was the same for all PCRs with the exception of the primer combinations. PCR mixes contained 0.5 µl of each forward and reverse primer at 50 µM, 25 µl of PCR mastermix (Promega, Southampton, UK) and DNA template (5 µl), in a total reaction volume of 50 µl. The PCR cycling conditions were different for each primer pair (targeted species) and were as follows:

- *S. aureus*: an initial 5 min at 94°C, then 35 cycles of 94°C for 40 s, 50°C for 40 s and 72°C for 1 min with a final elongation step of 72°C for 10 min.
- *S. mutans*: an initial 2 min at 95°C, then 35 cycles of 95°C for 30 s, 54.5°C for 30 s and 72°C for 1 min, and 72°C for 1 min.
- *P. aeruginosa*: an initial 5 min at 95°C followed by 35 cycles of 94°C for 45 s, 58.4°C for 45 s and 72°C for 1 min, ending with 5 min at 72°C.
- *P. gingivalis*: initial denaturation at 94°C for 3 min, then 36 cycles of 94°C for 45 s, 55°C for 30 s, 72°C for 45 s and a final elongation step of 10 min at 72°C.

PCR products were resolved by standard gel electrophoresis in 1.5% (w/v) agarose gels at 70V/cm^2 for 1 h in 0.5 × Tris-Borate-EDTA (TBE; 0.1 M Tris Base; 0.09 M Boric Acid; 0.1 mM EDTA) buffer. The resulting amplicons were stained with Safeview® (NBS biologicals; Huntingdon, UK) and visualised under UV light using a GelDoc system (Bio-Rad).

2.3.6.3 PCR identification of isolated microorganisms

For microbial isolates obtained from clinical samples by culture rDNA was amplified using universal bacterial primers targeting the 16S rDNA, and *Candida* species were identified by PCR amplification of the 5.8S rDNA region (Table 2.2). PCR volumes were 50 µl and included 1 µl of each forward and reverse primers at 50 µM, 12.5 µl of Promega PCR MasterMix® and DNA template (5 µl).

PCR cycling for bacteria comprised of an initial denaturation step at 95°C for 5 min followed by 30 cycles each of 95°C for 45 s, 60°C for 60 s, and 72°C for 1 min. The primer extension step was extended by 5 s per cycle and employed a final extension cycle of 72°C for 5 min. PCR for *Candida* comprised 30 thermal cycles of 95°C for 1 min, 55°C for 1 min and 72°C for 2 min, followed by a final extension step at 72°C for 5 min.

Negative controls of sterile DNA-free water in place of template DNA were included with each PCR, and amplicon sizes were confirmed by agarose gel electrophoresis, as described previously.

PCR products (5 µl) were initially 'cleaned' using 2 µl of ExoSAP-IT® and this mixture was incubated for 15 min at 37°C, and a further 15 min at 80°C. The cleaned products were sent with their corresponding forward primer to the Sequencing Core Unit at the School of Bioscience (Cardiff University) for automated sequencing using BigDye® Terminator v3.1 (Life Technologies) and the 3730xl DNA analyser as the platform (Eurofins, Germany).

Sequences were identified using the Basic Alignment Search Tool from the National Centre for Biotechnology Information (NCIB) for microbes http://blast.ncbi.nlm.nih.gov/Blast.cgi. A 95% identity match was used as a minimum similarity for identification.

Table 2.1 Species specifics PCR primers used for identification of isolates and detection of target species in clinical samples.

Species	Target Gene	Primers	Product size	Reference
Staphylococcus aureus	Vick	**vicK1:** 5'-CTA ATA CTG AAA GTG AGA AAC GTA-3' **vicK2:** 5'-TCC TGC ACA ATC GTA CTA AA-3'	289 bp	(Liu *et al.* 2007)
Streptococcus mutans	Sm479	**Sm479F:** 5'-TCG CGA AAA AGA TAA ACA AAC A-3' **Sm479R:** 5'-GCC CCT TCA CAG TTG GTT AG-3'	479 bp	(Chen *et al.* 2007)
Pseudomonas aeruginosa	*ecf*X	**Ps.aeru_ECF1:** 5'-ATG GAT GAG CGC TTC CGT G -3' **Ps.aeru_ECF2**: 5'-TCA TCC TTC GCC TCC CTG -3'	528 bp	(Lavenir *et al.* 2007)
Porphyromonas gingivalis	16S rRNA	**P.ging_16S-1:** 5'-AGG CAG CTT GCC ATA CTG CG-3' **P.ging_16S-2:** 5'-ACT GTT AGC AAC TAC CGA TGT-3'	404 bp	(Ashimoto *et al.* 1996)

Table 2.2 PCR primers used for amplification of microbial rDNA.

Species	Target Gene	Primers	Product size	Reference
C. albicans	5.8S	**ITS1:** -5'- TCC GTA GGT GAA CCT GCGG 3' **ITS2:** -5' – TCC TCC GCT TAT TGA TAT GC 3'	540bp	(Williams *et al.* 2001)
Bacteria	16S	**D88 :** GAGAGTTTGATYMTGGCTCAG **E94 :** GAAGGAGGTGWTCCARCCGCA	1500bp	(Paster *et al.* 2001)

2.3.6.4 Genotyping isolates

For analysing genetic similarities of the same microbial species from different clinical samples of the same patient, RAPD and PFGE were used. Isolates analysed were those that were found to be present in dental plaque and at least one other sample (ETT and/or NBL) from a given patient. Using this criterion, 36 *C. albicans*, 15 *S. aureus* and 5 *P. aeruginosa* isolates were analysed.

2.3.6.5 RAPD fingerprinting

Genomic DNA extracts were initially quantified using a Nano-Vue spectrophotometer (GE healthcare, Little Chalfont, UK) and the DNA concentration standardised for each species by diluting the DNA in nuclease free water. *Pseudomonas aeruginosa* genomic DNA extracts were used at 10 ng/µl*, C. albicans* at 20 ng/µl and *S. aureus* between 20 ng/µl and 80 ng/µl Samples from the same patient did not have a concentration difference higher than 30 ng/µl.

RAPD primers with corresponding PCR cycles were selected from published studies and validated against isolates from target and other species (Tables 2.3, 2.4).

For all primers and species, a 50-µl final reaction volume was prepared. All reagents were obtained from Promega unless stated otherwise, and PCR was performed in a G-Storm (Somertone, UK) thermal cycler. The PCR mix was prepared with 1 µl of primers, 25 µl of PCR Mastermix and DNA template (2 µl).

PCR reactions for *S. aureus* were prepared using a master mix with a higher concentration of *Taq* polymerase that had previously been successful in obtaining distinctive band patterns (Emanuel 2011). Each reaction containing 5 U of Go Taq® Flexi DNA polymerase, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 50 mM primer, 20 µl of 5× colourless GoTaq® reaction buffer, 50 mM primer and 10 µl DNA template; 50-µl final volume).

Two primers with the best band profiling for each species were chosen, and electrophoresis conditions optimised (Table 2.5). All gels were prepared using 2% (w/v) agarose (Sigma, Poole, UK) in 0.5 x TBE buffer (Sigma), which was also the running buffer. A 15-µl volume of amplicons was loaded in the gel and a commercial DNA ladder was used as reference every 5 lanes (Table 2.5). RAPD-PCR products were visualised under UV illumination in a GelDoc system (Biorad).

Banding patterns were analysed using GelCompar II software (Applied Maths, Sint-Marterns-Latem, Belgium). Gels were normalised, and cluster analysis performed using Dice's coefficient and the UPGMA method, thereby calculating a dendrogram of genetic similarity.

2.3.6.6 Pulsed Field Gel Electrophoresis (PFGE)

The PFGE instrument (CHEF-DR II®; Biorad) was set up in accordance with the manufacturer's instructions. All reagents were obtained from Sigma unless otherwise stated. Pulse field grade agarose (Biorad) was used to prepare the gels at 1% (w/v) with 0.5 x TBE buffer (Sigma). To compensate for water evaporation during the melting of the agarose, the TBE buffer/agarose mix was weighed before and after melting, and adjusted accordingly. The agarose was cooled in a water bath at 60°C for 15 min before use.

The PFGE casting gel tray and electrophoresis chamber were placed on an even surface determined with the provided level marker to avoid curved or slanted lanes. The buffer used for conducting electrophoresis was 0.5 × TBE.

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Table 2.3 Primers for DNA fingerprinting of respiratory pathogens using random amplification of polymorphic DNA (RAPD).

Species	Primer	Sequence	Primer per 50 μl	PCR Cycle	Reference
Pseudomonas aeruginosa	272	5'-AGCGGGCCAA-3'	40 pmol	4 cycles: (5 min 94°C, 5 min 36°C, 5 min 72°C) 30 cycles (1 min 94°C, 1 min 36°C, 2 min 72°C) 10 min 72°C	(Mahenthiralingam *et al.* 1996)
Staphylococcus aureus	MN45	5'-AAGACGCCGT-3'	25 μM	5 min 94°C 40 cycles (30s 94°C 40s 35°C 1 min 72°C) 7 min 72°C	(Taghi Akhi *et al.* 2008)
Staphylococcus aureus	1	5'-GGTTGGGTGAGAATTGCA-3'	50 pmol	4 min 94°C	(van Belkum *et al.* 1995)
	7	5'-GTGGATGCG-3'		35 cycles (1 min 94°C, 1 min 25°C, 2 min 74°C)	
	ERIC2	5'-AAGTAAGTGACTGGGGTGAGCG-3'			

Table 2.4 Primers for DNA fingerprinting of *Candida albicans* using random amplification of polymorphic DNA (RAPD).

Species	Primer	Sequence	Primer per 50 µl	PCR Cycle	Reference
Candida albicans	1251	5'-TGGGTGTGTGGGTGTGTGGGTGTG-3'	0.5 µM	40 cycles (1 min 94°C 2 min 52°C, 3 min 74°C) 5 min 94°C	(Bartie *et al.* 2001)
Candida albicans	1245	5'-AAG TAA GTG ACT GGG GTG AGC-3'	0.5 µM	35 cycles (1 min 94°C,1 min 25° C, 2 min 72°C) 10 min 72°C	(Bartie *et al*. 2001)
	1246	5' ATG TAA GCT CCT GGG GAT TCA C-3'	0.5 µM		
Candida albicans	JWFR	5'-GGTCCGTGTTTCAAGACG-3'	1 µM	5 cycles (30s 94°C,2 min 52°C,2min 72°C)	(Leung *et al.* 2000)
	JWFF	5'-GCATATCAATAAGCGGA-3'	1 µM	45 cycles(30 s 94°C, 2 min 57°C,2 min 72°C) 15 min 72°C	
Candida albicans	T3B	5'-AGGTCGCGGGTTCGAATCC-3'	25 pmol	5 min 95°C 45 cycles (15s 94°C,30 s 52°C, 1.2 min 72°C) 6 min 72°C	(Thanos *et al.* 1996)

Table 2.5 Random amplification of polymorphic DNA (RAPD) primers and electrophoresis conditions used.

Species	Primer	Ladder	Volts	Time	Size of gel
Candida albicans	1251	Fermentas Ready Run Super Ladder ™ Low 100 bp (Termo Fisher Scientific)	50	4 h	40 wells
Candida albicans	1245 1246	O'range Ruler™ 100 and 500 bp (Termo Fisher Scientific)	40	9 h	40 wells
Staphylococcus aureus	A1	O'range Ruler™ 100 and 500 bp	60	6 h	40 wells
Staphylococcus aureus	A7	O'range Ruler™ 100 and 500 bp	50	6 h	20 wells
Pseudomonas aeruginosa	A1	O'range Ruler™ 100 and 500 bp	60	6 h	40 wells
Pseudomonas aeruginosa	A7	O'range Ruler™ 100 and 500 bp	40	4.5 h	40 wells
Pseudomonas aeruginosa	1251	100 bp Promega ladder	40	3 h	16 wells

2.3.6.7 *Staphylococcus aureus* PFGE

The PFGE method used for *S. aureus* was as previously described (O'Brien *et al.* 2007). Briefly, isolates were cultured on MSA for 24 h at 37°C and a single colony inoculated into 5 ml of Brain Heart Infusion (BHI; BD Oxford, UK) broth. Inoculated BHI was incubated without shaking overnight at 37°C. One-ml of broth was centrifuged at 6000×*g* for 5 min and the cells washed (×2) by re-suspending in 2 ml of 50 mM EDTA followed by centrifugation. The cells were then re-suspended in 1 ml of "EC buffer" (6 mM Tris, 1 M sodium chloride, 10 mM EDTA, 0.5% (w/v) Brij 58, sodium deoxycholate and 0.5% (v/v), pH 7.5). Fifty µl of the cell suspension was then transferred to a sterile microcentrifuge tube and mixed with 50 µl of EC buffer containing 500 µg of lysostaphin and 100 µl of melted 2% (w/v) clean cut agarose (Biorad, Hertfordshire, UK). The preparation was maintained at 56°C, and the cell suspension was then mixed and transferred to disposable plug moulds (Biorad). Agarose plugs were allowed to set for a minimum of 10 min before being transferred to 24 well tissue culture plates (Starsted, Leicester, UK) with 500 µl of EC buffer. The suspended plugs were incubated at 37°C overnight for cell lysis. After incubation, the EC buffer was removed and 300 µl of "EST buffer" (5 mM Tris, 0.5 M EDTA and 1% (w/v) sarkosyl pH 7.5) and 20 µl of proteinase K (20 mg/ml) was added to the wells. This preparation was incubated overnight at 50°C to promote protein removal. The plugs were washed to remove existing buffer and 1 ml of 50 mM EDTA added. This preparation was incubated at room temperature with gentle shaking for 30 min, and the step was repeated three times. Plugs were then stored at 4°C until required. For restriction endonuclease digestion, plugs were bisected and one half of the plug transferred to a clean 1.5-ml microcentrifuge tube containing 1ml of sterile water and incubated for 30 min with gentle shaking at room temperature. The plug was then transferred to a clean 24-well tissue culture plate and 200 µl of sterile water containing 40 U of *SmaI* restriction enzyme (Promega) added, prior to overnight incubation at 25°C.

The digested plugs were gently placed into the gel wells, and a 2 mm slice of a molecular weight standard (PFGE Lambda ladder; New England Biolabs, Hertfordshire, UK) was also used every 5 to 7 lanes. The plugs and ladder were sealed in the wells using melted agarose and allowed to set for 10 min. The loaded gel was placed in the PFGE cell, and 2L of TBE buffer added. The electrophoresis settings employed were 6 V/cm^2 for 18 h with pulsing times of 1-40 s at 14°C.

2.3.6.8 *Candida albicans* PFGE

The method used was largely as previously described (Wilson *et al*., 2001) but was slightly modified in these studies. *Candida albicans* isolates were cultured on SDA agar (Lab M) at 37°C for 24 h. After incubation, a single colony was inoculated in 5 ml of YNB broth (Difco, BD, Oxford, UK) supplemented with 500 mM of sucrose (Fisher Scientific, Loughborough, UK). The inoculated broth was incubated overnight at 37°C. After incubation, cells were recovered by centrifugation (4000 rev/min; 10 min; Thermoscientific IEC CL10) and the harvested cells washed twice in 1.5 ml of 50 mM EDTA, pH 8.0. The cell pellet was resuspended in 100 µl of cell suspension buffer (1250 U/ml lyticase, 10 mM Tris, 20 mM NaCl, 50 mM EDTA; pH 8.0) and 100 µl of melted 2% (w/v) clean cut agarose (Biorad). This preparation was maintained at 56°C, and the cell suspension was mixed and transferred to disposable plug moulds (Biorad). Agarose plugs were allowed to set for a minimum of 10 min prior to transfer into a 24-well tissue culture plate (Starsted) with 500 µl of lyticase buffer (1250 U/ml lyticase, 10 mM Tris, 50 mM EDTA; pH 8.0). The tissue culture plates were sealed with Parafilm M® and incubated at 37°C for 72 h for cell lysis. The buffer was then removed and replaced with 1.5 ml of proteinase K buffer (1 mg/ml proteinase K; Promega), 0 01 M Tris, 50 mM EDTA; pH 8.0) followed by incubation at 50°C for 72 h for protein removal. Plugs were washed (×4) at 30 min intervals with 1.5 ml of wash buffer (20 mM Tris, 50 mM EDTA; pH 8.0). The plugs were then stored at 4°C until required. Agarose plugs were

divided in half, with one half being transferred to the wells of a 0.9% (w/v) agarose gel (Biorad) prepared with 0.5×TBE buffer. *Saccharomyces cerevisiae* DNA size standards (Biorad) served as molecular weight markers. The wells of the PFGE gel were sealed with melted agarose and allowed to set for 10 min. Three switching intervals were assessed (see below) to optimise resolution of subsequent banding profiles.

1. Initial switch time of 300 s at 4 V/cm^2 for 24 h, followed by switching intervals of 1000 s at 2.7 V/cm^2 for 42 h, with no final switch time.
2. Initial and final switch time of 300 s at 4 V/cm^2 for 24 h followed by an initial and final switch 1000 s switch time at 2.7 V/cm^2 for 42 h.
3. Initial and final switch time 120 s for 24 h followed by initial and final switch time 240 s for 36 h at 3.5 V/cm^2.

After electrophoresis, gels were stained using 25 µl/100 ml of Safeview® (NBS Biologicals, Cambridgeshire, UK) for 2 h. The gels were then de-stained in water for 20 min and imaged using a GelDoc system (Biorad). Gels were analysed with the GelCompar software as described in section 2.3.7.1.

2.4 Results

Dental plaque and NBLs were obtained from all 21 recruited patients. Table 2.6 presents demographic information, ITU diagnosis, days of hospital stay and days of intubation at the start of the study. It was not possible to collect ETTs from 3 patients due to hospital transfer, whilst 2 ETTs were obtained from one patient (Table 2.6) below.

Table 2.6 Number of samples of dental plaque, non-directed brochial lavages (NBL) and endotracheal tubes samples collected per patient.

Patient	Plaque	NBL	ETT
01	5	2	1
02	5	2	1
03	5	2	1
05	2	1	1
08	4	1	1
09	5	2	1
10	6	2	1
11	5	2	1
13	4	2	1
14	3	1	2
16	4	2	0
17	3	1	0
19	3	1	1
20	5	2	1
21	6	1	1
24	3	1	1
25	7	2	0
27	6	2	1
28	3	1	1
Total	**84**	**30**	**19**

Table 2.7 Characteristics of the 21 patients participating in this study.

Patient	Sex	Age	Diagnosis	Days between hospital stay and ITU	Days of intubation at time of recruitment	Antibiotic therapy at start of study
P01	M	53	Sepsis	2	2	Y
P02	F	61	Urosepsis	1	6	Y
P03	M	70	Pneumonia/ Pneumocystis Pneumonia (PCP)	4	2	Y
P05	M	43	Substance overdose/aspiration pneumonia	0	2	N
P06	F	55	Respiratory Failure	0	2	N
P07	F	37	Aneurysm	8	3	Y
P08	F	26	Ventricular fibrillation arrest postpartum	0	9	Y
P09	F	64	Respiratory failure	3	1	Y
P10	F	68	Head injury	1	0	N
P11	F	55	Urinary sepsis	4	1	Y
P13	M	52	Respiratory/renal failure	3	4	Y
P14	M	29	Head injury	0	0	N
P16	F	64	Type 2 respiratory failure	0	12	Y
P17	M	55	Respiratory failure	1	8	Y
P19	M	45	Sepsis and respiratory failure	16	7	Y
P20	F	23	Alcoholic liver disease /pneumonia	10	0	Y
P21	M	32	Cardiac arrest	0	4	N
P24	F	44	Sepsis	3	1	Y
P25	F	49	Cardiogenic shock	0	3	Y
P27	M	39	Type 1 respiratory failure	1	5	Y
P28	M	52	Septic shock	13	0	Y

2.4.1 Identification of isolates cultured from clinical samples

Isolate identification targeted 5 microbial species, namely *S. mutans, C. albicans, P. gingivalis, S. aureus* and *P. aeruginosa*. Isolates presumptively identified using differential and selective agar media were subjected to a range of further phenotypic identification tests (Appendix II). Definitive identification involved sequencing of rDNA amplicons and comparison of resulting sequences with those held within the NCIB database. Sequences with <95% identity to database sequences were not considered reliable and were repeat sequenced or not identified.

Tables 2.8 and 2.9 present a summary of the identification of cultured isolates based on phenotypic testing and sequencing. Of the target microorganisms, the most frequently isolated species was *C. albicans* with 38 isolates from 19 patients, followed by *S. aureus* with 17 isolates from 7 patients, and 5 *P. aeruginosa* was recovered from 2 patients. *Streptococcus mutans* was cultured on one occasion, and *P. gingivalis* was not cultured. Non-target species detected included non-*S. aureus Staphylococcus* species (N=11), *Prevotella intermedia* (N=5), non-*S mutans Streptococcus* species (N=4), *Escherichia coli* (N=4) and *Klebsiella pneumoniae* (N=2).

Staphylococcus aureus isolates were tested for meticillin resistance. Four of the 17 isolates were deemed as Meticillin Resistant *S. aureus* (MRSA), with 3 originating from different clinical samples from a single patient (Appendix II).

Based on the identification of cultured microorganisms, it was apparent that for 11 patients, a shared (and targeted) microbial species occurred in the dental plaque and also both the NBLs and ETTs. For 8 further patients, a shared microbial species was found in dental plaque and either the NBL or ETT.

Table 2.8 Microbial species isolated and confirmed with 16S rRNA sequencing (patients 1 to 13).

Patient	Site	Strain
P01	Plaque	*Staphylococcus haemolyticus*
		Candida albicans
		Propionobacterium propionicum
	NBL	*Candida albicans*
		Staphylococcus epidermis
	ETT	*Candida albicans*
P02	Plaque	*Candida albicans*
		Propionobacterium acnes
	NBL	*Candida albicans*
	ETT	*Candida albicans*
P03	Plaque	*Pseudomonas aeruginosa*
	NBL	*Pseudomonas aeruginosa*
	ETT	*Pseudomonas aeruginosa*
P05	Plaque	*Staphylococcus epidermis*
		Candida albicans
		Escherichia coli
	NBL	*Escherichia coli*
P06	Plaque	*Staphylococcus aureus*
		Prevotella intermedia
		Candida albicans
	NBL	*Staphylococcus aureus*
	ETT	*Staphylococcus aureus*
P07	Plaque	*Prevotella intermedia*
	NBL	*Streptococcus intermedius*
	ETT	*Candida albicans*
		Streptococcus intermedius
		Streptococcus gordonii
P08	Plaque	*Staphylococcus aureus*
		Escherichia coli
	NBL	*Staphylococcus aureus*
	ETT	*Candida albicans*
P09	Plaque	*Staphylococcus haemolyticus*
		Candida albicans
	ETT	*Staphylococcus haemolyticus*
		Candida albicans
P10	Plaque	*Candida albicans*
		Enterococcus faecalis
		Prevotella intermedia
	NBL	*Candida albicans*
		Staphylococcus aureus
	ETT	*Staphylococcus aureus*
P11	Plaque	*Candida albicans*
	NBL	*Candida albicans*
	ETT	*Candida albicans*
P13	Plaque	*Candida albicans*
	NBL	*Candida albicans*
	ETT	*Candida albicans*

Table 2.9 Microbial species isolated and confirmed with 16S rRNA sequencing (patients 14 to 28).

Patient	Site	Strain	Patient	Site	Strain
P14	Plaque	*Staphylococcus aureus*	P24	Plaque	*Pseudomonas aeruginosa*
		Candida albicans			*Candida albicans*
		Prevotella intermedia		NBL	*Pseudomonas aeruginosa*
	NBL	*Candida albicans*			*Candida albicans*
	ETT	*Candida albicans*		ETT	*Candida albicans*
P16	Plaque	*Staphylococcus haemolyticus*	P25	Plaque	*Prevotella intermedia*
		Candida albicans			*Streptococcus intermedius*
	NBL	*Staphylococcus aureus*		NBL	*Candida albicans*
P17	Plaque	*Candida albicans*	P27	Plaque	*Candida lusitaniae*
P18	Plaque	*Candida albicans*			*Staphylococcus epidermis*
		Prevotella intermedia			*Enterococcus faecium*
		Shigella sonnei		NBL	*Staphylococcus epidermis*
P19	Plaque	*Candida albicans*		ETT	*Candida albicans*
		Staphylococcus aureus			*Staphylococcus epidermis*
	ETT	*Candida albicans*			*Enterococcus faecium*
		Escherichia coli	P28	Plaque	*Candida albicans*
P20	Plaque	*Staphylococcus aureus*			*Staphylococcus aureus*
		Candida albicans			*Klebsiella pneumoniae*
	NBL	*Candida albicans*		NBL	*Klebsiella pneumoniae*
	ETT	*Candida albicans*			*Staphylococcus aureus*
P21	Plaque	*Streptococcus mutans*		ETT	*Candida albicans*
		Staphylococcus aureus			*Staphylococcus aureus*
	NBL	*Staphylococcus aureus*			*Staphylococcus lugdunensis*
	ETT	*Staphylococcus aureus*			

2.4.2 Molecular analysis

2.4.2.1 DNA Extraction

Successful extraction of bacterial DNA was confirmed by PCR targeting bacterial 16S rDNA genes (D88-E94 primers) and amplicon detection by standard gel electrophoresis. DNA extracts positive for PCR amplicons were obtained for all plaque samples, however no PCR products were obtained for DNA extracts of 13/21 NBLs (Patient numbers 1, 2, 8, 9, 10, 11, 13, 14, 16, 17, 19, 20 and 24). Similarly, no PCR products were detected in DNA extracts from 5 ETTs (Patients 2, 9, 11, 13, and 27). Additionally, 3 ETTs were not recovered from patients 16, 17, and 25. As a consequence, a total of 13 ETTs DNA extracts were available for species-specific detection using PCR.

2.4.2.2 Species-specific PCR

Bacterial DNA extracted directly from clinical samples was analysed by PCR using species-specific primers for *S. mutans, P. gingivalis, S. aureus* and *P. aeruginosa* (Figure 2.3). The identity of cultured clinical isolates was also confirmed using species-specific PCR (Figure 2.4), and this also served to validate selected primers.

Porphyromonas gingivalis amplicons were detected in 15 dental plaque samples, 6 ETTs and one NBL. Similarly, PCR products from *S. mutans* were detected in 8 dental plaque samples, one NBL and one ETT. *Staphylococcus aureus* PCR products were found in 13 dental plaque samples, 4 NBLs and 8 ETTs. PCR products from *P. aeruginosa* were not detected in any samples (Table 2.9). The summary of target species detected by culture and species-specific PCR on clinical samples is shown in Table 2.11.

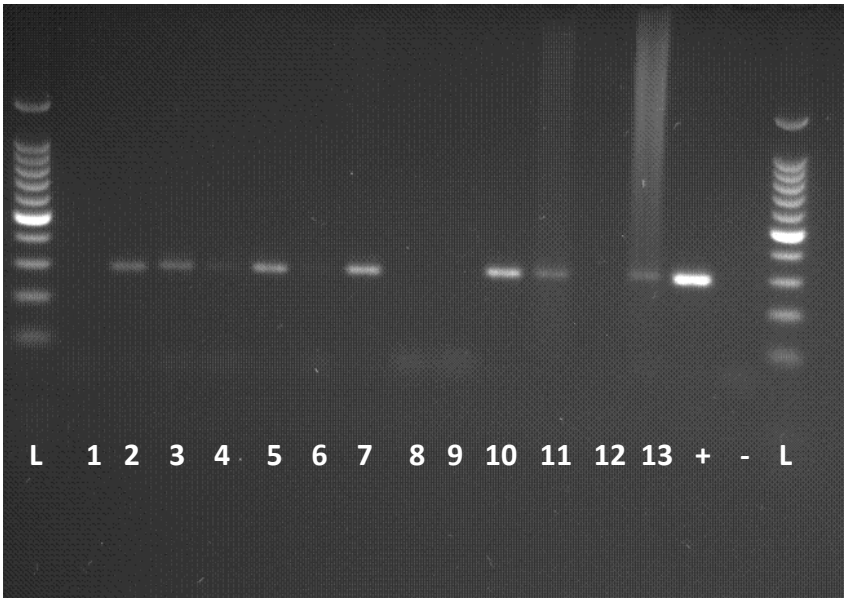


Figure 2.3 1.5% Agarose gel showing species specific PCR for detection of *Porphyromonas gingivalis*.

Lane 15: negative marker. Lane 14: Positive marker, Lanes 1 to 13: DNA extracts from dental plaque. Product size: 404 bp. Although *P. gingivalis* was not recovered by culture it was detected by PCR in the plaque, NBLs and ETTs. Target species were identified more often by PCR than by culture.

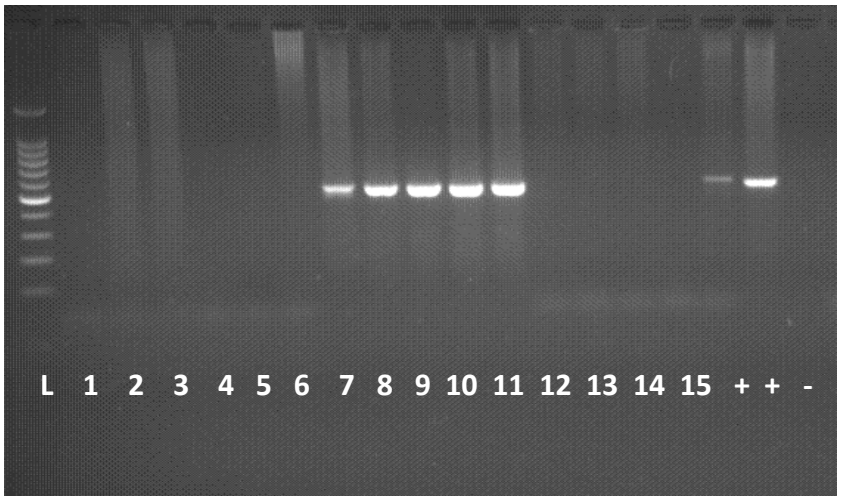


Figure 2.4 1.5% Agarose gel showing species specific PCR for detection of *Pseudomonas aeruginosa.*

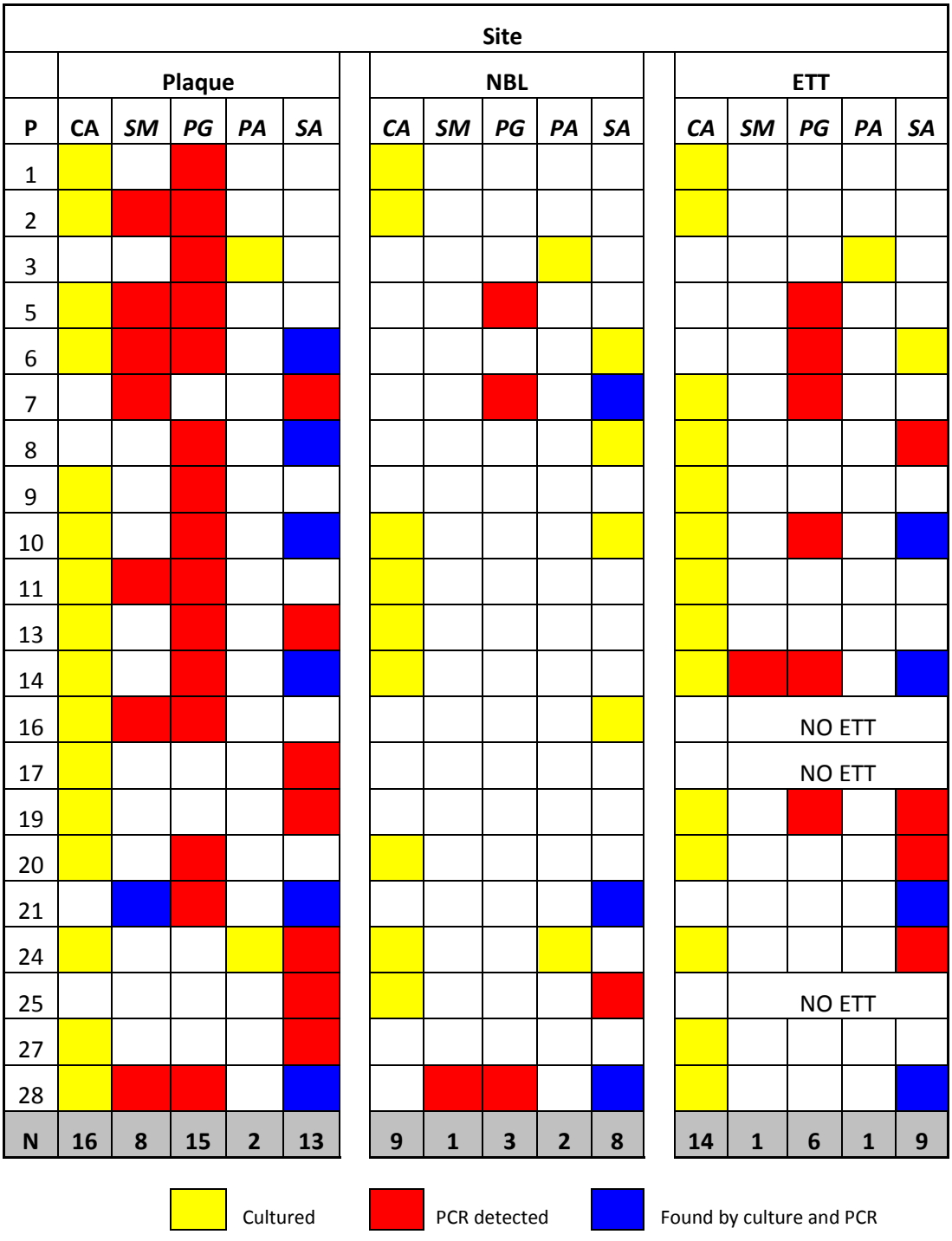
Lane 18: negative marker, Lane 16 and 17: Positive marker, Lanes 1 to 15: gDNA from clinical isolates. Product size: 528 bp.

Table 2.10 Species Specific PCR detection of target microorganisms. Non-directed bronchial lavage (NBL), Endotracheal tube (ETT), *S. mutans* (SM), P. gingivalis (PG), *P. aeruginosa* (PA) and *S. aureus* (SA).

	PLAQUE				NBL				ETT			
Patient	**SM**	**PG**	**PA**	**SA**	**SM**	**PG**	**PA**	**SA**	**SM**	**PG**	**PA**	**SA**
1		X										
2	X	X										
3		X										
5	X	X				X				X		
6	X	X		X						X		
7	X			X		X		X		X		
8		X		X								X
9		X										
10		X		X						X		X
11	X	X										
13		X		X								
14		X		X					X	X		X
16	X	X								NO ETT		
17				X						NO ETT		
19				X						X		X
20		X										X
21	X	X		X				X				X
24				X								X
25				X				X		NO ETT		
27				X								
28	X	X		X	X	X		X				
N	7	15	0	13	1	3	0	4	1	6	0	9

Table 2.11 Summary results for detection of target species by culture and PCR.

Candida albicans (*CA), Streptococcus mutans (SM), Porphyromonas gingivalis (PG), Pseudomonas aeruginosa (PA) and Staphylococcus aureus (SA)* by culture and PCR in plaque, NBLs and ETT of each patient (P).



2.4.3 Genotyping of target species isolates

2.4.3.1 Random Amplification of Polymorphic DNA (RAPD)

Combinations of RAPD primers were selected based on their ability to generate discriminatory profiles. All isolates of a target species from dental plaque and at least one of the respiratory samples (NBL or ETT) were genotyped using this approach.

Using primer 1251, RAPD profiles for *C. albicans* isolates from single patients, resulted in 8 identical matches between the dental plaque isolate and isolates from either ETTs or/and NBLs. Similarly, using the primer pair 1245-1246, 7 identical profiles between the plaque and respiratory isolates were evident (Figures 2.4 and 2.5).

Staphylococcus aureus isolates from 6 patients were analysed using either the A1 or A7 primer. In the case of primer A1, RAPD profiling revealed identical matches between all the isolates for 4 patients. In a further patient (P21), identical profiles for the isolate from the dental plaque and NBL was evident, and these differed to the profiles for the ETT isolate. The A7 primer did not produce matches for isolates from the same patients (Figure 2.7).

For genotyping the 5 *Pseudomonas aeruginosa* isolates from patients 3 and 24, the A1, A7 and 1251 primers were used. Primers initially tested for *P. aeruginosa* from the available literature failed to provide discriminatory bands for these isolates, therefore the primers originally selected for other species were used. Primer 1251 generated 2 different RAPD profiles for isolates from 2 patients with 100% identity. Primer A1 also showed identical profiles for isolates of patient 24 and for the ETT and NBL isolates of patient 3; the band profiling with this primer was less discriminatory than seen with primer 1251. Primer A7 showed identical profiles for isolates from patient 24 and >90% similarity for isolates from patient 3 (Figure 2.8).

2.4.3.2 Pulsed-field Gel Electrophoresis (electrophoretic karyotyping)

Candida albicans band profiles generated by PFGE exhibited a high level of discrimination with distinct patterns observed for majority of isolates. Following cluster analysis and dendrogram construction, identical profiles between isolates for 5 patients were evident (Figure 2.9).

Staphylococcus aureus PFGE analysis showed distinct band profiling for isolates from different patients and identical patterns for isolates obtained from individual patients. Isolates from 2 patients (patients 10 and 14) were not viable at the time of the experiments and therefore could unfortunately not be analysed (Figure 2.9).

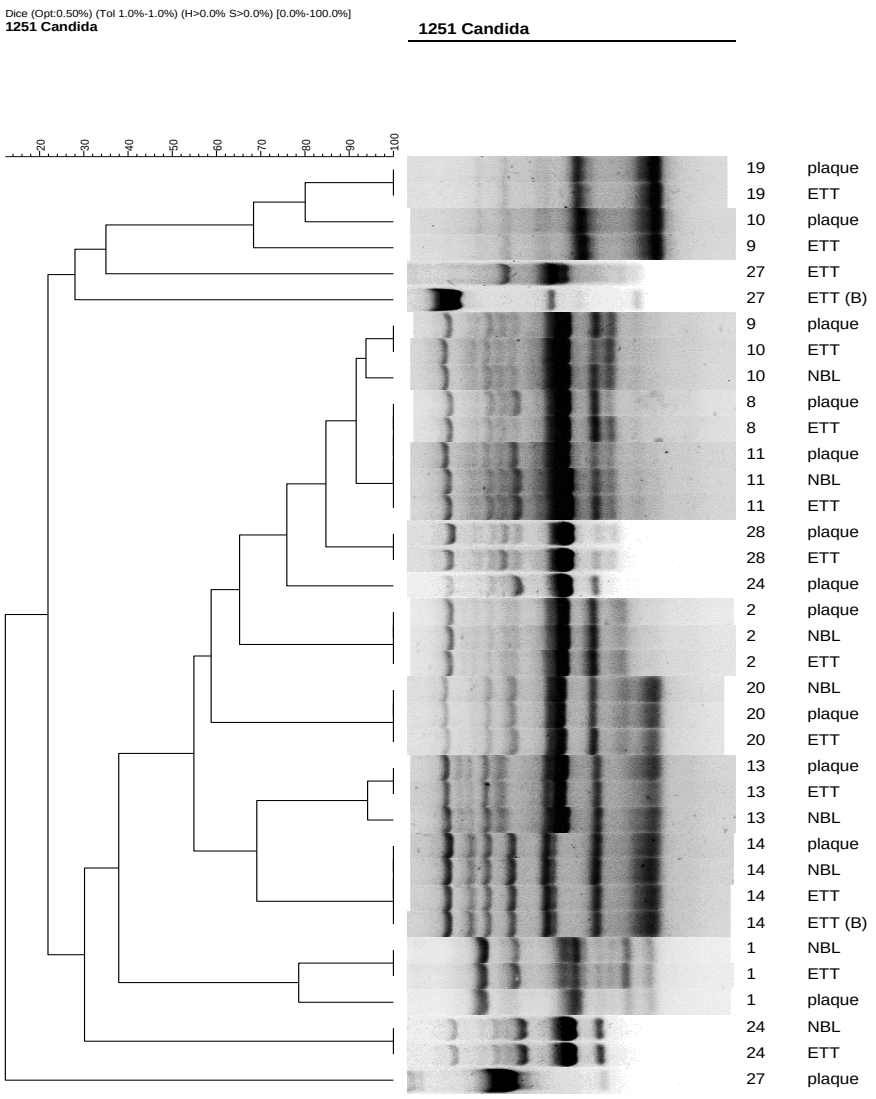


Figure 2.5 RAPD dendrogram of *Candida albicans* isolates using primer 1251.

Identical profiles from dental plaque isolates and a respiratory sample endotracheal tube (ETT) and or non-directed alveolar lavages (NBL) were detected in 8 patients (2, 8, 13, 11, 14, 19, 20).

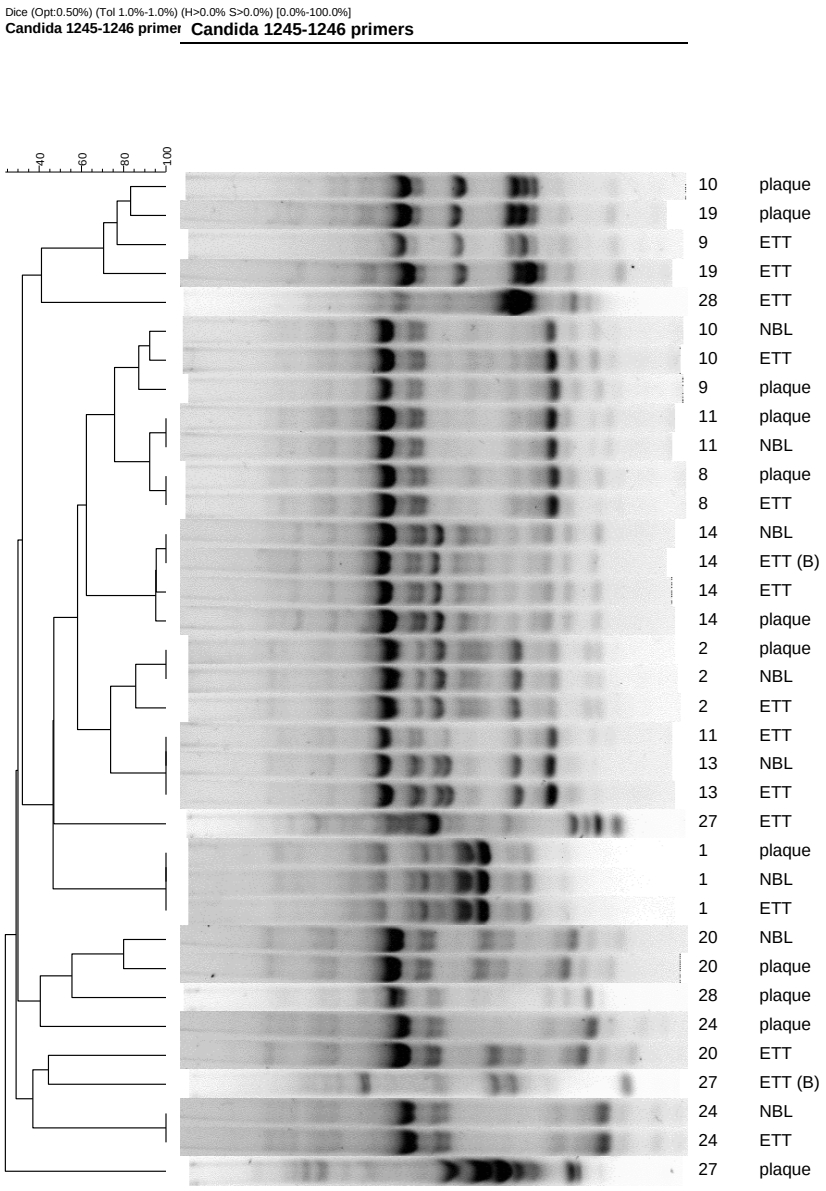
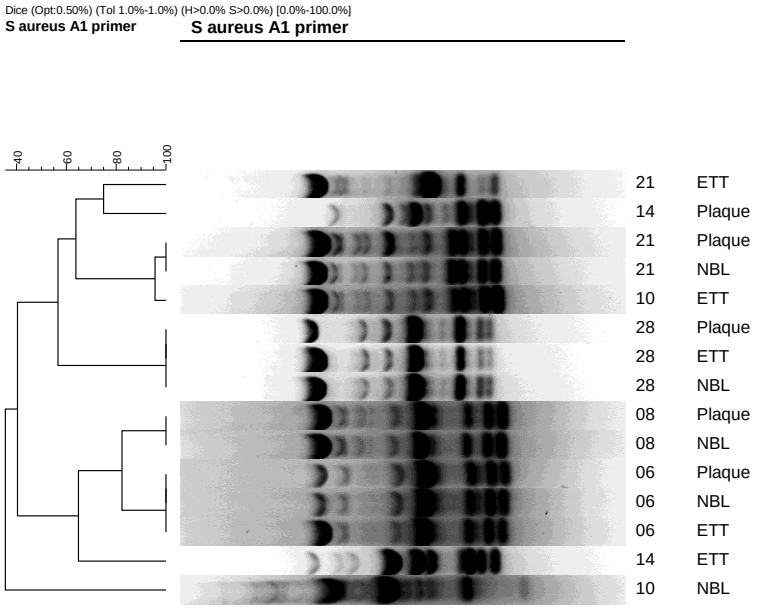


Figure 2.6 RAPD dendrogram of *Candida albicans* isolates using primer 1245-1246.

Identical profiles from dental plaque isolates and a respiratory sample endotracheal tube (ETT) and or non-directed alveolar lavages (NBL) were detected in 5 patients (Patients 1, 2, 8, 11, 14).



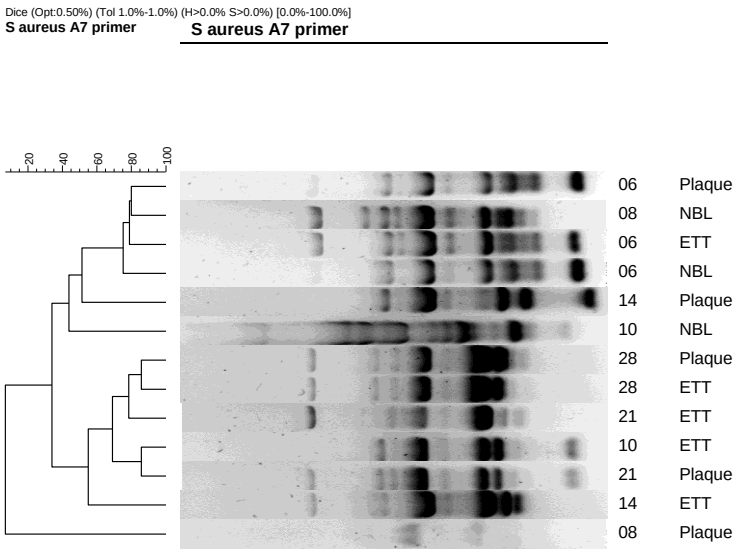


Figure 2.7 Random amplification of polymorphic DNA (RAPD) profiles for *Staphylococcus aureus* isolates RADP profiles.

Isolates from dental plaque, non-directed bronchial lavages (NBL) and endotracheal tubes (ETT). Primers A1 (top) and A7 (bottom).

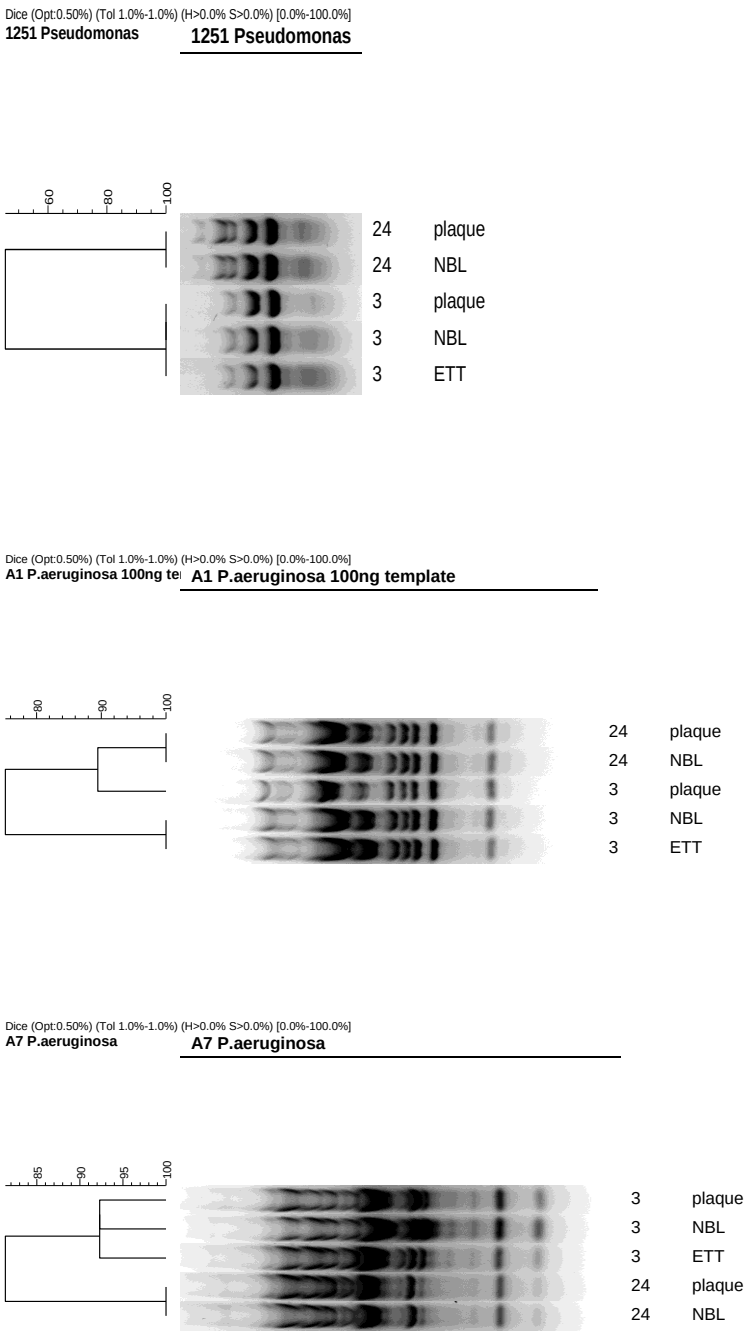


Figure 2.8 Random amplification of polymorphic DNA (RAPD) profiles for *Pseudomonas aeruginosa* isolates.

Isolates from dental plaque, non-directed bronchial lavages (NBL) and endotracheal tubes (ETT). Primers A1 (top), A7 (middle) and 1251 (bottom).

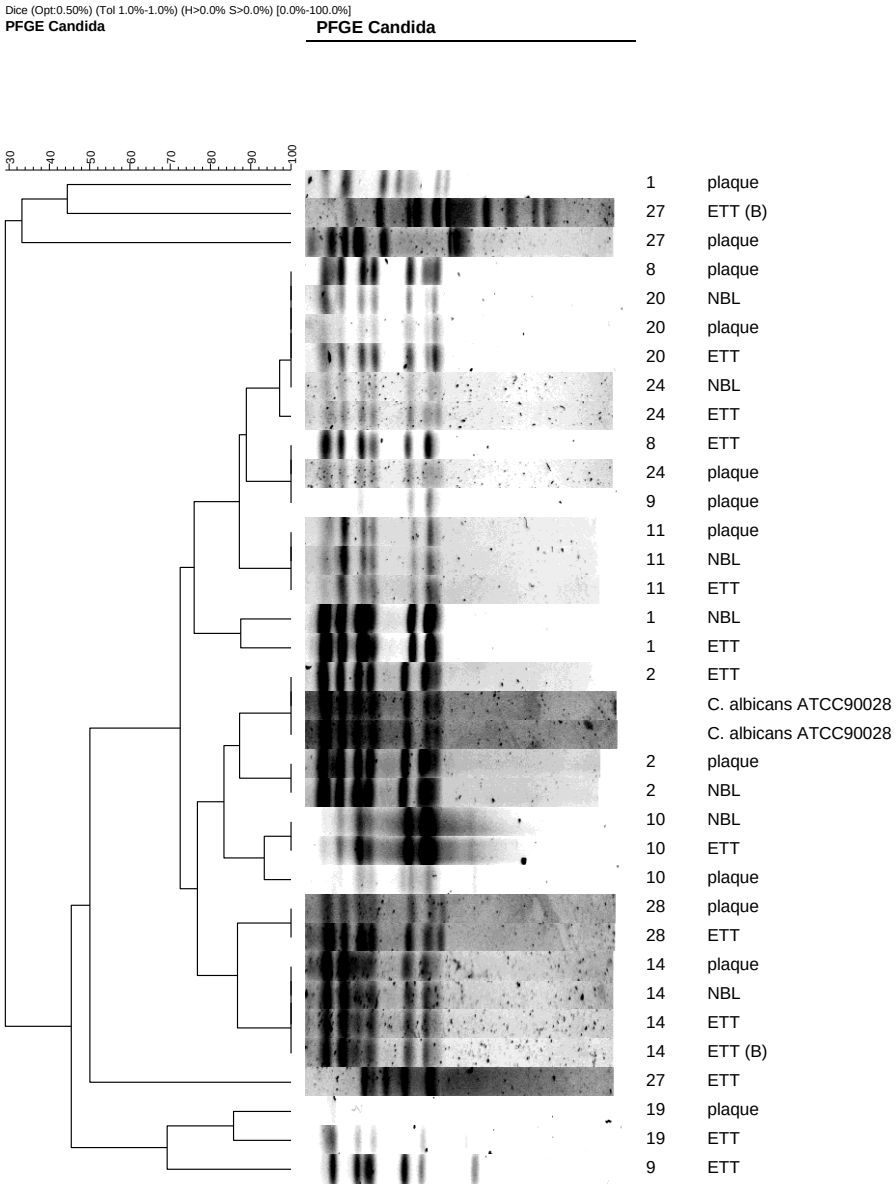


Figure 2.9 Dendogram of *Candida* isolates using pulse field gel electrophoresis.

Identical patterns from dental plaque, non-directed bronchial lavages (NBL) and/or endotracheal tubes (ETT) were evident in samples from 6 patients (2, 8, 11, 14, 20, 28).

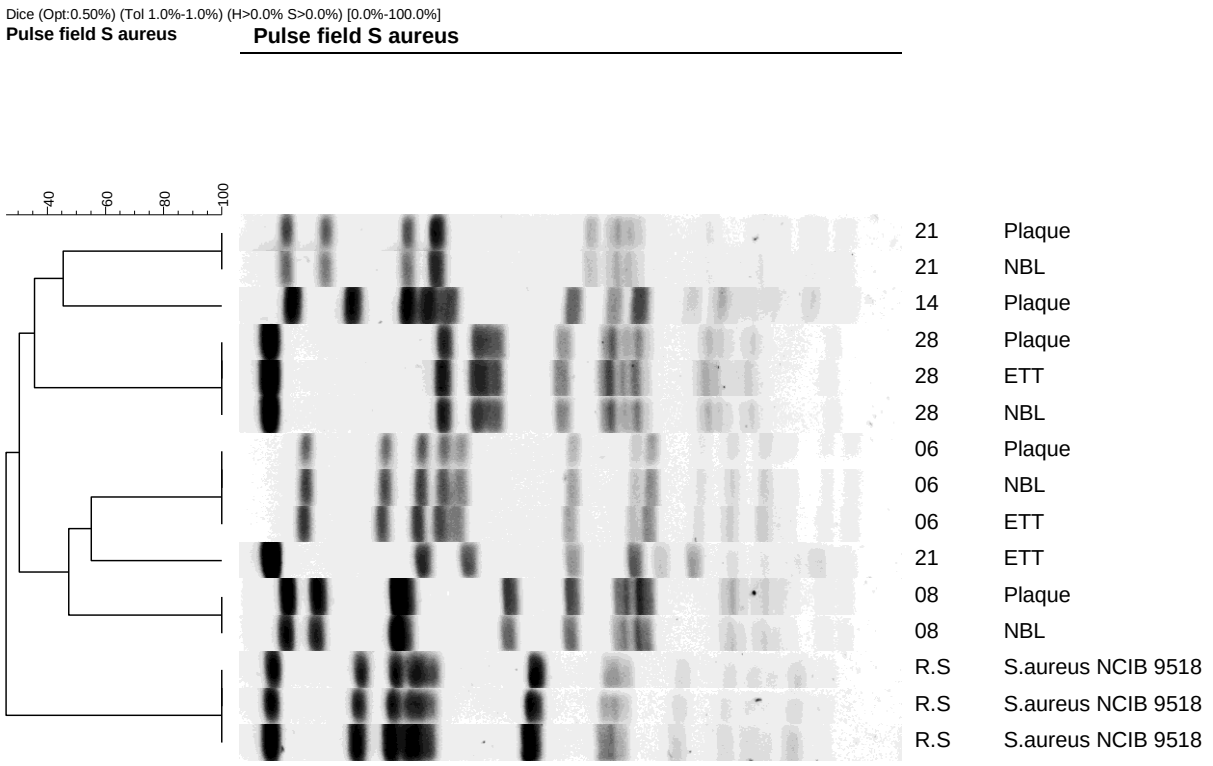


Figure 2.10 Dendrogram of *Staphylococcus aureus* isolates using pulse field gel electrophoresis.

Identical patterns from dental plaque, non-directed bronchial lavages (NBL) and/or endotracheal tubes (ETT) were evident in samples from 4 patients (6, 8, 21, 28).

2.5 Discussion

VAP is the most common nosocomial infection in the intensive care setting and its pathogenesis is still not fully understood, although multiple factors are thought to contribute (Chastre and Fagon 2002). Two of these factors namely oral colonisation and ETT biofilms, were the subject of this work.

The oropharynx has been identified as a potential source of pathogens that can cause infection elsewhere, and this is particularly important in the critically ill receiving mechanical ventilation where an ETT provides an interface between ventilator and lower airways. Unfortunately the endotracheal cuff does not provide a perfect seal to the lungs and microorganisms can transit from the oral cavity into the lower airways. Furthermore, the formation of a biofilm in the lumen of the ETTs creates a second potential reservoir of pathogens that are protected from the patient's defence system. However, there remains insufficient evidence on the route used by microorganisms to infect the lungs.

This study aimed to demonstrate the presence of key target microorganisms representative of the oral microflora (*Streptococcus mutans*, *Porphyromonas gingivalis* and *Candida albicans*) and the main respiratory pathogens (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) at three different sites in mechanically ventilated patients (*i.e.* dental plaque, ETT and NBL) and establish genetic similarities between isolates recovered. To date, this is the first time that a single study has simultaneously assessed the microbiology of these three sites.

The results from culture and PCR analyses showed shared species were present in the dental plaque and at least one more sample NBL and/or ETT. This finding supports the hypothesis that there is movement of microorganisms between these sites. In the case of respiratory pathogens, initial colonisation of the dental plaque is likely prior to translocation to sites in

the ETT and/or lower airways. It was particularly noticeable that all target species were more frequently found in dental plaque than the other samples (Table 2.11), which could add support to this hypothesis. The factors that contribute to microorganisms moving to other sites and the reasons why plaque colonisation is promoted by respiratory pathogens remain to be determined.

The most frequently isolated microorganism was *C. albicans* which was prevalent in 65% (39/60) of the samples, even though its detection was based solely on culture approaches. PCR detection was not possible to perform for this microorganism as yeast DNA extraction protocols differed to those used for bacteria making it difficult to perform both experiments on the same sample.

Candida albicans is considered part of the normal oral microflora and is found in the mouths of approximately 50 % to 75% of the healthy population (Ariyawardana *et al.* 2007; Javed *et al*. 2013). This fungal species is a well-recognised opportunistic pathogen in the oropharynx, vagina and importantly it can cause systemic candidiasis in the severely immunocompromised (Bassetti *et al.* 2006; Vincent *et al.* 1998). However whilst it does not seem to cause infection in the respiratory tract (el-Ebiary *et al.* 1997; Rello *et al.* 1998), in a multicentre study involving 803 patients bronchial *Candida* colonisation was identified as an independent risk factor for pneumonia, particularly involving *Pseudomonas* (Azoulay *et al.* 2006). This finding was later supported in a retrospective study that included 639 patients and reported poorer clinical outcomes including longer hospital stay and higher hospital mortality when *Candida* colonisation had occurred (Delisle *et al.* 2008).

Candida albicans and *Pseudomonas aeruginosa* are both adept biofilm producers and are found simultaneously in cystic fibrosis patients and in biofilms formed in medical devices (El-Azizi *et al.* 2004; McAlester *et al.* 2008). The interactions between these two microorganisms are complex. Some studies have found that *P. aeruginosa* inhibits *C. albicans* growth in the

host (Gupta *et al.* 2005; Kaleli *et al.* 2007; Kerr 1994) and *in vitro* studies report that *P. aeruginosa* alters the cell wall of *C. albicans* hyphae by adhering to the hyphae and releasing the antifungal pyocyanin, resulting in cidal effects (Brand *et al.* 2008; Kerr *et al.* 1999). Infection of *C. albicans in vitro* biofilms with *P.aeruginosa* in endotracheal tube sections resulted in a marked depletion of the *C. albicans* cells (Chapter 4, section 4.4.1). In contrast, Ader *et al*., (2011) showed that short term *Candida* colonisation of the mouse lung reduced *P. aeruginosa* load and also lung injury. Interestingly, antifungal treatment reversed this effect (Ader *et al.* 2011). Another murine model based study showed that *C. albicans* initiates alveolar innate immunity by the activation of innate lymphoid cells, macrophages, natural killer cells and dendritic cells. This induces secretion of interleukins 17 and 22 and production of antimicrobial peptides, protecting the host against subsequent *P. aeruginosa* infection (Mear *et al.* 2014). In contrast, in a burned mice model reported increased mortality when *C. albicans* infection was preceded by *P. aeruginosa*, this was thought to be due to proteolytic activity generated by the bacteria (Neely *et al.* 1986). Recently, in an acute lung injury murine model, it was reported that *P. aeruginosa* type III secretion system induced IL-18 secretion causing substantial neutrophil recruitment and host cell damage, and decreased IL-17 secretion which reduces the clearance of pathogens (Faure *et al.* 2014). In this present study, samples containing *C. albicans* were considerably more often encountered, compared with *P. aeruginosa*, but this might reflect the normal commensal colonisation of the former species.

Interestingly, the oral species *P. gingivalis* and *S. mutans* were detected by PCR in both NBL and ETT samples. *Streptococcus mutans* was only cultured from one dental plaque sample, which is lower than expected based on oral carriage rates reported as high as 70% to 93% (Li *et al.,*2005, Mortazavi and Akhlaghi 2012). However, it is noted in the literature that its incidence is closely related to the both the presence of caries and the extent of the carious

lesions (Hong and Hu 2010; O'Sullivan and Thibodeau 1996), which were not recorded in this study. It is also likely that the oral microflora of this patient group may differ significantly from the general population as there is no consumption of foods and patients exhibit a reduced salivary flow with altered salivary pH (Sands 2016). However, using PCR *S. mutans* was detected in the dental plaque of 8 patients as well as one ETTs and one NBL. It is important to remember that this approach does not distinguish between live and dead microorganisms and opens the question as to whether this species and other microorganisms are viable away from the oral cavity in mechanically-ventilated conditions. The significance of *S. mutans* colonisation of the ETT was not determined, but it is a renowned biofilm producer in the presence or absence of sucrose, it produces glucans, glucan binding proteins, antigen I/II surface proteins and wall-associated protein A which aid in attachment to the tooth surface, collagen, fibronectin and cell to cell aggregation (Chapter 1 Section 1.5.4). Indeed *S. mutans* is considered one of the pioneer microorganisms in the dental plaque biofilm (Krzyściak *et al.* 2014). It is therefore possible that its presence in the ETT could generate a 'biofilm favourable' surface for attachment of other microorganisms, including VAP causing agents.

Porphyromonas gingivalis is a particularly fastidious and strictly anaerobic species. The bacterium is more prevalent in subgingival plaque and likely to be most sensitive of the targeted species to loss of viability during transport. These reasons might explain why it was not isolated in this work although its detection was achieved by PCR. Importantly, PCR revealed the presence of *P. gingivalis* at all three sites sampled and its incidence in this study (N=6) in ETTs was similar to that reported previously (N=5) by Cairns *et al*., (2011). This is the first time this species has been reported being present within NBL specimens. In a recent study, Porto *et al.,* (2016) employed qPCR to detect periodontal pathogens in the ETTs of intensive care patients and found *Aggregatibacter actinomycetemcomitans, P. gingivalis*

and *Tannerella forsythia*. Finding periodontal pathogens in ETTs demonstrates the microbial complexity and diversity of the biofilms present at these sites. Additionally, although these organisms are not generally regarded as respiratory pathogens, there has been reported pneumonia cases caused by *A. actinomycetemcomitans* and *P. gingivalis* (Benedyk *et al.* 2016; Shilo *et al.* 2015). Furthermore, periodontal disease was identified in a metanalysis as a significant and independent factor for chronic respiratory disease (Zeng *et al.* 2012). The cytokines and enzymes induced from inflamed periodontal tissues may also relocate to the lungs and trigger local inflammatory processes and lung infections (Paju and Scannapieco 2007).

It has been widely documented that oral hygiene deteriorates during hospitalisation (Fourrier *et al*. 1998; Needleman *et al.* 2012; Sachdev *et al.* 2013; Scannapieco *et al*. 1992). Importantly, the changes in mass and complexity of the dental plaque may be conducive for colonisation by respiratory pathogens that may later translocate to the lower respiratory tract (Scannapieco 1999). Multiple culture based studies frequently find respiratory pathogens in the dental plaque of mechanically ventilated patients (26 to 65%) (Fourrier *et al.* 1998; Heo *et al*. 2008; Sachdev *et al*. 2013; Sumi *et al.* 2007). However, culture independent studies report this to be higher, Sands *et al* (2016) analysed by next generation sequencing dental plaque samples from mechanically ventilated patients at the start, during and after intubation. This study reported that during mechanical ventilation a significant 'microbial shift' occurred, 9/13 patients had >2 respiratory pathogens. Importantly, after removal of the ETT, the relative abundance of potential respiratory pathogens decreased and samples returned to a predominantly oral microbiota with a higher relative abundance of species like Prevotella spp., (Sands *et al.* 2016). In health, *P. aeruginosa* incidence has been estimated as <7% for (Morrison and Wenzel 1984; Rivas Caldas *et al.* 2015) and between 3 to 50% for *S. aureus*, however, when present is often considered to be a transient

coloniser rather than a permanent resident (Eick *et al.* 2016; Ohara-Nemoto *et al.* 2008). This contrast with the results of this study when the main respiratory pathogens associated with VAP, namely *P. aeruginosa* and *S. aureus,* were found more frequently in the dental plaque, as well as present in ETTs and NBLs of this studied patient group. To illustrate this, 66% (14/21) of the patients had one of the respiratory pathogens in the dental plaque and in 47% (10/21) of the NBLs and ETTs.

Porto *et al.,* (2016) found that higher intraoral levels of *A. actinomycetemcomitans* correlated with increased levels of this species in the ETTs. This would suggest that improved dental plaque control may promote lower microbial colonisation of ETTs. Additionally, a reduced incidence of respiratory disease in hospitalised patients has been shown to occur after implementation of oral hygiene regimes (Koeman *et al.* 2006; Mori *et al.* 2006).

One of the difficulties encountered in this work was obtaining bacterial DNA from NBL and ETT samples. Some of these samples presented with a high viscosity and were resistant to the different lysing methods applied. This may have resulted in an underestimation in the level of colonisation of the studied target species in these samples. A further issue was that in some cases where a microorganism was isolated, it was not subsequently detected by PCR. This situation occurred for all 5 *P. aeruginosa* isolates and one *S. aureus* isolates, however 3 of those samples later analysed with next generation sequencing and the species where detected then (Chapter 3).

One of the aims of this work was to demonstrate the genetic relationship between isolates found in the dental plaque, ETTs and NBL of the mechanically ventilated patient and it was found that in the majority of cases the genotype of isolates from the dental plaque was the same as from isolates from the NBLs and/or ETTs of the same patient. This was evident when both RAPD and PFGE were employed. RAPD profiles showed identical matches for 8/13 *C. albicans* from colonised patients*,* 4/6 *S. aureus* from colonised patients and 2/2 *P.*

aeruginosa from colonised patients. A limitation of RAPD is that being an enzymatic reaction results may vary depending on the quality and concentration of template DNA, concentrations of PCR components, and the PCR cycling conditions, for these reasons its results are often considered laboratory dependant (Mbwana *et al.* 2006; Williams *et al.* 1990). PFGE was been found to have more discriminatory power e and be more reproducible than RAPD (Werner *et al.* 2003) and for this reason was chosen to complement the results found by RAPD. In this study, PFGE showed the same results as encountered for *S. aureus*, the matches for *C albicans* strains were 5/13. No PFGE was performed for *P. aeruginosa* as it was considered that with isolates from only 2 patients the resources required were not justifiable. The results from this study supported the hypothesis that dental plaque served as reservoir for respiratory pathogens in this patient group.

Previous reports have found similar results using different molecular techniques (Bahrani-Mougeot *et al*. 2007; El-Solh *et al.* 2004; Heo *et al*. 2008; Heo *et al.* 2011). El-Solh *et al.,* (2016) used PFGE to analyse isolates from of bronchoalveolar fluid and dental plaque from 49 patients, including *S. aureus*, enteric Gram- negative bacilli and *P. aeruginosa* and found 9/13 isolates were identical (El-Solh *et al*. 2004). Recently, Heo *et al.,* (2008) used PFGE and MSLT to analyse respiratory pathogens from 100 intensive care patients. Targeted isolates were *S. aureus, P. aeruginosa, E. coli* and other Gram-negative bacilli, and in 18 patients the same strains was isolated from all three clinical sites (oral, tracheal secretions, and bronchoalveolar lavage). This same study found that not only did the isolates from the same patient have the same genetic profile, but also the same clone of *P. aeruginosa* was shared between 3/6 patients, indicating that its source was a common environmental one (Heo *et al.* 2008) in this study this was observed for *C. albicans* isolates in 2/14 patients (Figure 2.9). *Candida albicans* colonisation was also studied for the same cohort of patients and it was found that over 60% of patients had this fungus at least one site, 14 patients were colonised

in at least 2 sites and identical genetic profiles were determined for 12 patients (Heo *et al.* 2011).

All of these results clearly suggest that the route of colonisation to the lower airway follows mouth-trachea/ETT biofilm-lung, highlighting the importance of oral care in order to minimize colonisation of the dental plaque by potential pathogens endogenous to critically ill patients.

2.6 Conclusions

- The most frequent microorganism isolated for this cohort of patients was *C. albicans*, the oral bacteria *S. mutans* and *P. gingivalis* were found primarily by PCR in the dental plaque, as well as from ETT biofilms and NBLs
- The respiratory pathogens *S. aureus* and *P. aeruginosa* were found in the dental plaque as well as ETT biofilms and NBLs; it was also noted that these microorganisms were more frequently found in the dental plaque.
- When isolates of the same species were isolated from dental plaque and at one other site they were often found to have an identical genetic profiles by RAPD and PFGE.

3. Biofilm community profiling using metataxonomics

3.1 Introduction

The oral cavity has been implicated as a source of respiratory pathogens that can lead to pneumonias, including ventilator-associated pneumonia (VAP) (Garrouste-Orgeas *et al.* 1997; Scannapieco 1999). As previously mentioned (Chapter 1; Section 1.3.3), VAP is the most common nosocomial infection in intensive care settings and it has been linked with high patient mortality (up to 69%), extended hospital stay (5 to 7 days) and increased economic costs (£22000 per case). As such, in order to help formulate prevention strategies it is important to understand the contributory factors associated with VAP.

A primary contributory factor for VAP is the placement of an endotracheal tube (ETT), which is an essential medical device through which gaseous exchange occurs in mechanically ventilated patients (Hamilton and Grap 2012). However, the ETT will inadvertently impede certain natural host defence processes normally displayed by the respiratory tract, including cough reflexes and mucocilliary clearance. The necessity to create a seal between the ETT and the trachea using an inflated cuff means that pooling of microbial laden secretions invariably occurs above the cuff (Blot *et al.* 2014; Rello *et al.* 1996). These secretions may subsequently aspirate to the lower airway via microchannels in the cuff material (Figure 2.1), and the microorganisms in these secretions may then directly cause VAP or be drawn into the ETT lumen were they develop as biofilms (Brennan *et al.* 2004; Rodrigues *et al.* 2009; Rumbak 2005). Such biofilm formation on ETT surfaces has been known for a number of years (Inglis *et al.* 1989; Lee *et al.* 2012; Vandecandelaere *et al.* 2012) and these biofilms have been shown to harbour both respiratory pathogens and microorganisms normally associated with the oral cavity (Cairns *et al.* 2011; Perkins *et al.* 2010; Vandecandelaere *et al.* 2012). The presence of oral microorganisms in the ETT could be an important component in facilitating colonisation by respiratory pathogens in the ETT biofilm by providing

adherence sites and favourable conditions for attachment. Importantly the ETT biofilms are protected from the host immune system and administered antibiotic therapy.

Dental plaque itself is naturally diverse and dynamic in terms of its microbial constituents, and typically contains between 500 and 700 bacterial species (Dewhirst *et al.* 2010). An estimated 96% of these species belong to the phyla *Firmicutes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes*, and *Fusobacteria*. The remaining 4% of taxa are formed by *Euryarchaeota, Chlamydia, Chloroflexi,* SR1*, Synergistetes, Tenericutes,* and TM7 (Dewhirst *et al*. 2010). *Streptococcus* species are the most abundant in the mouth, followed by *Lactobacillus*. Dynamic changes in the microbial ecology of dental plaque arises following receipt of various medications, an altered diet, or due to the presence of an underlying systemic condition (Adler *et al.* 2013; Marsh 2006; Szymanska *et al.* 2014). Respiratory pathogens are not normally present in the oral cavity, although they have been found in the plaque of some healthy adults (*S. aureus* 3% to 30%, *P. aeruginosa* 6%) (Eick *et al.* 2016; Lister *et al.* 2009), where they are likely to be transient rather than endogenous colonisers. Importantly, however, a number of studies into the dental plaque of hospitalised and institutionalised patients have suggested that during mechanical ventilation, dental plaque can rapidly become colonised by respiratory pathogens (Fourrier *et al.* 1998; Sachdev *et al.* 2013; Scannapieco *et al.* 1992). Sands *et al.* (2016) collected dental plaque at the start, during and after mechanical ventilation with an endotracheal tube. The authors described a microbial "shift" that occurred during mechanical ventilation towards a plaque that contained respiratory pathogens including *P. aeruginosa* and *S. aureus*. Importantly after extubation the dental plaque of the majority of patients returned to one that was dominated by traditionally accepted oral species, and the respiratory pathogens were reduced in prevalence and abundance (Sands *et al.* 2016). The reasons why mechanical ventilation may induce such microbial changes remain unclear, but could relate to difficulties in maintaining

adequate oral hygiene in these patients, a reduced salivary flow, or be a feature of underlying disease and associated treatments. Regardless, the colonisation of the mouth by respiratory pathogens would likely represent a significant risk factor for subsequent VAP.

Most evidence to date for oral microbial involvement in VAP has been based on culture methods, which have also been used to analyse bronchial lavages, protected brush specimens or non-directed bronchial lavages (NBLs) in VAP diagnosis. In a suspected VAP patient, culture analysis tends to initially focus on microorganisms most frequently associated with VAP including *Staphylococcus aureus*, certain *Streptococcus* species, *Pseudomonas aeruginosa*, Enterobacteriaceae, *Haemophilus* and *Acinetobacter* species (Chastre and Fagon 2002). Whilst microbial culture permits characterisation of phenotypic traits for individual isolates (including antibiotic susceptibility), a significant proportion of bacteria remain unculturable using standard microbiological methods. For example, in environmental samples, it has been estimated that over 90% of bacteria cannot be grown *in vitro* (Wade 2002) and even though dental plaque is one of the most studied biofilms, it is estimated that over half of its microbial community is unculturable (Paster *et al.* 2001). Therefore, the microbial composition of such communities cannot be fully determined without use of culture-independent molecular techniques.

Given the increased accessibility of culture-independent methods, including next generation sequencing (NGS), a more comprehensive characterisation of dental plaque and clinical samples is now possible (Xie *et al.* 2010). NGS was introduced commercially in 2005 with the Genome Analyser, where a single sequencing run produced one gigabase of data (Mardis 2008). By 2014, the output of NGS increased to 1.8 terabases using the latest platform HiSeqX Ten (Illumina). The output increase has also had an inverse proportional effect on the cost of sequencing, making it much more accessible for small research projects. The high

efficiency of this technology has transformed our ability to determine the microbial diversity of complex microbial communities including those of biofilms (Oulas *et al.* 2015).

In recent years, molecular approaches have been developed that combine the sensitivity of polymerase chain reaction (PCR) with the specificity of sequencing to gain detailed information and understanding of the interactions between microbial species in particular communities. Microbiomics is a collective term, which encompasses the molecular tools available to achieve profiling of microbial communities, including uncultivable components (Culligan *et al.* 2013). One aspect of this toolkit uses inventories of 16S rRNA genes to provide a "snapshot" of bacterial diversity and relative abundance within a sample (Gee *et al.* 2004; Ziesemer *et al.* 2015). NGS differs from traditional Sanger sequencing as it is based on spatially separated, clonally amplified DNA templates or single DNA molecules on a flow cell where the process is extended across millions of fragments. Similar to previous technologies, a DNA polymerase (*i.e*. *Bst*) catalyses fluorescently labelled dNTPs into a DNA template strand during a series of PCR cycles and in each cycle, the incorporated nucleotides are identified by fluorophore excitation (Nakazato *et al.* 2013). NGS employs adapter sequences that allow for selective amplification by PCR, and this eliminates the need for bacterial cloning to achieve amplification of genomic fragments, in fact for some platforms (Helicos and Pacific Biosystems) the amplification of DNA fragments is not required before sequencing (Mardis 2008). Figure 3.1 explains the Illumina platform used in this work.

This research has been partially published in:

Marino P J, Wise M P, Smith A, Marchesi J R, Riggio M P Lewis M A O, Williams D W. Community analysis of dental plaque and endotracheal tube biofilms from mechanically ventilated patients. *Journal of Critical Care.* 2017 (*in press*).

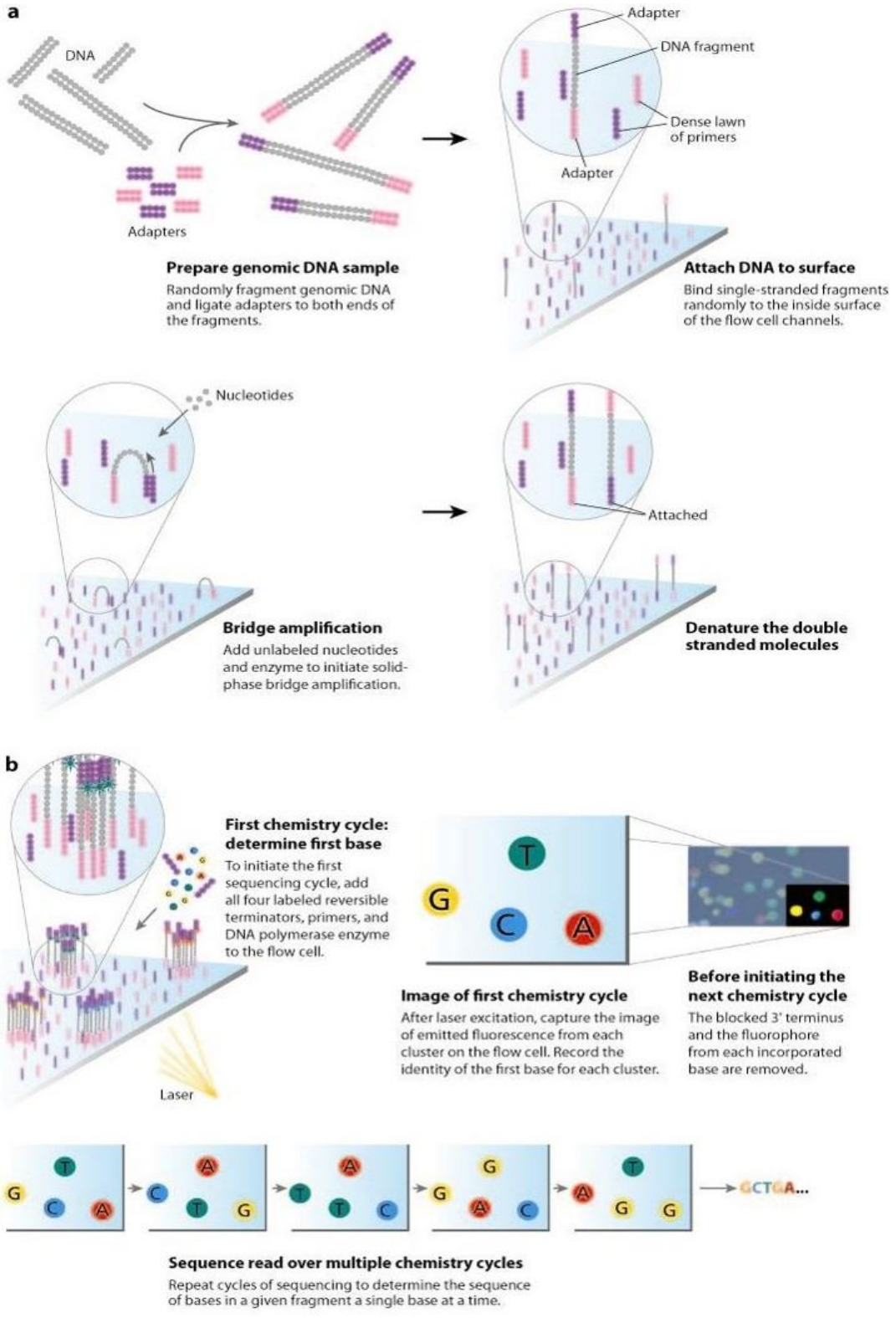


Figure 3.1. The Illumina sequencing-by-synthesis approach. Reproduced with permission from Mardis (2008).

3.2 Objectives

In this experimental chapter, for the first time the microbiota of dental plaque, ETT biofilms and NBLs from mechanically ventilated patients is characterised using metataxonomics. These sites could potentially be related, similarities in the microbiomes of these three sites could demonstrate that dental plaque plays an important role in the colonisation of the lower airways by respiratory pathogens and in the development of biofilm in the lumen of ETTs

3.3 Materials and Methods

3.3.1 Collection and processing of clinical specimens

Ethical approval for collection of clinical samples was obtained from the Research Ethics Committee for Wales (Reference # 08044240; Appendix I). A total of 21 patients were recruited (Trial registration: Clinical Trials.Gov NCT01154257 14th June 2010) for a trial that compared toothbrushes and foam swabs as methods of delivering oral care for mechanically ventilated patients (Chapter 5) and following receipt of informed and written consent from patients or relatives complying with the Mental Capacity Act 2005, these patients provided dental plaque, NBLs and ETT samples for analyses.

Inclusion criteria were that patients had to be aged >18 years, had >20 teeth, and had an expectation of mechanical ventilation with an ETT for >24 h. Samples from 12 patients (7 male and 5 female, aged 23 to 70 years old; Table 3.1) were selected for NGS. Patients were recruited for the study for a minimum of 2 days and a maximum of 7 days. At the time of recruitment, the typically prescribed antibiotics were tazocin or meropenem to cover both Gram-positive and Gram-negative bacteria.

Dental plaque was obtained from the upper and lower first molars, first bicuspid and central incisors on each side of the mouth using sterile endodontic paper points (size ISO45).

Sampling was initiated at the distal part of the buccal aspect of the tooth with 1 mm of paper point placed into the gingival sulcus. Using a slow and continuous motion, the paper point collected dental plaque by being drawn towards the operator. Paper points were placed in 1 ml of microbiological transport medium (Reduced Transport Fluid) (Loesche *et al.* 1972) prior to processing for DNA extraction.

NBL specimens were obtained twice weekly from patients as part of routine screening at the time of the study. This involved insertion of a suction catheter with a 20-mL syringe of saline attached into the lung via the ETT until resistance was met. Saline was instilled and slowly withdrawn immediately. The ETT itself was recovered for analysis after extubation. The ETT was wrapped in a sterile paper towel damped with sterile saline solution and sealed; all the samples were processed within 4 h. The central part of the ETT was cut to provide a 1 cm section from which the biofilm was recovered by scraping with a sterile surgical blade. Recovered biofilm was resuspended in 1 ml of phosphate saline buffer (PBS). All samples were stored at -80°C prior to DNA extraction.

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Table 3.1 Demographics of the 12 patients participating in this study

Patient	Sex	Age	Diagnosis	Days between hospital stay and ITU admission	Days of intubation at time of recruitment	Antibiotic therapy at start of study
P01	M	53	Sepsis	2	2	Y
P03	M	70	Pneumonia/ Pneumocystis Pneumonia (PCP)	4	2	Y
P05	M	43	Substance overdose/ Aspiration pneumonia	0	2	N
P08	F	26	Ventricular fibrillation arrest postpartum	0	9	Y
P10	F	68	Head injury	0	0	N
P14	M	29	Head injury	0	0	N
P20	F	23	Alcoholic liver disease and pneumonia	10	0	Y
P21	M	32	Cardiac arrest	0	4	N
P24	F	44	Sepsis	3	1	Y
P25	F	49	Cardiogenic shock	0	3	Y
P27	M	39	Type 1 respiratory failure/Pneumonia	1	5	Y
P28	M	52	Septic shock	13	0	Y

The initial reason for mechanical ventilation in 7 patients (5, 8, 10, 14, 21, 24, 25) was for a reduced conscious level, and in the other 5 patients, it was for respiratory failure as a result of pneumonia (3, 20, 27) or extrapulmonary sepsis (1, 28). F indicates female; M, male; Y, yes; N, no

3.3.2 DNA extraction

DNA was extracted from the samples using the Gentra Puregene® Yeast/Bacteria kit employing the Gram-positive bacterial protocol as described by the manufacturer (Qiagen, Manchester, UK). Pre-treatment of highly viscous NBLs and ETT biofilms involved addition of an equal volume of Sputasol® (Oxoid, Altrincham, UK) to the specimen, which was incubated at 37°C whilst being rotated at 100 rev/min for 2 h (Stuart orbital incubator SI500). Samples that remained viscous following Sputasol® treatment had 50 µl sterile glass beads (425–600 µm in diameter, Sigma) added and were homogenised for 30 s in a mini bead beater (Stratech Scientific, Newmarket, UK) before proceeding with the DNA extraction protocol. Purified DNA was stabilised in a DNA eluting solution (Qiagen).

DNA extraction was confirmed by PCR of the bacterial 16S rRNA gene using the bacterial primers pair of D88 GAGAGTTTGATYMTGGCTCAG and E94 GAAGGAGGTGWTCCARCCGCA (Paster *et al.* 2001). For 25 µl reactions, the PCR mix contained 12.5 µl of PCR master mix (Promega), 1 µl of DNA template, and 0.5 µl of each forward and reverse primers at 50 µM. PCR thermal cycling parameters were an initial denaturation step of 95°C for 1 min, followed by 26 cycles of 94°C for 45 s, 50°C for 45 s and 72°C for 90 s (Thermocycler G-Storm, Somertone, UK). Amplicons were visualised by standard gel electrophoresis as described earlier (Chapter 2 section 2.3.6.2).

3.3.3 MiSeq sequencing

Sequencing of targeted bacterial 16S rRNA gene regions, was undertaken by Research and Testing Laboratory (Austin, USA) using the Illumina MiSeq. Bacterial primers (28F; GAGTTTGATCNTGGCTCAG and 388R; TGCTGCCTCCCGTAGGAGT). Sequences of approximately 250 base pairs, overlapping at the V4 region of the 16S rRNA gene were generated.

3.3.4 Phylogenetic identification and data analysis

The 16S rRNA gene sequences were analysed using the bioinformatics software package Mothur (Kozich, 2013) and the MiSeq SOP Pipeline. 16S rRNA gene sequence reads were quality checked and normalised to the lowest number of reads in Mothur. To maintain normalisation and minimise artefacts, singletons and any Operational Taxonomic Units (OTUs), which were not found on more than 10 occasions in any sample were collated as OTU singletons and OTU_rare phylotypes. Using the Vegan package of the R statistical package (R Development Core Team, 2008), analysis was performed on the datasets contained within the files generated by Mothur (all OTUs were defined using a cut off value of 97%). The Unifrac weighted distance matrix was analysed in R using non-metric multidimensional scaling (NMDS) ordination and the shared OTU file was used to determine the number of times that an OTU was observed in multiple samples, and was used for multivariate analysis in R. OTU taxonomies (from phylum to genus) were determined using the RDP MultiClassifier script to generate the RDP taxonomy (Wang *et al.* 2007). Alpha and beta indices were calculated from these datasets with Mothur and R using the Vegan package.

3.4 Results

From the 12 participating patients, 34 samples were obtained (Table 3.2) with one dental plaque sample from each patient, 12 ETTs from 10 patients and 10 NBLs from 7 patients. There were samples from all three sites for 5 patients (P05, P10, P14, P20 and P21). The raw number of sequence reads was 2248956 and this was subsampled down to 9385 per sample.

The number of OTUs was 127 for plaque, 125 for ETTs, and 83 for NBLs

3.4.1 Evaluation of diversity microbiome diversity between samples sites

Chao, Shannon, and analysis of variance (ANOVA) were used to measure similarities in diversity of the whole microbiome of dental plaque, NBLs and ETTs. Analyses revealed no significant differences in the microbiomes at the three different sites (Shannon P=0.306, Chao P=0.685; (Figure 3.2). Although pairwise comparisons (P values adjusted using the Bonferroni correction) did show a statistical difference between the microbial communities of NBL samples compared with dental plaque (P=0.003) and ETTs (P=0.027). No significant difference was evident between ETT and dental plaque biofilm communities. NMDS was used to visualise the position of each sample's community in a multidimensional space and showed overlaps between the microbial communities of dental plaque, NBLs and ETTs (Figure 3.3).

Table 3.2 Number of samples and number of days of mechanical ventilation at time of collection.

Patient code	Number of samples		
	Plaque	NBL	ETT
P01	1 (6)	0	1 (10)
P03	1 (7)	2 (3,6)	0
P05	1 (4)	1 (3)	1 (5)
P08	1 (12)	0	2 (13)
P10	1 (4)	1 (3)	1 (11)
P14	1 (3)	1 (1)	2 (2,4)
P20	1 (3)	2 (1)	1 (8)
P21	1 (6)	1(6)	1 (12)
P24	1 (4)	0	1 (6)
P25	1 (6)	0	1 (12)
P27	1(8)	2 (7)	0
P28	1 (2)	0	1 (3)

Number in parenthesis indicates days of mechanical ventilation at time of sample collection, NBL indicates non directed bronchial lavage, ETT indicates endotracheal tube.

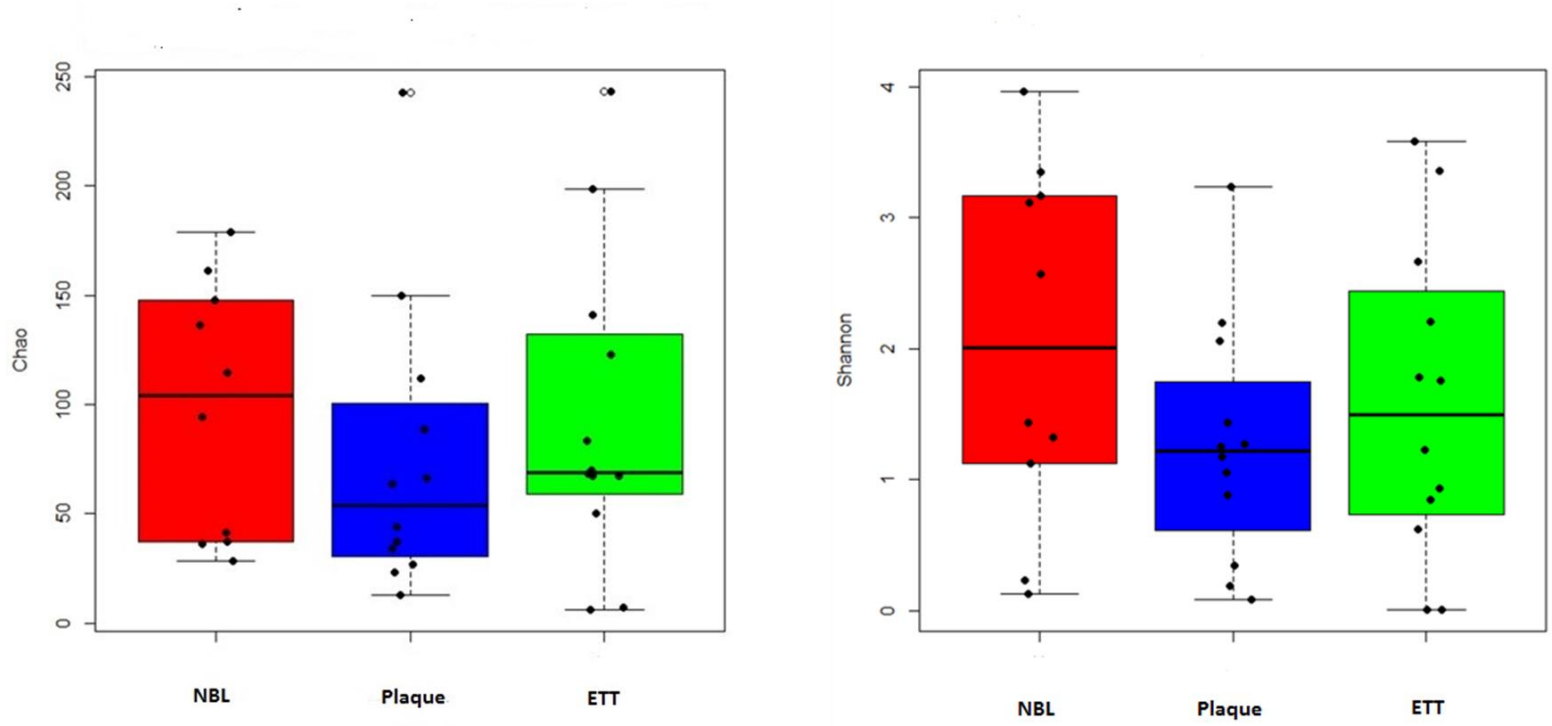

Figure 3.2 Diversity analysis for non-directed bronchial lavages (NBL), dental plaque and endotracheal tubes (ETT) using Chao and Shannon analyses.

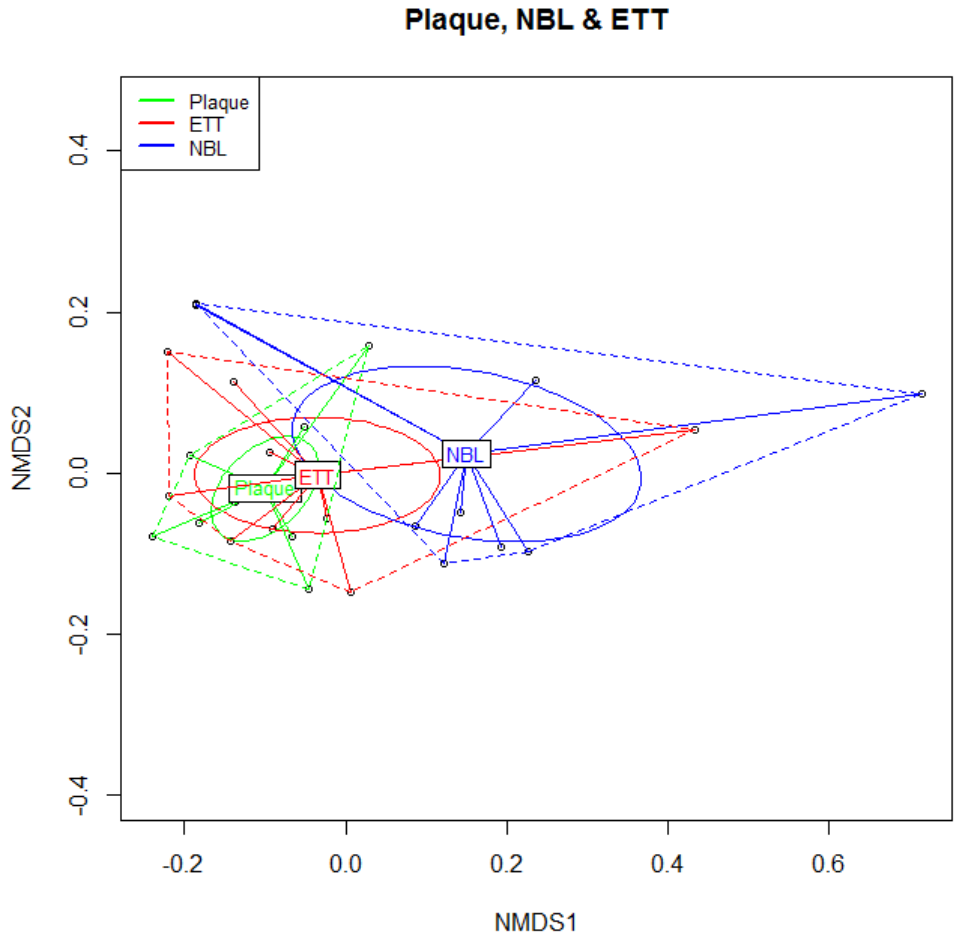

Figure 3.3 Nonmetric multidimensional scaling analysis illustrating the position of microbial communities of dental plaque, non-directed bronchial lavages (NBL) and endotracheal tube biofilms (ETT).

3.4.3 Similarities between samples

Similarities between samples were analysed using the Bray-Curtis index. A constructed dendrogram (Figure 3.4) showed identification of four major sample clusters, and each cluster contained dental plaque, ETT and/or NBL samples. There were eight sub-clusters of four pairs, where the microbial composition of dental plaque and either NBL or ETT from the same patient was indistinguishable (P28, P03, P24, P27). Moreover, the same level of similarity was observed for samples from different patients on 7 occasions, forming pairs between dental plaque and NBLs (N=2), dental plaque and ETT (N=1), ETT and NBL (N=1), and grouping same site samples from different patients NBLs (N=2) and ETT (N=1). One cluster of three 'identical' dental plaque samples (P10, P14, P20) and a further 4 pairs of closely related samples from the same patient were apparent. Interestingly, samples from all three sites for patients 21, 10 and 14 appeared distantly related.

3.4.4 Bacterial composition

Analysis of the most representative species was based on the 100 most abundant species, with 58.5% of species identified at a cut off value of 97%. Analyses of the first 20 species showed that overall, the most commonly detected species was *S. aureus,* followed by *P. aeruginosa, S. pneumoniae* and *H. influenzae.* Importantly all these species are potential respiratory pathogens. This group was followed with species normally associated with the urinary (*Enterococcus hirae*) and gastrointestinal (*Shigella dysenteriae*) tracts. The remaining 13 species were typical oral microorganisms.

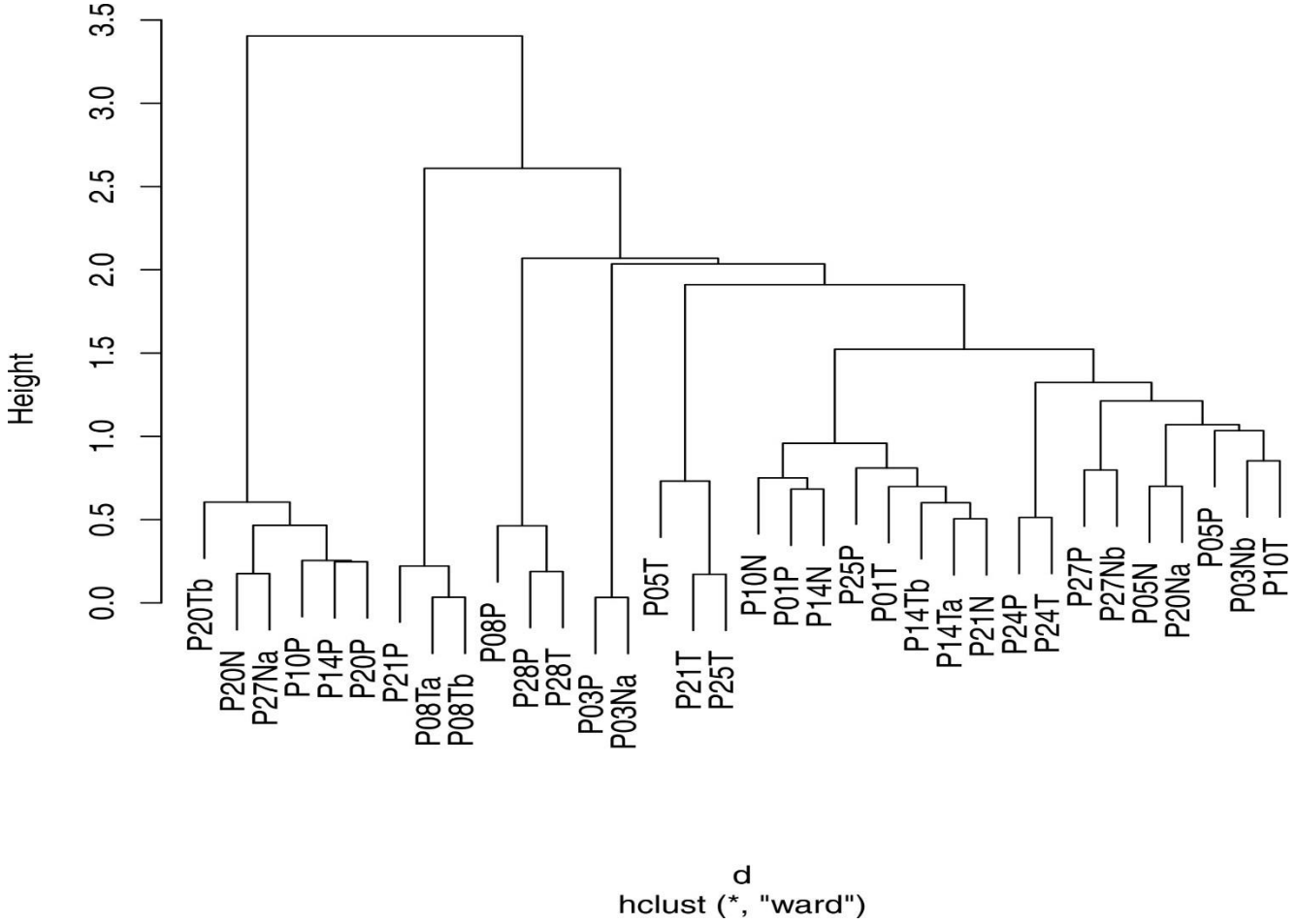

Figure 3.4 Bray Curtis dissimilarity dendrogram of sequences from dental plaque (P), non-directed bronchial lavage (N) and endotracheal tubes (T)

The top 100 species were further analysed per sample site. In dental plaque (Figure 3.5), *S. pneumoniae* was the most abundant species followed by *E. hirae,* (although most OTUs for this species originated from only one patient; P27) and *Fusobacterium nucleatum*, an oral microorganism recognised for its role in bacterial coaggregation. Importantly, the respiratory pathogens *S. aureus* and *P. aeruginosa* were also abundant in dental plaque samples. For ETTs, *S. aureus, H. influenzae* and *S. pneumoniae* were the three most abundant species, but the presence of oral microorganisms in these biofilms was also evident (Figure 3. 6). Finally, in NBLs, and similar to the ETTs, typical oral species such as *F. nucleatum, S. oralis* and *P. melaninogenica* were detected (Figure 3.7).

The top 20 species per individual patient was analysed by heat maps, and revealed that most microorganisms were simultaneously present in all available samples. Interestingly, in some cases (*S aureus* in P14, P20 and P21) the OTUs were higher in the NBL and/or the ETTs than in dental plaque (Figures 3.8 -3.10).

An attempt was made to evaluate if antibiotic therapy at the start of the intubation period had an impact on the microbiome but no differences were found. Similarly dental plaque and gingival scores available from the toothbrushes/foam swabs trial (Chapter 5) had no impact on the colonisation of respiratory pathogens.

From this cohort of patients three were admitted to the ICU with pneumonia (P03, P20 and P27) and two were diagnosed with VAP during the study (P21, 25) in accordance with the clinical pulmonary infection score (>6 points; Table 1.5) (Pugin 2002). The most abundant species found in ETT and/or NBLs are listed in table 3.3, all species were also found in dental plaque with lower OTU reads.

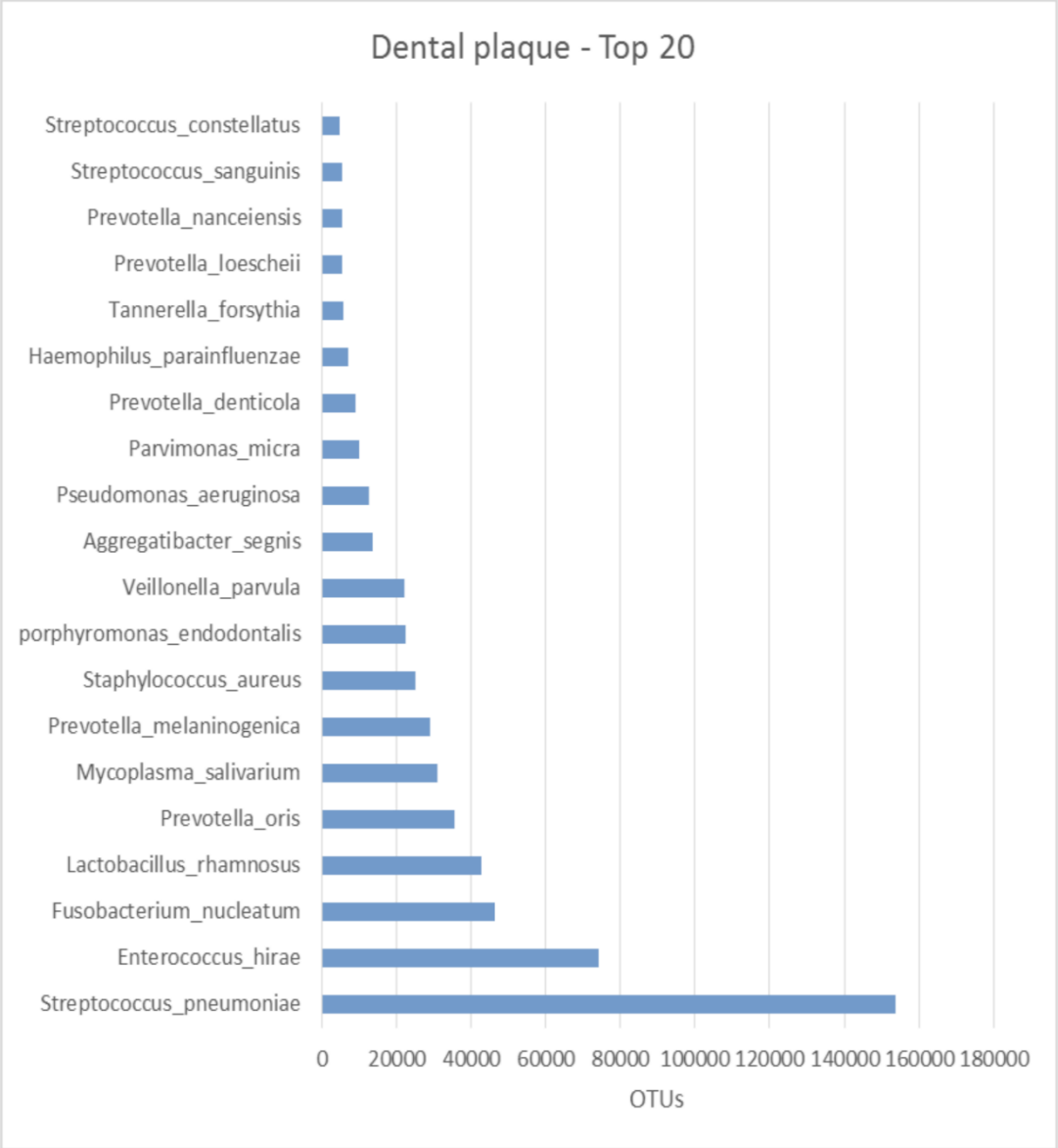

Figure 3.5 The 20 most abundant species in dental plaque

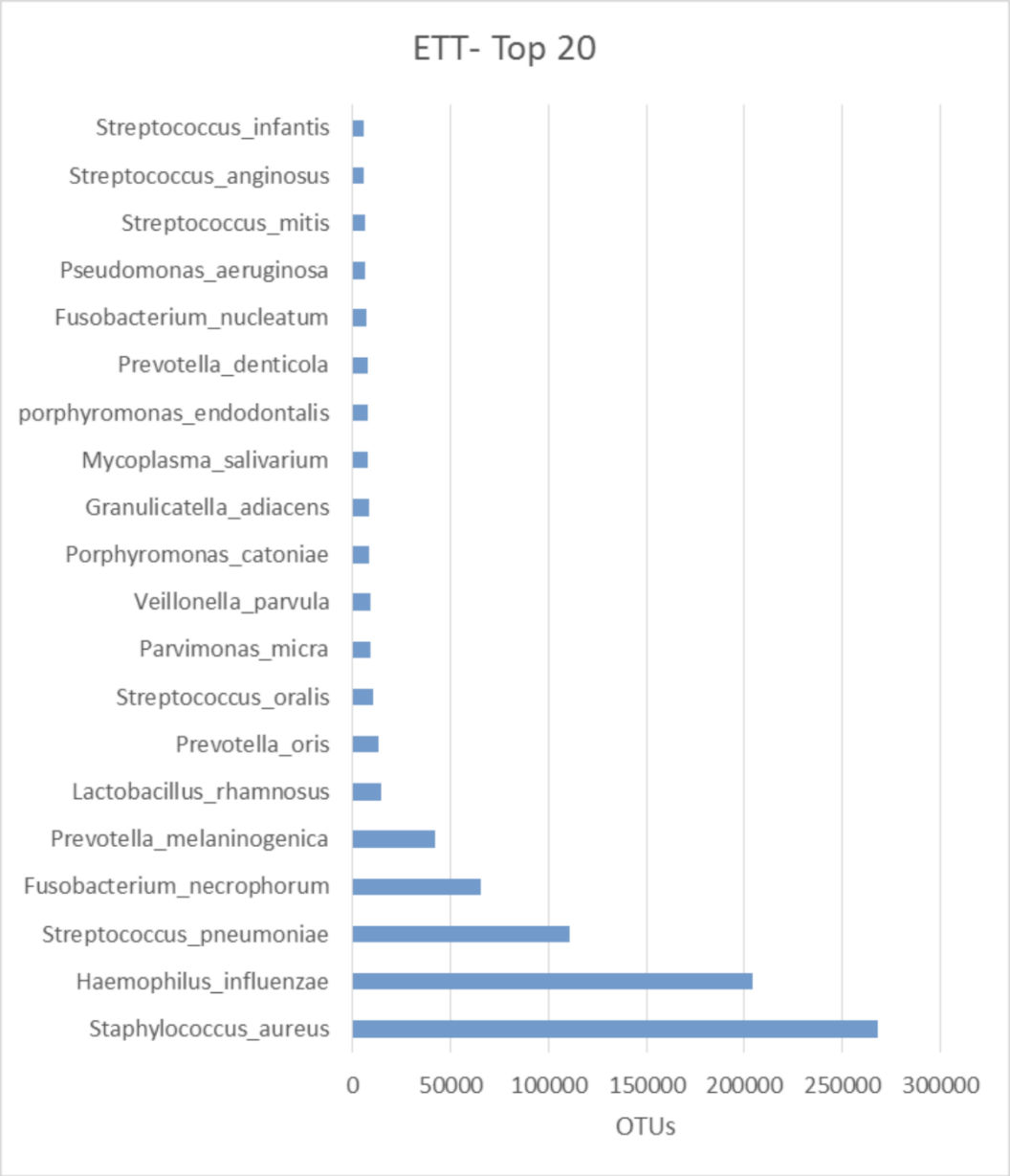

Figure 3.6 The 20 most abundant species in endotracheal tube biofilms (ETT).

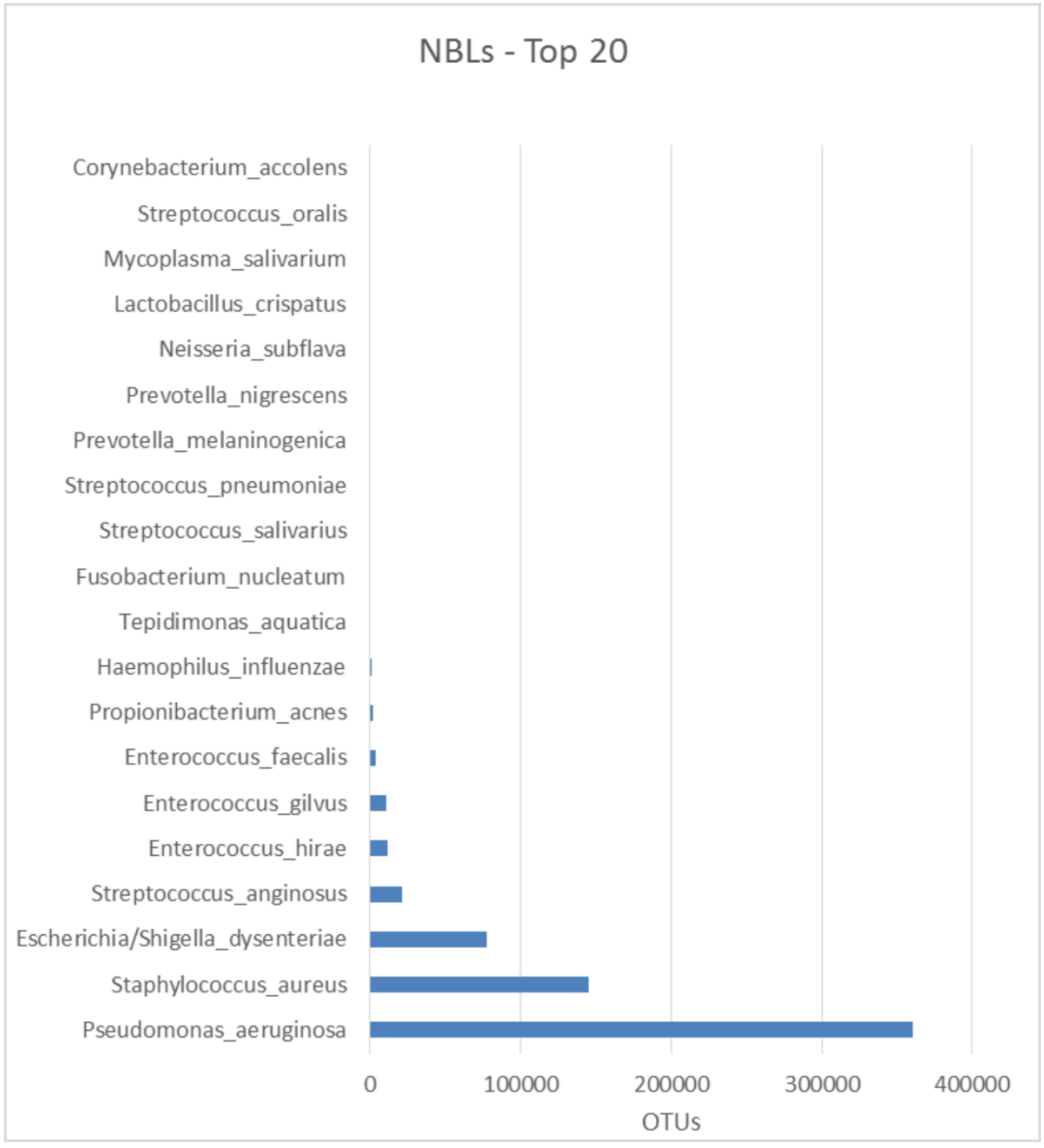

Figure 3.7 The 20 most abundant species in non-directed bronchial lavages (NBLs).

Not visible values start from 702 to 209 OTUs

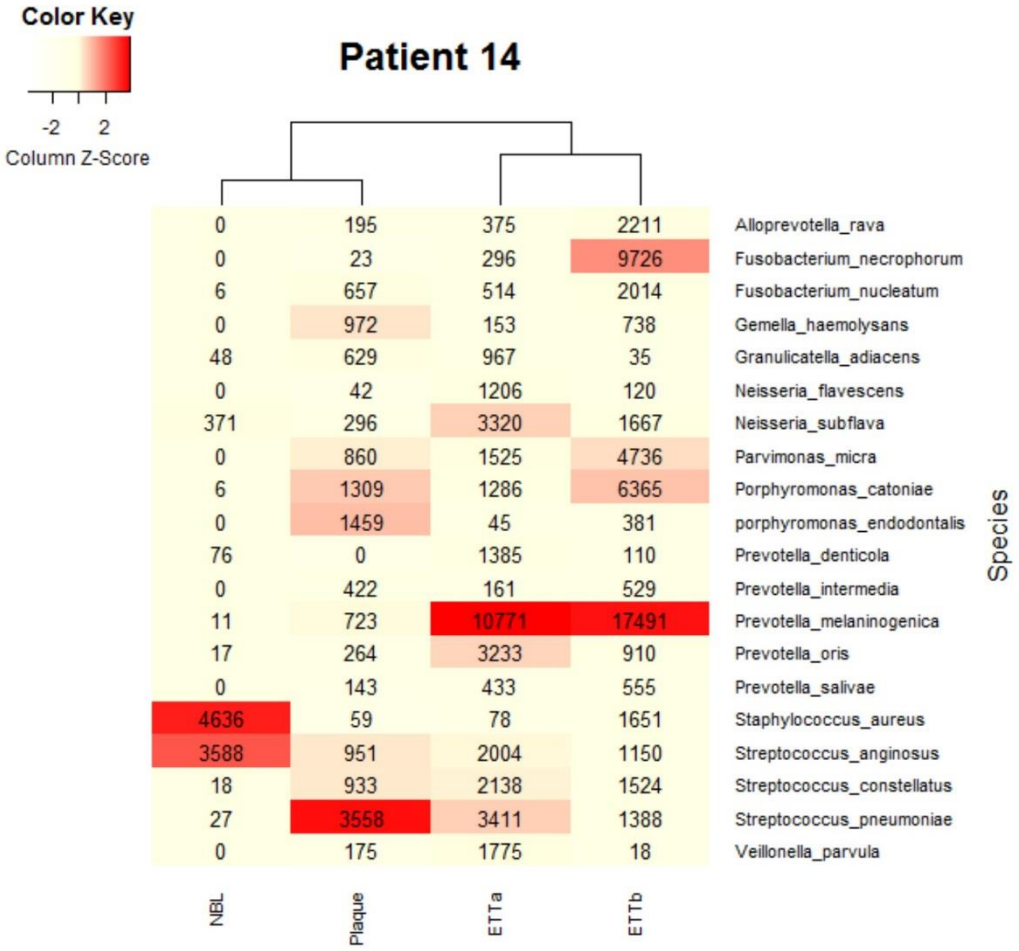

Figure 3.8 Heat map for patient 14 shows shared species in the dental plaque, non-directed bronchial lavage (NBL) and endotracheal tube (ETT). Numbers indicate operational taxonomic unit reads.

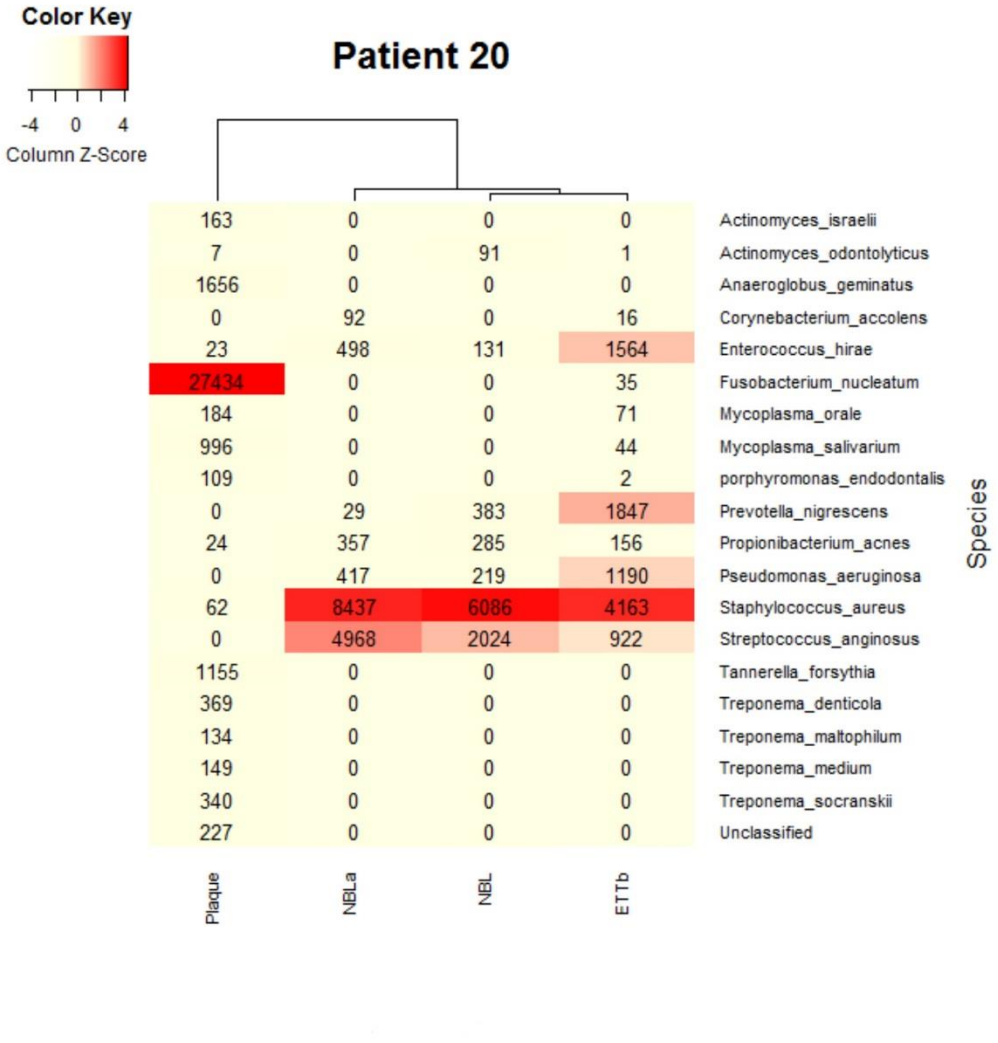

Figure 3.9 Heat map for patient 20 shows shared species in the dental plaque, non-directed bronchial lavage (NBL) and endotracheal tube (ETTb). Numbers indicate operational taxonomic unit reads.

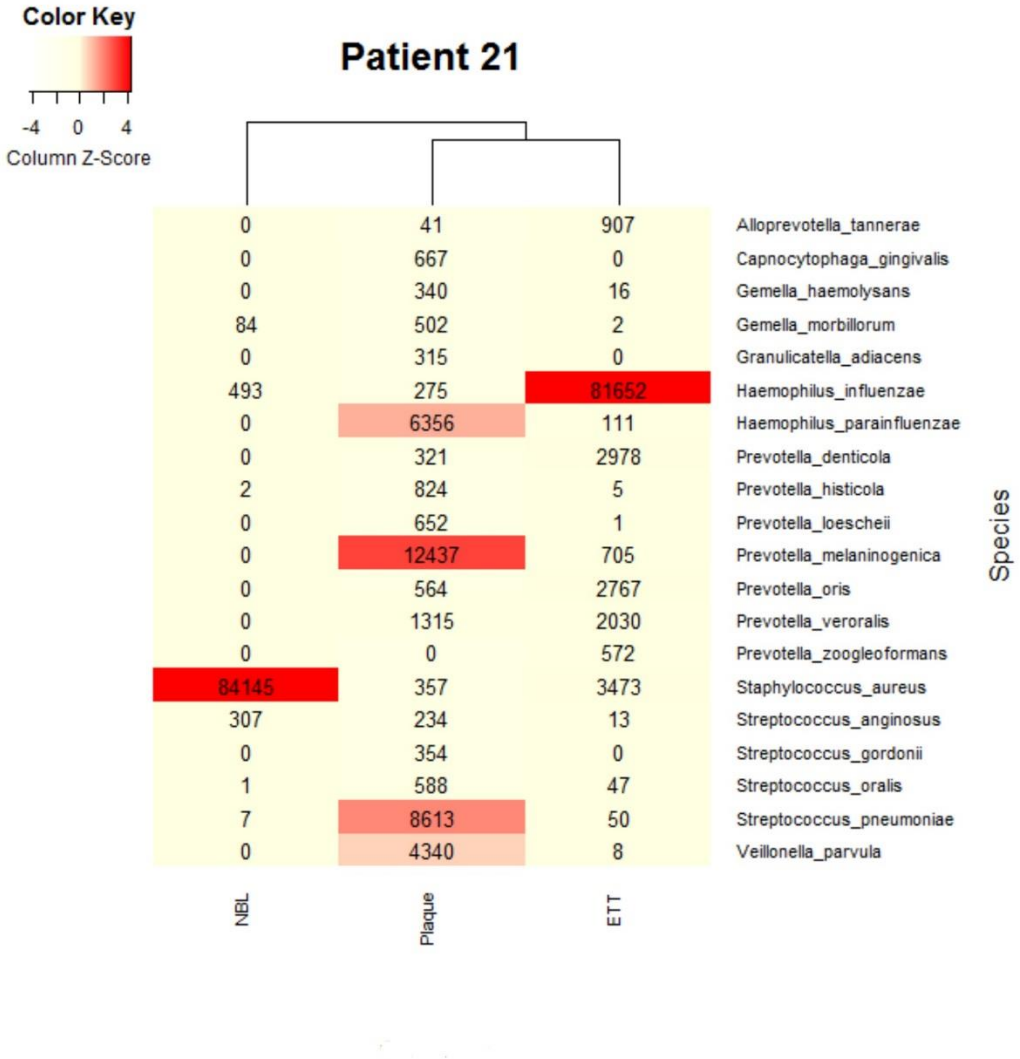

Figure 3.10 Heat map for patient 21 shows shared species in the dental plaque, non- directed bronchial lavage (NBL) and endotracheal tube (ETT). Numbers indicate operational taxonomic unit reads.

Table 3.3 Most abundant species in patients with diagnosed pneumonia.

Patient	**Organism**	**OTUs per site**		
		Plaque	**ETT**	**NBL**
3	*Pseudomonas aeruginosa*	5	N/A	183678
20	*Staphylococcus aureus*	62	4163	8437
21*	*Staphylococcus aureus*	357	3473	84145
25*	*Haemophilus influenzae*	176	94809	N/A
27	*Staphylococcus aureus*	665	N/A	25114

Operational taxonomic unit (OTU) reads of the most abundant species found in endotracheal tubes (ETT) and /or, non- directed bronchial lavage (NBL). *Patients were diagnosed with ventilator-associated pneumonia during the course of the study. N/A: not applicable as no sample was analysed.

3.5 Discussion

The colonisation of dental plaque and ETT biofilms by respiratory pathogens has been identified as risk factors for VAP in mechanically-ventilated patients (Fourrier *et al*. 1998; Sachdev *et al*. 2013; Scannapieco *et al*. 1992). VAP is an important nosocomial disease that prolongs hospital stay for up to 7 days, increases mortality rates and carries significant cost to the health care systems (Kollef *et al.* 2012; Melsen *et al.* 2013).

Culture-dependant and independent approaches have shown the presence of respiratory pathogens in dental plaque and similarly, microbial species normally associated with the oral cavity have been detected in lower airways specimens and ETT biofilms (Cairns *et al*. 2011; Perkins *et al*. 2010; Vandecandelaere *et al*. 2012).

The underlying hypothesis proposed for microbial aspiration to the lower airway is that subglottic secretions pool above the ETT cuff and subsequently leak past the cuff via microchannels created by folding of the cuff material. These secretions contain microbial components from the oropharynx and due to the forces of mechanical ventilation some of these will be drawn into the ETT lumen and form biofilms. If the secretions contain respiratory pathogens then this presents an imminent risk for the development of VAP.

In these studies, next generation sequencing (NGS) has been used for the first time, to characterise the microbiota from three separate, but potentially associated sites within mechanically ventilated patients. The sites were the oral cavity (dental plaque samples), the lumen of the ETT and the lower airway (NBL samples). Samples were studied simultaneously to establish whether high levels of similarities existed in terms of microbiome in the samples.

Metataxonomics is an NGS technique that allows characterisation of a microbial community through analysis of conserved and variable sequences of the bacterial 16S rRNA gene (Culligan *et al.* 2014; Marchesi and Ravel 2015). This culture-independent technique

overcomes the shortcomings of culture, which relate to the failure to detect many microorganisms that are unculturable *in vitro*. Indeed, currently an estimated 50% of oral microorganisms are thought to be unculturable (Paster *et al*. 2001). Metataxonomics also avoids the need for a cloning step which was a necessity when analysing mixed species populations using the Sanger sequencing method (Didelot *et al.* 2012).

The lower airways were previously deemed to be sterile until the development of molecular approaches. Now, multiple studies have reported that the lower airways microbiota is similar to that of the oropharynx and upper respiratory tract (Beck *et al.* 2015; Cui *et al.* 2014). *Prevotella* and *Veillonella* species have also been reported amongst the most prevalent detected species accounting for >20% of OTUs (Hilty *et al.* 2010).

Clearly, it is not possible to fully appreciate complex microbial communities without the use of culture independent methods, of which, metataxonomics offers the most advantages in terms of output, cost and effort.

In these studies, samples (n=34) of dental plaque, NBLs and or ETTs were obtained from 12 mechanically ventilated patients for analysis. It was not possible to obtain all samples simultaneously for a single patient, but given that biofilms develop over time, we did not consider this to be detrimental in our comparison of dental plaque and ETT biofilm communities. It could be argued that NBL samples might be more variable with temporal change, which could be regarded as a potential limitation to the study. Whilst metataxonomics have previously been used to characterise the microbiology in ETT biofilms (Vandecandelaere *et al*. 2012), dental plaque (Sands *et al*. 2016) and lavage samples (Yang *et al.* 2015), this represents the first study to simultaneously compare the microbiome from all three sites within a given patient.

In the normal oral microbiome the *Streptococcus* genus, which comprises several key species (*e.g. S. mitis, S. oralis, S. salivarius, S. sanguinis*) is the most abundant (Dewhirst *et al.* 2010).

Interestingly, in the studied patient group, the most common OTU (153,665; Figure 3.5) in dental plaque belonged to *S. pneumoniae* which is the most common causative organisms for community acquired pneumonia (CAP) (AlonsoDeVelasco *et al.* 1995). High prevalence of *S. pneumoniae* in dental plaque is compatible with health, as was shown by a study comparing the dental plaque of healthy young, healthy elderly and elderly requiring nursing care (N=97), all groups had high prevalence of *S. pneumoniae* (60 to 77%) (Abe *et al.* 2001). This contrasts with another study on healthy independent elderly including 265 individuals in which the organism was not detected (Ogawa *et al.* 2012). Importantly, this organism is opportunistic by nature (Siegel and Weiser 2015) therefore it presents a risk for vulnerable groups as is the case for most patients in ICU. The species *S. sanguis* was the second most prevalent streptococcal OTU but this was noticeably lower than for *S. pneumonia* (5,322). Of particular note, was the detection of other respiratory pathogens in dental plaque including *H. influenzae*, *S. aureus* and *P. aeruginosa*. Not only were these species found in dental plaque, but they were also amongst the top 20 of the most abundant species and all patients had at least one of these species. These findings are similar to those previously reported by Sands *et al.* (2016), where NGS showed that 9/13 mechanically ventilated patients had >2 respiratory pathogens in their dental plaque.

Previous studies analysing the dental plaque of elderly institutionalised and critically ill patients using microbiological culture have also reported colonisation with respiratory pathogens (Abe *et al.* 2001; El-Solh *et al.* 2004; Sachdev *et al*. 2013). Even though these respiratory pathogens are frequently recovered from dental plaque by culture, reported incidence in mechanically ventilated patients varies from 26% to 60% when culture is used (Heo *et al.* 2008; Sachdev *et al.* 2013). These levels are much lower that the results from this work (100%), and emphasises the sensitivity of the NGS approach employed. A key outcome from these analyses was that dental plaque was confirmed to be a reservoir of respiratory

pathogens in these patients at risk of acquiring VAP. From this group of patients two patients were diagnosed with VAP, likely caused by *S. aureus* and *H. influenzae*, both of which were present in dental plaque as well as the ETT and NBL.

In terms of species abundance, the NBLs from the studied patients were dominated by *P. aeruginosa* and *S. aureus* with 360,581 and 145,915 OTUs, respectively. This result is comparable to a recent study where bronchial lavages and ETT aspirates from 120 suspected VAP patients were analysed by culture and real time PCR, the most common isolates were *S. aureus, P. aeruginosa* and *H. influenzae* (Clavel *et al.* 2016).

The presence of oral species in the lower respiratory tract, whether they are normal inhabitants or transient colonisers could be significant as they may promote biofilm formation, in Chapter 4 of this thesis it was found that in the presence of *S. mutans*, *S. aureus* produced a more abundant biofilm and exhibited enhanced expression of biofilm genes. In NBLs, amongst the top 20 species was *Fusobacterium nucleatum*, which is renowned as a large 'bridging' bacterium that co-aggregates with numerous other species of bacteria there by playing a role in biofilm stability (Shen *et al.* 2005). The presence of this species in the NBLs may promote respiratory pathogens to thrive in associated biofilms, further *in vitro* studies may demonstrate synergistic effects between *F. nucleatum* and respiratory pathogens. In a recent study involving 83 cases of CAP and 94 cases of healthcare-associated pneumonia (HCAP) , bacterial 16S rRNA genes in bronchial lavage samples were analysed using PCR, cloning and sequencing (Akata *et al* 2016). It was found that a higher percentage of oral streptococci were present in patients with aspiration risk, comorbidities or a previous history of pneumonia (Akata *et al.* 2016).

Interestingly, *E. hirae* was amongst the top 5 species found overall, however this bacterium was only found in two patients, one of whom had very high OTU reads in their dental plaque (71730) and NBL (9808). This bacterium is mainly associated with disease in mammals and

birds and is considered a rare pathogen of humans (Larsson *et al.* 2014). However, there have been recent reports of urinary tract infections and severe bacteraemia caused by this species (Bourafa *et al.* 2015; Dicpinigaitis *et al.* 2015).

Biofilms have been shown to develop in 80% of ETTs independently of the duration of mechanical ventilation (Feldman *et al.* 1999; Wilson *et al.* 2012). Such biofilms have been shown to possess complex structures that incorporate a fibrin network, blood cells, bacteria and yeast (De Souza *et al.* 2014). Importantly, ETT biofilms are an identified risk factor for VAP (Adair *et al.* 1999; Gil-Perotin *et al.* 2012).

In this present study, the biofilms from 11 ETTs from 10 patients were analysed, and found to have a microbial diversity that generally correlated with dental plaque and NBLs. Of the top 20 species found in ETT biofilms, 10 and 12 species were also present in the top 20 of NBLs and dental plaque, respectively. Five of these species were amongst the top 20 species for all samples and these were *S. aureus, S. pneumoniae, Prevotella melaninogenica, Mycoplasma salivarium* and *P. aeruginosa*. The results support those previously reported by Cairns *et al*. (2011) which showed that of 24 ETTs analysed by Denaturating Gradiant Gel Electrophoresis (DGGE), a high microbial diversity was evident with an average of 6 bands present per ETT biofilm. Of note is that in DGGE, each band can represent more than one bacterial species (Zijnge *et al.* 2006). Additionally, with species-specific PCR, the key respiratory pathogens of *S. aureus* and *P. aeruginosa*, along with the oral microorganisms. *S. mutans* and *P. gingivalis* were detected in this study (Cairns *et al.* 2011). Similarly, Perkins *et al.* (2010) studied biofilms from 8 ETTs using both quantitative PCR and surveys of the 16s rRNA gene and reported that 70% of sequences belonged to genera commonly associated with the oral cavity. This study also highlighted that *Streptococcus* and *Actinomyces* species were found in 7 samples even though some ETTs had been in place for <24 h. Species of these two genera are primary colonisers in dental plaque and have proven co-aggregation

interactions (Palmer *et al.* 2003). In the group of patients presented in this chapter, members of the *Actinomyces* genus were detected in 10/12 plaque samples, 9/11 ETTs and one NBL.

In neonatal ETT biofilms, *Klebsiella*, *Streptococcus*, and *Pseudomona*s are reported as being the most frequent bacterial genera (Li *et al.* 2015)**.** In these investigations, it was proposed that the quorum-sensing AI-2 molecule, produced by *K. pneumoniae* and *Streptococcus* species could facilitate *P. aeruginosa* aggregation in ETT biofilms (Li *et al*. 2015).

In this study, the microbial diversity between samples was similar. It was evident, that when all community profiles were compared for 4 patients (P03, P24, P28, P27), the microbiome of either the ETT or the NBL had highest similarity to the dental plaque from the same patient. This would suggest that any inter-patient variation between dental plaque communities was on occasion, greater than the variation between different sample types from the same patient, demonstrating that the microorganisms were likely to have a common origin. Since the most microbiologically 'diverse' sample was dental plaque, it would not be unreasonable to suggest that that dental plaque represents the main reservoir of bacteria present in the other sample types. Also, the oropharynx is in constant exposure to the environment, and in hospitalised patients this may contribute to oral colonisation by pathogenic bacteria.

Since the microbiome of the lower airways and ETTs was similar to that of dental plaque, there may be scope for VAP surveillance by sampling the mouth, instead of the lower airways. This would certainly be a less invasive procedure for patients in cases where VAP is not suspected.

The oral health status of patients admitted into intensive care is generally poorer than that of the general population (Chapter 5; Table 5.7). For mechanically-ventilated patients, oral hygiene tends to deteriorate with time (Fourrier *et al.* 1998; Jones *et al.* 2011; Sachdev *et al.* 2013; Scannapieco 2006). Despite a greater

recognition that improving oral care can be a means of preventing infection as opposed to merely improving comfort (Dale *et al.* 2013), there remains a need to improve attitudes and protocols of oral care in mechanically-ventilated patients.

As is evident in Table 3.2 the samples analysed in this study were not obtained on the same day. Simultaneous sample taking was not always possible; ETTs could only be obtained after extubation was clinically indicated. Similarly, NBLs were only obtained up to twice a week for non VAP suspected patients, therefore it was decided that whenever possible a “mid trial” plaque sample was to be analysed. Although it is possible this may present potential for error in interpretation, it was not considered detrimental for the investigation as similarities in the microbiome of the different sites were observed. However the timing of colonisation of dental plaque by respiratory pathogens was not an aim of this investigation and cannot be concluded from its results.

A limitation of this study is that in a small number of subjects there was great variability in age, clinical diagnosis, underlying medical conditions and exposure to antimicrobial agents. These variables are likely to have an impact in the oral microbiome but could not be analysed independently,a great higher number of patients would be required to factor these confounging factors.

Another limitation of this present work was that the metagenomic analysis only involved bacteria, but of course viruses and yeast are also likely to co-exist in these samples. Also, although NGS offers high sensitivity, the method is not able to determine whether the bacteria are viable, which is of course determined by traditional culture, which also offers the advantage of being able to test isolates for their antibiotic sensitivity.

3.6 Conclusions

- The microbiomes of dental plaque, NBL and ETT biofilms were similar in terms of their microbial diversity. It was noted that the similarities between samples from a given patient were higher than between the sample type of different patients.
- Microorganisms traditionally regarded as members of the oral microbiome were frequently found in all sample types.
- A high abundance of respiratory pathogens was evident in dental plaque, ETT and NBL.
- These findings support the role of dental plaque as a reservoir for pathogens that disseminate to the lower airways and ETT biofilms.

4. Characterisation of *in vitro* endotracheal tubes biofilms

4.1 Introduction

It is widely recognised that microorganisms primarily exist within biofilms, which are typically multi-species communities adhered to a surface and encased in extracellular polymeric substances (EPS) (O'Toole *et al.* 2000). Biofilm microorganisms are notably distinct from their equivalent free-living or planktonic counterparts. In these different growth types, differential gene expression has been reported (Jefferson 2004) with biofilm cells also frequently exhibiting higher (up to 1000 fold greater) resistance to antimicrobials and host defences (Luppens *et al.* 2002). Cells within complex mixed species biofilms are effectively shielded against host defence mechanisms, whilst EPS is often attributed with impeding antimicrobial penetration by diffusion limitation and sequestration (Mah *et al.* 2003).

Biofilms are known to instigate numerous human diseases, and are currently thought to be responsible for over 60% of human infections. Examples of such biofilm infections include chronic middle ear infections, chronic sinusitis, chronic otitis, and respiratory infections including ventilator-associated pneumonia (VAP), (Donlan and Costerton 2002).

VAP is the most common nosocomial infection in patients receiving intensive care, and in terms of hospital acquired infections (HAIs) is second only to urinary tract infections in its prevalence. VAP has been proposed to account for more than half of the antibiotics prescribed in the intensive care unit (ICU) (Zilberberg and Shorr 2010). The high incidence of VAP combined with an attributable increased hospital stay and mortality (Melsen *et al.* 2013; Safdar *et al.* 2005) justify efforts aimed at increasing our understanding of the pathogenesis of this infection and the development of prevention strategies.

The most common causative microorganisms for VAP are *Pseudomonas aeruginosa* (24%), *Staphylococcus aureus* (20%) Enterobacteriaceae (14%), *Haemophilus* species (10%), *Acinetobacter* species (8%) and *Streptococcus* species (8%) (Chastre and Fagon 2002).

One commonly attributed aetiological factor for the development of VAP is aspiration of oropharyngeal secretions into the lungs. In mechanically-ventilated patients, the endotracheal tube (ETT) facilitates gas exchange, however its presence impairs natural barrier defence mechanisms including the cough reflex and mucociliary clearance. Once the ETT is inserted, a polyvinyl chloride (PVC) balloon referred to as a cuff (Figure 1.2), is inflated to 1.5 to 2 times the diameter of the trachea in an attempt to seal the lower airways (Pneumatikos *et al.* 2009). However, as the cuff inflates the material folds, creating microchannels that allow passage of pooled oropharyngeal secretions above the cuff (Hamilton and Grap 2012). If these secretions contain respiratory pathogens, then there is a clear risk for subsequent infection.

In institutionalised and hospitalised patients, a deterioration in oral hygiene occurs (Fourrier *et al.* 1998; Jones *et al.* 2011; Sousa *et al.* 2014) and it has been shown that the dental plaque can become colonised by respiratory pathogens (Binkley *et al.* 2009; Ewan *et al.* 2010). This phenomenon has also been reported in mechanically-ventilated patients, with *P. aeruginosa*, *Acinetobacter* species and *S. aureus* repeatedly being recovered from dental plaque and saliva in these patients (Sachdev *et al.* 2013; Sands *et al.* 2016b; Zuanazzi *et al.* 2010). Indeed, this finding was also reported in Chapters 2 and 3 of this thesis.

The presence of an ETT is an identified risk factor for VAP and a recent study reported an increased risk of VAP (RR 7.41, $P < 0.001$) for intubation periods exceeding 8 days (De Souza *et al.* 2014). The ETT also supports the growth of biofilms in its lumen and these biofilms have been observed in ETTs after as little as 12 h intubation (Adair *et al.* 1999). As the ETT biofilm is not exposed to the patient's immune system or administered antibiotics, this biofilm provides a protected reservoir of pathogens (Bauer *et al.* 2002). In an observational study of 75 patients where ETTs and endotracheal aspirates were collected, respiratory pathogens were detected in both sample types (Gil-Perotin *et al.* 2012). Importantly, the

study reported that higher treatment failure and relapse (100% vs 29%, P = 0.021; 57% vs 14%, P = 0.133) occurred when the causative organism was present in the ETT as well as the ETT aspirate (Gil-Perotin *et al.* 2012).

Molecular studies of ETT biofilms have identified respiratory pathogens as well as oral microbes including species of *Streptococcus*, *Lactobacillus*, *Candida* and *Porphyromonas* (Cairns *et al.* 2011; Perkins *et al.* 2010). Perkins *et al*. (2010) showed that the most common bacterial genus found in ETT biofilms was *Streptococcus* and that 70% of the sequences found in biofilms were typical of the oral microflora. Similarly, a study using pyrosequencing to characterise 4 ETT biofilms showed a high diversity and prevalence of oral bacteria including *Prevotella* and *Peptostreptococcus* species (Vandecandelaere *et al.* 2012). Importantly, bacteria recovered from ETTs have also shown increased antibiotic resistance (Sands *et al*. 2016b).

Oral microbes are known to be adept biofilm producers. This feature is illustrated by *Streptococcus mutans* which produces extracellular polysaccharides in the form of glucans, which allow it to firmly adhere to the smooth tooth surface (Kuramitsu *et al.* 2007). *Streptococcus mutans* also produces glucan binding proteins (*i.e.* GbpA, -B, -C and –D) that are thought to play important roles in subsequent cell-cell aggregation and biofilm development (Lynch *et al.* 2013). Therefore it is plausible that oral microorganisms could 'precondition' the ETT with a biofilm that is conducive to colonisation by respiratory pathogens.

4.1.1 Microbial interaction

In polymicrobial biofilms, the intimate relationship in which microorganisms are spatially distributed means that interactions will inevitably occur. This may be through sharing of

metabolic end products between species, where the donor of the metabolite exhibits accelerated growth along with the recipient (Kouzuma *et al.* 2015). Additionally, microbial cells can display multiple phenotypes within a biofilm, and are able to interact with each other through quorum sensing (Burmølle *et al.* 2006). It is inevitable that interactions between respiratory pathogens and oral microorganisms will arise when both are present in the same biofilm.

Multiple synergistic effects can occur from interactions in multispecies biofilms. One of these is the coaggregation of cells, an effect that has been studied frequently in dental plaque. In terms of shared metabolic products, the oral bacterial species *Treponema denticola* produces succinate and this facilitates growth *Porphyromonas gingivalis* in biofilms (Grenier 1992). *P. gingivalis* is a primary coloniser of the subgingival tooth surface and confocal laser scanning microscopy (CLSM) of dental plaque has shown close proximity of *T. denticola* to *P. gingivalis,* rather than to the enamel, suggesting the later might facilitate *T. denticola* colonisation (Yamada *et al.* 2005). Similar relationships have been described between *Streptococc*us and *Actinomyces* species (Palmer Jr *et al.* 2003), and between *Tannerella forsythia* and *Fusobacterium nucleatum* (Sharma *et al.* 2005). Similarly, an increased biofilm streptococcal biomass has been shown to occur in an *in vitro* open flow cell system mucosal model in the presence of *C. albicans*. The underlying mechanism for this was deemed to be enhanced coaggregation, rather than growth stimulation (Diaz *et al.* 2012).

In the case of respiratory pathogens, there is also evidence of bacterial interactions. An *in vitro* experiment showed a 26 and 106-fold increase in numbers of *Acinetobacter baumannii* and *Pseudomonas aeruginosa,* respectively, when cultured with *Stenotrophomonas maltophilia*. *S. maltophilia* is an opportunistic Gram-negative bacterium that colonises humid indwelling medical devices such as ETTs and urinary catheters (Chang *et al.* 2015) and unsurprisingly, is frequently linked to medical device infections (Varposhti *et al.* 2014).

Another phenomenon in mixed biofilms is conjugation, where physical contact between bacterial cells allows exchange of genetic material (also known as horizontal gene transfer) occurs, and it is thought to be facilitated by plasmid expressed factors that promote biofilm formation by planktonic bacteria (Ghigo 2001).

Bacterial interaction can also result in antagonistic effects in biofilm growth due to the production of bacterial toxins (Leriche *et al.* 2003). In dual species *in vitro* biofilms of *P. aeruginosa* and *C. albicans, P. aeruginosa* can degrade the hyphal cell wall of *C. albicans* by release of phenazines, namely pyocyanin and 1-hydroxyphenazine, which are toxic to *C. albicans* (Harriott and Noverr 2011; Kerr *et al.* 1999). Hogan *et al*., (2004) demonstrated that *P. aeruginosa* was also able to kill *C. albicans* hyphae, through a process involving the homoserine lactone quorum-sensing molecule, 3-oxo-C12.

4.1.2 Gene expression in biofilms

Multiple *in vitro* studies reveal that microorganisms exhibit different gene expression when growing in a biofilm, compared to planktonic growth (Cvitkovitch *et al.* 2003; Jefferson 2004). Such changes in gene expression allow bacteria to attach to surfaces, produce extracellular polymeric matrices and survive under limited nutrient content and suboptimal oxygen levels (Dotsch *et al.* 2012). Changes in gene expression also alter the virulence of microorganisms, for example, hyphal growth by *C. albicans* facilitates host tissue invasion and protects it from phagocytosis (Gow *et al.* 2012). The presence of certain microorganisms may influence both biofilm formation and virulence of other microbes. There are multiple *in vitro* and *in vivo* studies showing that oral streptococcal species including *S. gordonii*, *S. oralis* and *S. mutans* enhance the virulence of *C. albicans* (Bamford *et al.* 2009; Cavalcanti *et al.* 2015; Diaz *et al*. 2012; Falsetta *et al.* 2014). It has also been proposed that excretion of lactate by streptococci provides a carbon source that promotes hyphal growth by *C. albicans* (Holmes *et al.* 1996; Jenkinson *et al.* 1990). *Streptococcus sanguinis* and *Actinomyces* species

can also influence virulence of *C. albicans,* through up regulation of genes encoding for secreted aspartyl proteinases (SAPs) and hyphal wall protein in *in vitro* biofilms (Cavalcanti *et al*. 2015).

Staphylococcus aureus and *C. albicans* often co-exist in other biofilm infections including chronic wounds, intraperitoneal and lung infections. Interestingly, studies of 24 h *in vitro* biofilms comprising of both *C. albicans* and *S. aureus* on catheter discs, have demonstrated increased pathogenicity of *S. aureus* (Peters *et al.* 2010). Peters *et al.* (2010) found that 27 proteins were up regulated in these dual species biofilms and these were mainly involved in growth, metabolism, or stress responses. Another study from the same group, used high resolution scanning electron microscopy (SEM) and fluorescence *in situ* hybridisation using peptide nucleic acid probes (PNA FISH) to show *S. aureus* attachment to *C. albicans* hyphae. This interaction appeared to relate to the *C. albicans* Als3 protein, as *S aureus* attachment was reduced in *C. albicans* mutants lacking this protein. Importantly hyphal attachment by *S. aureus* also aided invasion of epithelial cells by the bacterium (Peters *et al.* 2012b).

As previously mentioned, *P. aeruginosa* and *S. aureus* are frequently involved in the development of VAP (Section 4.1 and Chapter 1 Section 1.3.4) and as such are a focus of this research. A selection of genes required for different stages of biofilm formation by *P. aeruginosa* and *S. aureus* are presented in tables 4.1 and 4.2.

4.1.2.1 Gene expression in *Pseudomonas aeruginosa* biofilms

Pseudomonas aeruginosa is a Gram-negative bacterium that is recognised as a major human pathogen and is frequently linked to nosocomial infections including VAP. *Pseudomonas aeruginosa* can adhere to abiotic surfaces, host tissues, and to each other and is considered an adept biofilm producer. Infections caused by *P. aeruginosa* are often difficult to treat due to increased antibiotic resistance (Obritsch *et al.* 2005; Vallet *et al.* 2001).

One important *P. aeruginosa* virulence factor is the production of alginate. Alginate is a viscous exopolysaccharide comprising of linked D-mannuronic and L-guluronic acid. Alginate production is associated with biofilm formation by *P. aeruginosa*, bacterial adherence, protection of cells against phagocytosis and the neutralisation of oxygen free radicals (Matsukawa and Greenberg 2004; Ramsey and Wozniak 2005).

Alginate production is regulated by environmental factors through a response regulator involved in a signal transduction system, and by a gene cluster containing the sigma factor *algU* (Edwards and Saunders 2001). The *algU* gene is auto-regulated by the negative regulators *mucA* and *mucB* and inactivation of these regulators results in enhanced *algU* expression and alginate production (Edwards and Saunders 2001).

Although alginate is the most important factor in *P. aeruginosa* biofilm formation, alginate-independent biofilm formation occurs and is mediated by genes that increase levels of the non-alginate exopolysaccharide, Psl (Huse *et al.* 2013).

Table .5.1 Selected *Pseudomonas aeruginosa* genes involved in different stages of biofilm formation.

Biofilm stage	Gene	Function	Reference
Initial adhesion	Type IV Pili	Twitching motility	(O'Toole and Kolter 1998)
	Chaperone usher pathway (cup A, B, C)	Assembly of fimbria	(Vallet *et al*. 2001)
Attachment	cupA	Adhesion to inert surfaces	(Vallet et al. 2001)
Coaggregation	Psl	Cell to cell attachment	(Jackson et al. 2004)
EPS and maintenance of biofilm	algA-algD operon	Alginate synthesis	(Ramsey and Wozniak 2005)
	Muc A, MucB	Regulates the algA-D operon	
	Pel	Production of glucose matrix	(Pulcrano et al. 2012)
			(Friedman and Kolter 2004)
Maturation	Rpo	Adapt to environmental stresses	(Schuster et al. 2004)
	2070, 5033	Biofilm formation and antibiotic resistance	(Zhang *et al.* 2013)
Dispersal	nirS	Sensing of environmental clues and detachment	(Morgan et al. 2006)
	bdlA		(Barraud *et al*. 2009)
		Nitric oxide signalling	

Table 4.2 Selected *Staphylococcus aureus* genes involved in different stages of biofilm formation.

Biofilm stage	Genes	Function	Reference
Initial adhesion	*MSCRAMMs* *(Microbial surface components recognizing adhesive matrix molecules)*	Bind to fibrinogen or fibronectin	(Patti *et al.* 1994)
	Bap	Induce biofilm formation	(Cucarella 2001)
Attachment	fnbA, fnbB	Fibronectin binding proteins	(Arciola *et al.* 2005)
	AtLE, dltA, *Bap*	adhesion to polystyrene surfaces	(Cucarella 2001; Hall-Stoodley *et al.* 2004)
Coaggregation	Agr	Quorum sensing	(Yarwood and Schlievert 2003)
	Aap	Cell to cell adhesion	(Conrady *et al.* 2013)
EPS; biofilm maintenance	IcaABCD	Polysaccharide intercellular adhesins (PIA)	(Heilmann et al. 1996)
	icaR	Environmental regulation, regulation of ica operon	(Conlon *et al.* 2002)
Maturation	Beta toxin Hlb	Production of nucleoprotein matrix	(Huseby *et al.* 2010)
Dispersal	*luxS*	Reduces cell to cell adhesion	(Mirani *et al.* 2013)

4.1.2.2 Gene expression in *Staphylococcus aureus* biofilms

Staphylococcus aureus is a Gram-positive, coccus-shaped bacterium that is frequently associated with a wide variety of human infections including chronic wounds, respiratory tract infections (including VAP), bacteraemia, endocarditis and food poisoning (Lowy, 1998). In terms of gene expression, the *agr* system of *S. aureus* regulates production of several virulence factors that enable adaption to different stages of cell life *e.g.* the upregulation of cell-wall associated proteins during early stages of biofilm development (Pollitt *et al.* 2014). Based on autoinducing peptide specificity for the signal receptor *agrC*, strains can be classified into four specific *agr* groups (I-IV). Within a given group, each strain produces a peptide that can activate the *agr* response in the other member strains, whereas the autoinducing peptides produced by the different groups are usually mutually inhibitory (Shopsin *et al.* 2003). It appears that strains of a particular *agr* type would be related to a particular disease, *e.g.* group III to toxic shock syndrome, Group IV to exfoliative syndromes like staphylococcal scalded-skin syndrome, and group I to meticillin resistance and suppurative infections (Jarraud *et al.* 2002).

Another important component of *S. aureus* biofilm development is expression of polysaccharide intercellular adhesin (PIA), which facilitates adhesion to a substrate and cell to cell adhesion (Heilmann *et al*. 1996; O'Gara 2007). PIA is a linear polysaccharide of β-1, 6-linked glucosaminolglycans and its production is regulated by the *ica* locus. The locus is comprised of 4 intercellular adhesion genes (*icaA, icaB*, *icaC* and *icaD*) and one regulator gene, *icaR* (Figure 4.1) (Cafiso *et al.* 2004; O'Gara 2007). *IcaA* produces an N-acetylglucosamine transferase, *icaB* encodes a signal peptide that is secreted into the medium, and *icaC* and *D* encode membrane proteins (O'Gara 2007).

4.2 Objectives

Although co-occurrence of respiratory pathogens and oral bacterial species in biofilms has been reported in multiple clinical studies, including those of the oral cavity and respiratory tract, the effect that the two microbial types exert on each other has not been characterised. The research in this chapter will address this for specific aspects relating to virulence and biofilm development. Specifically the research aims to:

- Assess synergistic/antagonistic effects of oral microorganisms on respiratory pathogens in *in vitro* biofilms.
- Ascertain the spatial location and interaction between oral microorganisms and respiratory pathogens in mixed species biofilms.
- Determine the effects of oral microorganisms on the expression of respiratory pathogen virulence factors.

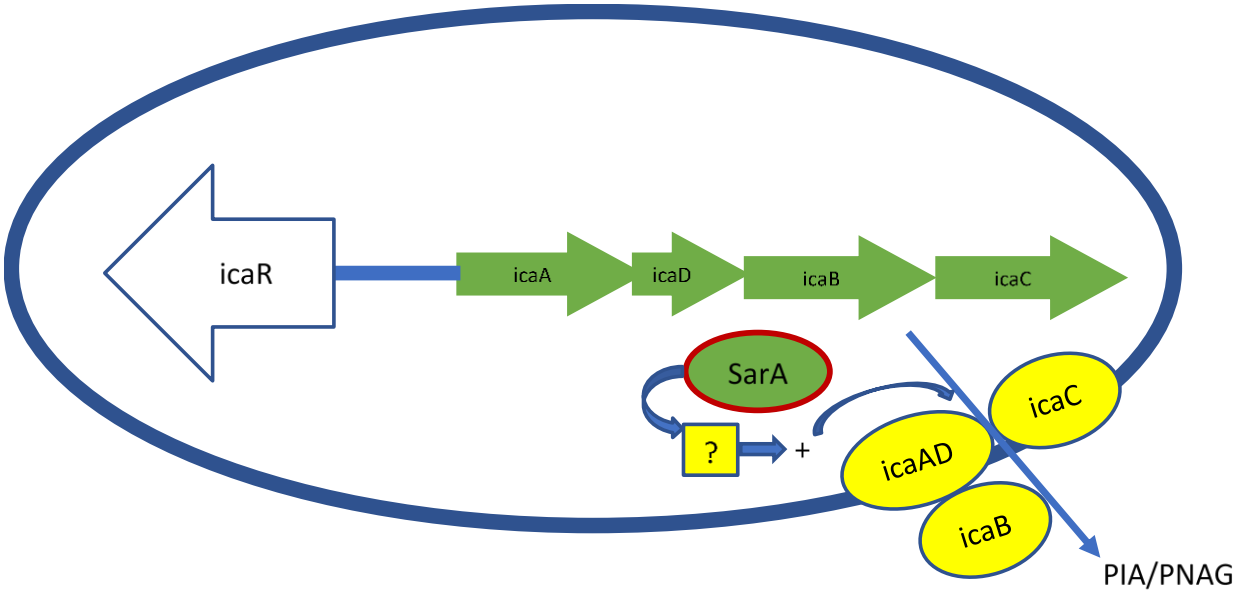

Figure 4.4.1 Schematic despiectionof the ica dependant biofilm mechanism in *Staphylococcus aureus*.

The green arrows indicate a positive regulatory pathway and the white arrow is the negative regulatory effect of i*ca R* locus. Production of the polysaccharide intercellular adhesion (PIA) and polymeric *N*-acetyl-glucosamine (PNAG) are mediated by the *ica* operon. Adapted from (O'Gara 2007).

4.3 Materials and Methods

4.3.1 Microorganisms and culture conditions

Reference strains of target microorganisms that were representative members of the oral microflora (*S. mutans, P. gingivalis* and *C. albicans*) and known VAP pathogens (*S. aureus* and *P. aeruginosa)* (Table 4.3) were used in these experiments. The identity of the reference strains was confirmed through PCR-mediated 16S rDNA sequencing. Sequences were identified using the Basic Alignment Search Tool (BLAST) from the National Centre for Biotechnology Information (NCIB) as previously described in Chapter 2 (Section 2.3.6.3).

Culture media was obtained from Lab M (Burry, UK) unless otherwise stated. Bacterial species were initially grown on blood agar (BA) for 48 h, except for the anaerobic oral bacterium *P. gingivalis*, which was cultured for a minimum of 4 days anaerobically on Fastidious Anaerobe Agar (FAA), and the yeast *C. albicans*, which was grown on Sabouraud dextrose agar (SDA).

4.3.2 Growth of *in vitro* biofilms in ETT sections

A 20-ml volume of fastidious anaerobe broth (FAB) was inoculated with a loop-full of each microorganism for 18 h at 37°C. *Porphyromonas gingivalis* cultures were incubated anaerobically for 48 h. A standardised (McFarland 0.5 turbidity) inoculum was generated from which serial decimal dilutions were prepared in Phosphate Buffered Saline (PBS). The diluted preparations were spiral-plated on BA, FAA or SDA using a Whitley Automated Spiral Plater (WASP) system (Don Whitley Scientific, Shipley, UK) as described above. This enabled determination of the numbers of viable cells in the inoculum.

Three sterile cylindrical (1 cm x 8 mm) lengths of ETT were placed in a universal container with 1 ml of the prepared inoculum and 9 ml of FAB.

Table 4.3 Test microorganisms used for *in vitro* ETT biofilm formation.

Species	**Source**	**Strain**	**16S sequence identity**
Candida albicans	American Type Culture Collection	90028	99%
Streptococcus mutans	Deutsche Sammlung von Mikroorganismen (DSM; German collection of microorganisms and cultures)	20523t	97%
Porphyromonas gingivalis	National Collection of Type Cultures	11834	99%
Staphylococcus aureus	National Collection of Industrial Bacteria	9518	96%
Pseudomonas aeruginosa	American Type Culture Collection	15682	98%

Three separate experimental conditions were used:

1. Mixed biofilms comprising of all test strains and incubation for 5 d.
2. Dual species biofilms, where single species biofilms of *S. mutans, C. albicans or P. gingivalis* were first generated by incubation for 5 d, followed by addition of a VAP pathogen and further incubation for 5 d.
3. Single species biofilms, served as controls for mixed and dual species biofilms and were incubated for 5 days.

Culture medium was replenished every 24 h to ensure constant nutrient availability and waste removal. After incubation, the broth was discarded and the three ETT sections were rinsed (×3) in sterile PBS and processed for culture analysis, fluorescent *in situ* hybridisation (FISH), Gram staining, and scanning electron microscopy (SEM).

4.3.2.1 Culture analysis

Using a dry sterile cotton swab, the biofilm was scraped from the lumen of the ETT and placed in 10 ml of PBS. The cotton swab was then agitated by vortex mixing in PBS for 5 s and 10-fold serial dilutions of the recovered biofilm cells prepared. From these dilutions, 50 µl was spiral-plated on to selective culture media for the species of interest *i.e.* FAA for *P. gingivalis*, SDA or CHROMagar® *Candida* (CHROMagar, Paris, France) for *C. albicans,* Mitis Salivarius Bacitracin agar (MSB; Difco, BD, Oxford, UK) for *S. mutans*, mannitol salt agar (MSA) for *S. aureus*, and *Pseudomonas aeruginosa* agar (PsA) for *P. aeruginosa*. Agars were incubated under appropriate gaseous conditions for up to 7d. Enumeration and colony identification of isolates from the plates was performed after 24 h for *P. aeruginosa*, 48 h for *C. albicans*, *S. aureus* and *S. mutans* and 7 d for *P. gingivalis*.

4.3.2.2 Preparation of ETT sections for light microscopy

ETT sections were placed in 35 mm tissue culture dishes (Lennox, Dublin, Ireland) and covered with 2% (w/v) agarose (Sigma, Poole, UK) to stabilise the biofilm. Once the agar had solidified, excess agarose was trimmed and the ETT sections immersed in 2% (v/v) paraformaldehyde for at least 24 h prior to embedding in paraffin wax and processing using standard histological techniques. Sections (20 µm thickness) were prepared from these ETT by Ms Kath Allsopp (Oral pathology, School of Dentistry, Cardiff University).

4.3.2.3 Fluorescent *in situ* hybridisation (FISH)

Biofilm cells on sections were stained using peptide nucleic acid (PNA) probes (Table 4.4) and analysed using confocal laser scanning microscopy (CLSM). Validation of PNA probe specificity was initially undertaken using planktonic cells of the reference strains. The method was as previously described by O'Keefe *et al*. (2001) and included some modifications (Malic 2008). *Porphyromonas gingivalis* was cultured in FAB, whilst *S. mutans, S. aureus* and *P. aeruginosa* were grown in Brain Heart Infusion (BHI; Oxoid, Hampshire, UK) and *C. albicans* in Sabouraud dextrose broth (SDB). All these species were incubated overnight at 37°C. *Porphyromonas gingivalis* was cultured anaerobically for 48 h.

A 1-ml volume of broth was pelleted by centrifugation (9,600 x *g*, 5 min) and re-suspended in PBS. Cell suspensions were re-centrifuged and suspended in PBS with 4% (w/v) paraformaldehyde (Sigma) and fixed for 1 h. The fixed cells were rinsed in PBS and re-suspended in 50% (v/v) ethanol and incubated for at least 30 min at -20°C.

A 100-µl volume of fixed cells was pelleted by centrifugation and the pellet was rinsed with PBS. The cells were then suspended in 100 µl of hybridisation buffer (25 mM Tris-HCL, pH 9.0; 100 mM NaCl; 0.5% (w/v) SDS) containing 150 to 500 nM of PNA probe. For *S. aureus* and *S. mutans*, incubation in 10 mg/ml of lysozyme (Grade VI from chicken egg white, Sigma) was performed prior to incubation with the probe. Additionally, formamide (Sigma) 30%

(w/v) was added to the hybridisation buffer containing the PNA probe specific for *S. aureus* and *S. mutans*.

The re-suspended cells in hybridisation buffer were incubated for 15 to 90 min at 55°C depending on the species. Cells were then centrifuged (13,000 x *g)* for 5 min and re-suspended in 500 µl of wash solution (10 mM Tris pH 9.0, 1 mM EDTA), incubated for 10 min and pelleted by centrifugation. This was procedure was repeated on a further two occasions. After the last wash, cells were re-suspended in 100 µl of wash solution and 5 µl of the cell suspension spread on a clean HistoBond®-coated microscope slide (Raymond A Lamb, UK) and allowed to dry. Vectashield® (Vector ltd, Orton Southgate, UK) mounting medium (2 µl) was applied to the specimen and the preparation was overlaid with a coverslip. The coverslip was sealed using nail varnish.

For *in vitro* biofilms, the paraffin embedded sections were de-waxed by gentle washing with xylene for 5 min, followed with absolute ethanol for 2 min and distilled water for 2 min; this process was performed twice. Sections were treated with 100 µl of lysozyme (10 mg/ml) and incubated for 30 min at 37°C, followed by a brief rinse with wash solution, which had been pre-warmed at 55°C. Then, a 150-µl volume of hybridisation buffer containing PNA probes was added and the sections placed in a dark humidified chamber and incubated for 90 min at 55°C. Sections were then suspended in wash solution with a magnetic stirrer for 30 min at room temperature, after which they were air dried prior to 2 µl of Vectashield® (Vector Laboratories, Peterborough, UK) being added and overlaid with a coverslip as described above.

Table 4.4 Peptide nucleic acid (PNA) probes used for fluorescent in situ hybridisation (FISH).

Probes	Target	Nucleotide sequence (5'→3')	Fluorescent label	nM *	References
Bac-Uni1	Bacterial Universal	OO-CTGCCTCCCGTAGGA	CY3	150	(Perry-O'Keefe *et al*. 2001)
Psaer	*P. aeruginosa*	OO-AACTTGCTGAACCAC	FITC	300	(Coull and Hylding-Nielsen, 2003)
Sta 16S03	*S. aureus*	OO-GCTTCTCGTCCGTTC	CY5	500	(Perry-O'Keefe *et al*. 2001)
Mut590	*S. mutans*	OO-ACT-CCA-GAC-TTT-CCT-GAC	Alexa 405	300	(Thurnheer, Gmür *et al.* 2001)
Candida	*C. albicans*	ACAGCAGAAGCCGTG	FITC	300	(Oliveira *et al.* 2001)

* Working concentration (nM)

4.3.2.4 Confocal Laser Scanning Microscopy (CLSM)

CLSM was accomplished with the assistance of Dr Anthony Hayes (School of Bioscience, Cardiff University). The stained sections were viewed using a Leica TCS SP2 AOBS spectral confocal microscope (Leica; Wetzalar, Germany) and scanning with the appropriate excitation laser lines for the fluorescent labels of each probe.

Single and mixed cultures of planktonic microorganisms were analysed with multiplex probe staining. For multichannel recordings, fluorochromes were scanned sequentially to eliminate spectral overlap.

4.3.2.5 Gram staining

ETT sections were de-waxed as described above and the microscope slides were flooded with 1% (v/v) crystal violet for 2 min. Followed by brief washing with distilled water, slides were flooded with 3% (v/v) Lugol's iodine for 2 min sections decolourised with 100% acetone for 2s, rinsed with distilled water and flooded with 1%(v/v) carbol-fushin as counterstain for 2 min, washed with distilled water and air dried. Sections were viewed using light microscopy (Cark Zeiss Ltd, UK).

4.3.2.6 Scanning electron microscopy

Sections were left in 9 ml of FAB, and for fixation of the biofilms 1 ml of 25% glutaraldehyde (to give final concentration of 2.5% v/v) was added and incubated at room temperature for 24 h. Samples were then transferred for microscopy in the medical microscopy unit of the School of Medicine (Cardiff University) and imaging was achieved with the assistance of Dr Jan Hobot.

Sections were washed in increasing concentrations of ethanol (50%, 70%, 90% and 100%) for 5 min each, then washed ($\times 3$) with hexamethyldisilazane and air dried overnight. Self-adhesive carbon tabs (25 mm diameter, G3348N, Agar Scientific) were placed onto an

aluminium stub and the ETT sections were then placed onto the tabs and sputter coated with gold for 8 min. The sections were viewed using a JEOL 840A SEM operated at 5 kV and a working distance of 12 mm. Digital images captured using SIS software at screen magnifications of x100.

4.3.3 Preparation of *in vitro* biofilms for gene expression analysis

4.3.3.1 Formation of biofilms in ETT sections

Biofilms were initially grown on ETT sections with media and culture conditions identical to those previously described (section 4.3.2). To optimise biofilm quantities and subsequent RNA yields, different ETT section lengths (1, 3, 6 and 12 cm of an 8 mm diameter ETT; Portex®, Smiths Medical, Kent, UK) were used.

4.3.3.1.1 Biofilms grown in 6-well plates

In vitro biofilms were also developed in 6-well plates with shorter incubation times in an attempt to increase RNA yields. The principle of the biofilms grown on ETT sections were maintained, however *P. gingivalis* was not included in these experiments. Bacteria were initially cultured aerobically at 37°C for 24 h on BA, and SDA was used for *C. albicans*.

One to 3 colonies of each microorganism were used to separately inoculate 10 ml of BHI, which was incubated aerobically and statically overnight at 37°C. Turbidity of the broth containing each microorganism was then standardised between 0.8 to 1.0 OD at 600 nm (Implen, Geneflow) and then diluted 10-fold in BHI to give approximately 10^7 CFU/ml. A 3-ml volume of this inoculum was added to the wells of tissue culture plates (9.5 cm^2 area per well; Starsted, Leicester, UK), which were incubated without agitation at 37°C for 24 h.

Single species biofilms incubated for 24 h were used as controls for mixed and dual species biofilms. Mixed biofilms, included all test strains incubated for 24 h. Dual species biofilms, involved initial development of a 24 h single species biofilm (*S. mutans* or *C. albicans*) and to

this, a VAP pathogen was added and incubated for a further 24 h Figure 4.2 illustrates the protocol followed for these biofilms

For mixed species biofilms, all microorganisms were prepared in BHI as above and then added together in equal volumes prior to inoculation of the tissue culture wells and 24 h incubation at 37°C. For dual species biofilms, the first 24 h of biofilm growth was either with single species of *S. mutans* or *C. albicans*. After this time, the broth was removed and a 3-ml volume of BHI with either *S. aureus* or *P. aeruginosa* was introduced gently to avoid disruption of the existing biofilm. This preparation was then incubated for a further 24 h.

4.3.3.2 Recovery of biofilms on ETT sections

Biofilm recovery was initially performed as described earlier for culture analysis (section 4.3.2.1). The method was later modified to replace the cotton swab with a sterile 25 cm cell scraper (Starsted, Newton, USA) to recover the biofilm from the ETTs whilst immersed in 3 ml of PBS in 35-mm tissue culture dishes (Iwaki® Sterling, Billingham, UK). Resuspended biofilms were transferred to a universal container and centrifuged at 4000 rev/min for 13 min (IEC CL 10, Thermo scientific), the supernatant was discarded and the biofilm cells resuspended in 2 ml of RNA Bacteria Protect® (Qiagen, Manchester, UK), vortex mixed for 10 s and incubated at room temperature for 5 min. After this treatment, the preparation was centrifuged (10,000 rev/min for 5 min; IEC CL 10, Thermo scientific), the supernatant discarded and the biofilm pellets were either frozen at -80°C or immediately processed for RNA extraction.

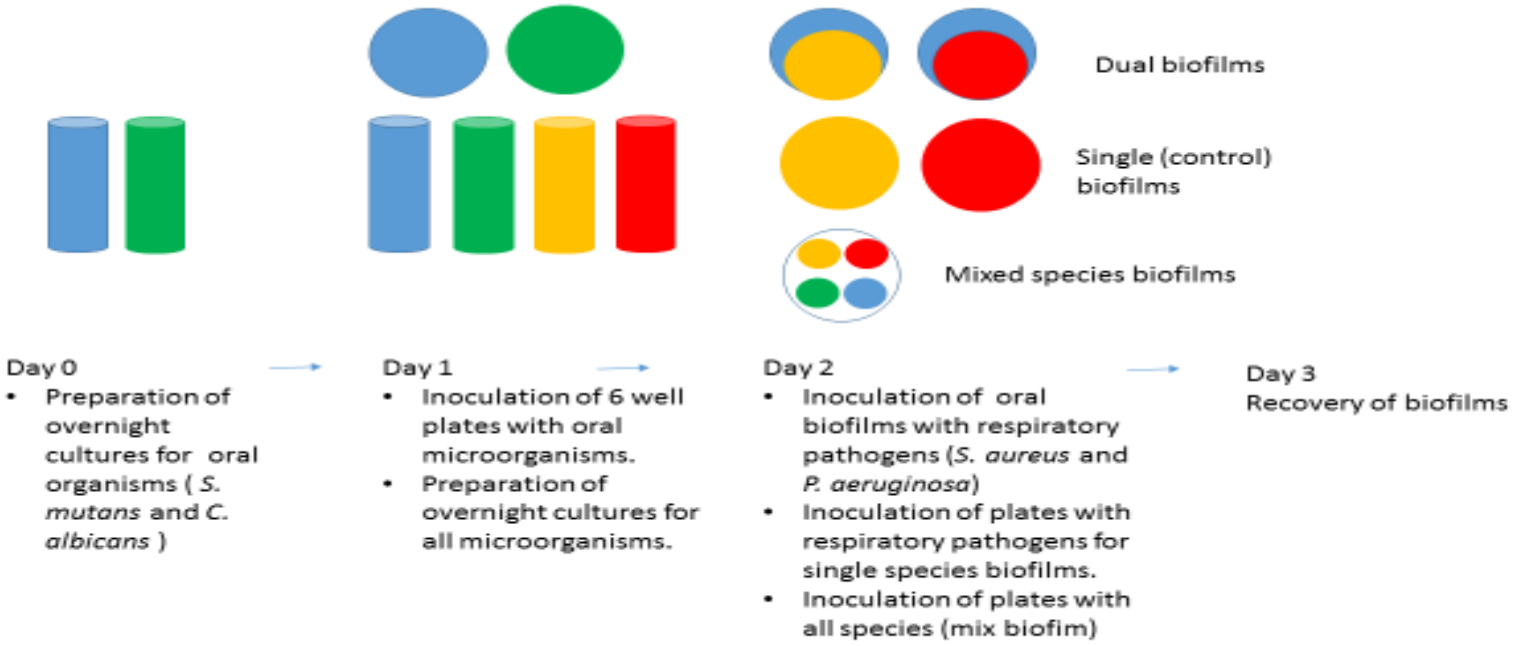

Figure 4.4.2 Schematic of *in vitro* biofilm protocol for gene expression analysis.

4.3.3.3 Recovery of biofilms from 6-well tissue plates

After incubation, broth was removed from the wells which were then gently washed with 3 ml of ice cold PBS. One-ml of RNA Bacteria Protect® (Qiagen) was placed in each well and a sterile cell scraper (Starsted) was used to disrupt the biofilm. Biofilm was resuspended by pipetting, vortex mixed and incubated for 5 min at room temperature followed by centrifugation at 6000 rev/min for 5 min (Heraeus PICO 27, Thermoscientific). The supernatant was discarded and the biofilm pellets stored at -80°C.

4.3.4 RNA extraction from *in vitro* biofilms

4.3.4.1 Cell lysis

For all protocols, an initial enzymatic disruption was performed. Frozen bacterial cells were thawed at room temperature prior to being suspended and vortex mixed in either 100 µl or 200 µl of Lysing buffer (LTE) (10 mM Tris-HCL; 1 mM EDTA; 10 mg/ml lysozyme, pH 8.0). For samples containing *S. aureus*, LTE buffer was supplemented with 200 µg/ml of lysostaphin (Sigma, Dorset, UK). Samples were then incubated with shaking (250 to 300 rev/min, RT, 90 min, Stuart, SI500). RNA extraction protocols were then performed as described below.

4.3.4.2 Qiagen RNA extraction protocol

The manufacturer's (Qiagen) recommended protocol for total bacterial RNA extraction was followed using the RNeasy®mini kit. In brief, to the initial lysate, 350 µl of RTL buffer (containing 10µl/ml of β-mercaptoethanol) was added and vortex mixed for 5 s before transferring into a Pathogen Lysis Tube® (Qiagen) with small glass beads. Biofilm cells were homogenised twice for 30 s using a Mini-Bead Beater (Stratech Scientific Ltd., Soham, UK).After cell disruption, the lysate was transferred to a clean 1.5 ml tube containing 350 µl of absolute ethanol (molecular grade; Sigma). The ethanol/lysate mix was then transferred to an RNAeasy column and centrifuged (10000 rev/min, 1 min; Heraeus PICO 27,

Thermoscientific). The flow-through was discarded and 350 µl of buffer RW1 added followed by centrifugation (10.000 rev/min, 1 min). The flow-through was again discarded and 80 µl of DNase 1 incubation mix (10 µl of DNase stock solution; 70µl of buffer RDD; Qiagen) was applied to the column membrane for DNA digestion and incubated (RT, 15 min). A 350-µl volume of RW1 buffer was added and incubated at room temperature for 5 min. After incubation, the column was centrifuged (10.000 rev/min, 1 min) and the flow through discarded. The membrane was washed twice by adding 500 µl of RPE buffer followed by centrifugation (10.000 rev/min, 1 min). The column was dried by centrifugation (10.000 rev/min, 1 min) before adding 30 µl of RNase free water to elute the RNA. The column was then placed in a clean collection tube and centrifuged (10.000 rev/min, 1 min) and this step was repeated using the RNA eluate to increase yield. Modifications of this method included addition of 100 µl of Proteinase K (Qiagen) to 1 ml of the LTE buffer; changing the enzymatic lysis incubation from 90 min RT to 30 min at 37°C in parallel samples; and the exclusion of glass beads.

4.3.4.3 Trizol RNA extraction protocol

To the initial lysate, 500 µl of TRIsolate reagent (NBS Biologicals, Cambridgeshire, UK) was added and mixed. The mix was homogenised using a tissue homogeniser (T 8 ULTRA-TURRAX® IKA®-WERKE, Staufen, Germany) for 10 s and incubated for 5 min at room temperature. The preparations were then centrifuged (12.000 × *g* for 10 min at 4°C) to remove any insoluble material like extracellular membranes, polysaccharides, and high molecular mass DNA. The clear supernatant was transferred to a clean 1.5 ml microcentrifuge tube, and a 100-µl volume of chloroform (>99%; Sigma) added. The preparation was vortex mixed for 15 s and incubated (15 min, room temperature). A phase lock gel microfuge tube (5 Prime, Hilden, Germany) was initially centrifuged (12.000 x *g*, room temperature, 30 s) to remove the gel, and the above preparation added and

centrifuged (12.000 x *g*, 4°C, 15 min). The upper aqueous phase containing RNA was transferred to a clean tube to which a 250-µL volume of 2-propanol (Fisher Scientific, Loughborough, UK) was added and vortex mixed. The preparation was then incubated for 10 min at room temperature and centrifuged (12.000 × *g*, 4°C, 10 min). The supernatant was discarded and the RNA pellet washed with 1 ml of 75% (v/v) ice cold ethanol prepared with nuclease free water and centrifuged (7.500 × *g*, 4°C, 5 min). The supernatant was discarded and the RNA pellet air dried for 15 min. To re-suspend the RNA, 30 µl of RNAse free water was added and the suspension incubated in a water bath (Grant, Shepreth, UK; 60°C, 15 min).

4.3.4.4 Combined Phenol/Qiagen RNA Protocol.

To the initial lysate, 350 µl of RLT buffer (containing 10µl/ml of β-mercaptoethanol) was added and this was vortex mixed for 15 s. To the mixture, 500 µl of phenol:chloroform:isoamyl alcohol (25:24:1 saturated with 10 mM Tris pH 8.0, 1 mM EDTA; Sigma) was added, mixed and transferred to a Pathogen Lysis Tube®(Qiagen) which contained small glass beads. The preparation was then disrupted in a Mini-Bead Beater (Stratech Scientific Ltd) for 1 min at maximum speed. Preparations were centrifuged (11.000 x *g*, room temperature, 5 min) and the upper aqueous transparent phase transferred to a 1.5 ml tube containing 700 µl of absolute ethanol, mixed and incubated (-20°C, 15 min) to precipitate RNA. The lysate was transferred to an RNAeasy column and centrifuged (11.000 x *g*, 1 min). The flow-through was discarded, and any residual DNA removed by addition of 80 µl of DNase 1 incubation mix (10 µl of DNase stock solution; 70µl of buffer RDD; Qiagen) to the column membrane and incubation (room temperature, 30 min). After this step, 350 µl of RW1 buffer was added for 5 min and then centrifuged (11.000 x *g*, 1 min). Wash steps with RPE buffer and RNA elution were performed as described earlier (Qiagen protocol) and

the RNA then resuspended in a 25-µl volume of nuclease free water. This method was also undertaken without the use of glass beads.

4.3.4.5 Quality, quantification and storage of RNA

The quantity of RNA in the eluates was measured using a spectrophotometer (Nano-Vue™, GE healthcare) and the A260/A280 ratio confirmed to be of ≥1.7. To check integrity of RNA extracts, visualisation of RNA following electrophoresis was also performed. Using this approach, intact RNA would show as two clear rRNA (28S and 18S) bands. For electrophoresis, an aliquot of approximate 500 ng of RNA was loaded into 1.5% (w/v) agarose gels prepared in 0.5 × Tris-Borate-EDTA (TBE; 0.1 M Tris Base; 0.09 M Boric Acid; 0.1 mM EDTA) buffer and stained with Safeview® (NBS biologicals; Huntingdon, UK). Gels were run at 70 V/cm^2 for 1 h and visualised under UV light using a GelDoc system (Bio-Rad). Extracted RNA was stored at -80°C.

4.3.4.6 Removal of DNA from RNA extracts

Elimination of genomic DNA from RNA extracts was necessary to avoid interference with expression analysis. To remove DNA, the RQ1 RNase-Free DNase (Promega) protocol was followed using the manufacturer's instructions. Reactions included 1 µg of RNA, 7 µl of water, 1 µl of RQ1 DNase and 1 µl of buffer (×10) within a PCR tube. Reactions were incubated at 37°C for 30 min, after which 1 µl of the provided 'Stop solution' was added. The mixture was then incubated in a water bath for 10 min at 65°C to deactivate the DNase activity. Treated samples were then immediately subjected to reverse transcription.

4.3.5 Preparation of cDNA libraries

All reagents used to prepare cDNA were obtained from Promega. A total of 1 µg of RNA was used for reverse transcription and this involved a two-stage process. Firstly, to 1 µg of RNA, 50 ng of random primers were added along with RNase free water to make a final volume of

15 µl. The mixture was briefly centrifuged and incubated at 70°C for 30 min. Preparations were then rapidly cooled on ice. The mixture was then incorporated to a 'master mix' containing 10 mM of dNTPs, 25 U of RNAsin, 1 µl of M-MLV enzyme, 5 µl of M-MLV reaction buffer (×5) and 2 µl of water, to generate a total volume of 25 µl. The mixture was incubated at 37°C for 1 h, and then cooled to 4°C, prior to storage at -20°C. Before use cDNA was diluted 1:5 in nuclease free water.

4.3.6 PCR on cDNA libraries

PCR targeted six virulence genes for *P. aeruginosa*, these included genes involved in adhesion (*cupA*) alginate synthesis (*algD, mucA, mucB*) and biofilm formation and antimicrobial resistance (2070, 5033) (Edwards and Saunders 2001; Mah *et al*. 2003; Zhang *et al*. 2013). The constitutively expressed gene encoding the 50S ribosomal protein L21 (*rplU*) was used as housekeeping control (Mah *et al*. 2003) (Table 4.5). Similarly for *S. aureus* genes related to adhesion (*fnbA*), polysaccharide intercellular adhesins (PIA) production (*icaC, icaR, icaB, icaA*), quorum sensing (*AgrI*) (Atshan *et al.* 2013; Cafiso *et al*. 2004; Malic 2008; Shopsin *et al.* 2003) and the 16S gene was used as control (Table 4.6).

PCR volumes were 20 µl and included 2 µl of each forward and reverse primers at 3 µM, 10 µl of Primer design MasterMix® and 5 µl of the diluted cDNA template.

PCRs were performed in a real-time PCR instrument (ABI 7000 prism or QuantStudio ®6 Flex; Applied Biosystems). Cycling conditions included an initial denaturation step (95°C ;2 min) followed by 40 cycles of denaturation (95°C ;15 s), annealing (55°C ;30 s) and extension (72°C; 30 s). All reactions were undertaken in triplicate for three biofilms prepared on separate occasions resulting in a total of 9 biofilms being analysed.

Table 4.5 Primers used to amplify virulence factors and control gene for *Pseudomonas aeruginosa*.

Gene	Primer sequence	Reference
rplU (housekeeping)	F: 5'-CGCAGTGATTGTTACCGGTG-3' R: 5'-AGGCCTGAATGCCGGTGATC-3'	(Mah *et al*. 2003; Palmer *et al.* 2007b)
cupA1	F: 5'-CATGCGCAGTGGTATTGGCCTTTG-3' R: 5'-GAACAGGGTGGTGAAATGCTCGTC-3'	(Mah *et al.* 2003)
algD	F: 5'-GCGACCTGGACCTGGGCT-3' R: 5'-TCCTCGATCACGGGGATC-3'	(Edwards and Saunders 2001)
mucA	F: 5'-GGAAACTCTGTCCGCTGTGATGGA-3' R: 5'-GGCTCGCGGTGCATGACG-3'	(Edwards and Saunders 2001)
mucB	F: 5'GCTGCCGACGCTTCCGACTGGCT-3' R: 5'-CGCTGTCCACGCGATGCC-3'	(Edwards and Saunders 2001)
5033	F: 5'GGCGTTCTGGTAGGAACCTG-3' R: 5'AGACCACGTTGCCGAAGCTG-3'	(Zhang *et al*. 2013)
2070	F: 5'CTCCGCGGTGGATCTCAACA-3' R: 5'GTCGAAGCGGCCTTCGTTCA-3'	(Zhang *et al.* 2013)

Table 4.6 Primers used to amplify virulence factors and control genes for *Staphylococcus aureus.*

Gene	Primer sequence	Reference
16S (housekeeping)	F 5'-GGGACCCGCACAAGCGGTGG-3'	(Atshan *et al.* 2013)
	R: 5'-GGGTTGCGCTCGTTGCGGGA-3'	
agrI	F: 5'-ATGCACATGGTGCACATGC-3'	(Shopsin *et al.* 2003)
	R: 5'-GTCACAAGTACTATAAGCTGCGAT-3'	
icaB	F: 5'-CACATACCCACGATTTGCAT-3'	(Malic 2008)
	R: 5'-TCGGAGTGACTGCTTTTTCC-3'	
icaAb	F: 5'-CGCACTCAATCAAGGCATTA-3'	(Malic 2008)
	R: 5'-CCAGCAAGTGTCTGACTTCG-3'	
icaRa	F: 5'-CCAAATTTTTGCGAAAAGGA-3'	(Malic 2008)
	R: 5'-TACGCCTGAGGAATTTTCTG-3'	
IcaC	F: 5'-CTTGGGTATTTGCACGCATT-3'	(Cafiso *et al*. 2004)
	R: 5'-GCAATATCATGCCGACACCT-3'	
fnbA	F: 5'-AAATTGGGAGCAGCATCAGT-3	(Atshan *et al.* 2013)
	R: 5'-GCAGCTGAATTCCCATTTTC-3'	

4.3.7 gDNA contamination assessment

To further confirm total elimination of gDNA, in addition to a cDNA preparation, a 'non-RT' cDNA control was prepared by following all the c-DNA library preparation steps but replacing the M-MLV enzyme with water. A PCR cycle was completed as above targeting the housekeeping gene for non-RT controls and samples. The PCR products were then visualised by gel electrophoresis

4.3.8 Statistical analysis

For statistical analysis the Graph Pad Prism ® statistical software was used. Culture analysis and qPCR results of mixed and dual biofilms were analysed with Wilcoxon matched pair signed ranks test with single biofilms of identical conditions used as controls.

4.4 Results

4.4.1 Biofilm culture

In vitro biofilms were created using different combinations of five microbial species as examples of typical oral commensal microorganisms (*S. mutans, P. gingivalis* and *C. albicans*) and key respiratory pathogens for VAP (*S. aureus* and *P. aeruginosa*). To assess biofilm formation, cells were recovered from the lumen of ETT sections and enumerated to estimate microbial growth. Single species biofilms were used as comparative controls and the average inoculum levels of each species used is presented in Table 4.7. The single/mixed biofilms were prepared and tested on multiple occasions (10 to 20 times).

In single species biofilm preparations, all tested microorganisms were found to generate ETT biofilms (Figure 4.3). In terms of cell numbers, the most extensive single species biofilms were produced by *P. gingivalis* (1.9 x 10^5 CFU/ml; SD 6.1 x 10^5 CFU/ml) and *P. aeruginosa* (1.7 x 10^5 CFU/ml; SD 1.9 x 10^5 CFU/ml). For all bacterial species, when comparing single species to mixed species biofilms, higher cell counts were encountered in the latter. The

most notable increase was evident for mixed species biofilms of *S. aureus* and *S. mutans* (p= <0.05). In the respective single species biofilms the cell counts were of the order of 1×10^4 CFU/ml. However, in mixed species biofilms, the counts were 100-fold higher (Figure 4.3). In contrast, in the case of *C. albicans*, lower counts occurred in mixed species biofilms compared with single species biofilms (1×10^1 CFU/ml compared to 1×10^2 CFU/ml; p<0.005). Interestingly, the strict anaerobic bacterium *P. gingivalis* was recovered from aerobic mixed species biofilm preparations in these studies.

In dual species biofilms, pre-existing biofilms formed by an oral microorganism were later infected with a respiratory pathogen. In these experiments, initial biofilms formed by *S. mutans, P. gingivalis* and *C. albicans* did not subsequently lead to significant differences in *P. aeruginosa* biofilm growth in ETT sections (p= >0.5; Figure 4.4).

Figure 4.5 shows dual species biofilms of *S. aureus* with oral species. It was evident that a significantly higher number of *S. aureus* CFU/ml was obtained when cultured with *S. mutans*, 2.2×10^5 CFU/ml compared to 3.8×10^4 CFU/ml (p= < 0.05). No significant changes in cell counts were seen in dual biofilms of *S. aureus* with *C. albicans* or *P. gingivalis (*p= >0.5).

To estimate the impact on biofilm formation by oral species in the presence of respiratory pathogens the number *S. mutans, C. albicans* and *P. gingivalis* recovered from ETT biofilm sections were also recorded in dual preparations with *S. aureus* and *P. aeruginosa*. Figure 4.6 shows no significant difference (p= >0.05) in *S. mutans* cell counts from the single species biofilms occurred when this species was cultured in dual biofilms with either *P. aeruginosa* or *S. aureus*. Of note was that numbers of *C. albicans* were significantly reduced when combination with *P. aeruginosa* (2.3×10^3 CFU/ml compared to 1.0×10^1 CFU/ml; p= 0.005; Figure 4.7). No statistical difference was seen in the counts of *C. albicans* in the presence or absence of *S. aureus* (p= >0.05). For *P. gingivalis* cultured in aerobic biofilms (Figure 4.8), the mean number of cells recovered from dual species biofilms (both *P. aeruginosa* and *S.*

aureus) was lower than in single biofilms. However, this finding was inconsistent and a statistical difference (p= >0.5) was not evident.

Table 4.7 Standardised inoculum used for development of endotracheal tube biofilms.

Microorganims	**Inoculum**
Streptococcus mutans	2.0×10^8
Porphyromonas gingivalis	1.7×10^7
Candida albicans	7.8×10^6
Staphylococcus aureus	4.3×10^8
Pseudomonas aeruginosa	8.0×10^7

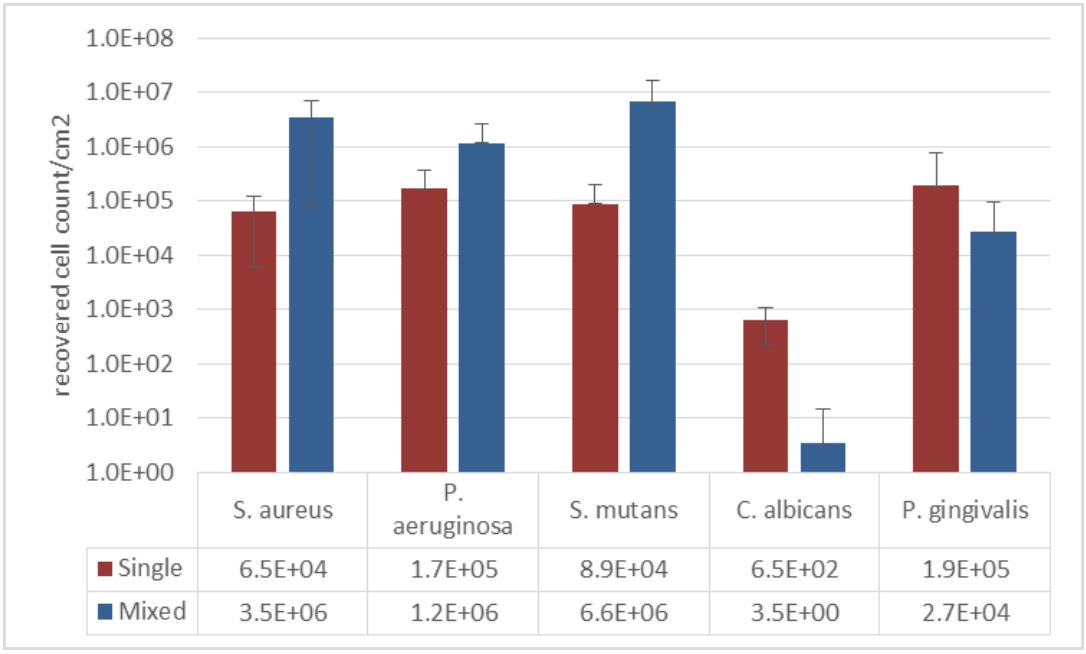

Figure 4.3 Microbial cells recovered from single and mixed species biofilms.

Average results shown for estimated number of recovered cells per cm^2 of biofilm grown in endotracheal tube sections. Bars represent standard deviation.

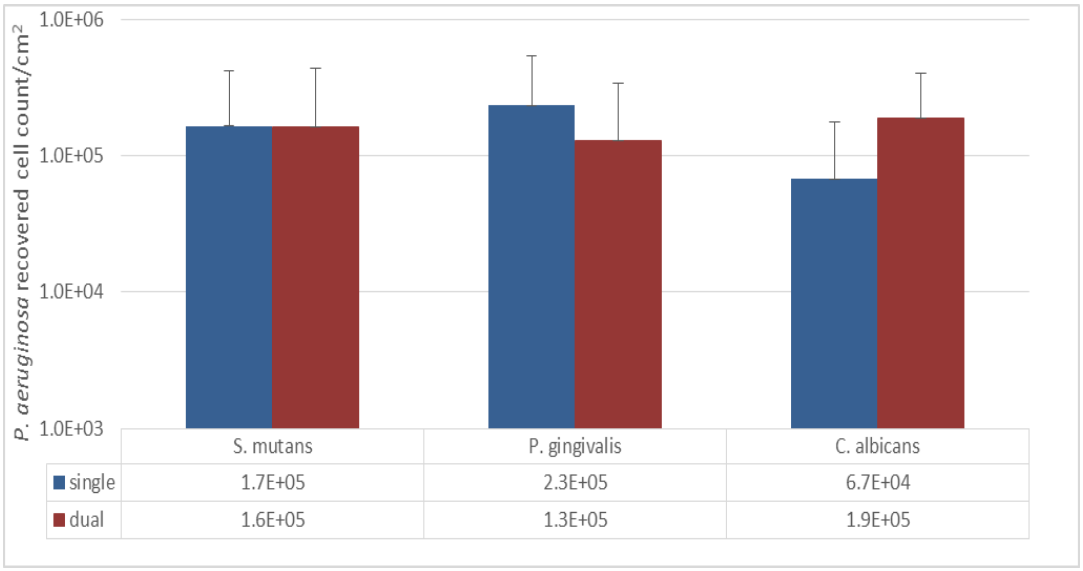

Figure 4.4 Recovery of *Pseudomonas aeruginosa* from dual species in vitro biofilms with oral microbial species

Mean results shown for estimated number of recovered viable cells per cm^2 of biofilm grown in endotracheal tube sections. Bars represent standard deviation.

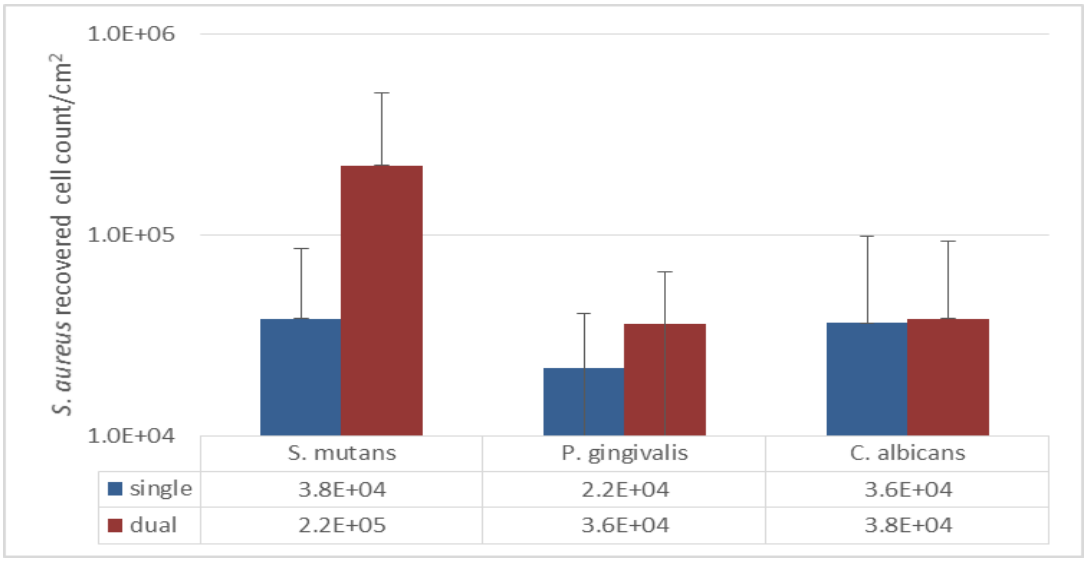

Figure 4.5 Recovery of *Staphylococcus aureus* from dual species *in vitro* biofilms with oral microbial species.

Mean results shown for estimated number of recovered viable cells per cm^2 of biofilm grown in endotracheal tube sections. Bars represent standard deviation.

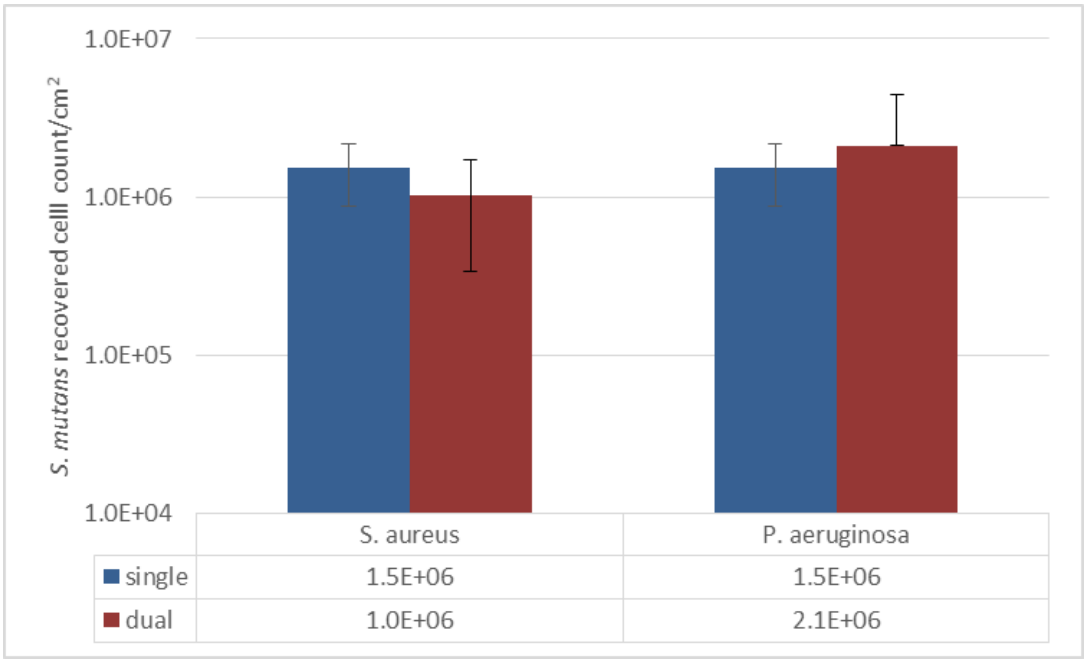

Figure 4.6 Recovery of *Streptococcus mutans* from dual species *in vitro* biofilms with *Staphylococcus aureus* or *Pseudomonas aeruginosa.*

Mean results shown for estimated number of recovered viable cells per cm^2 of biofilm grown in endotracheal tube sections. Bars represent standard deviation.

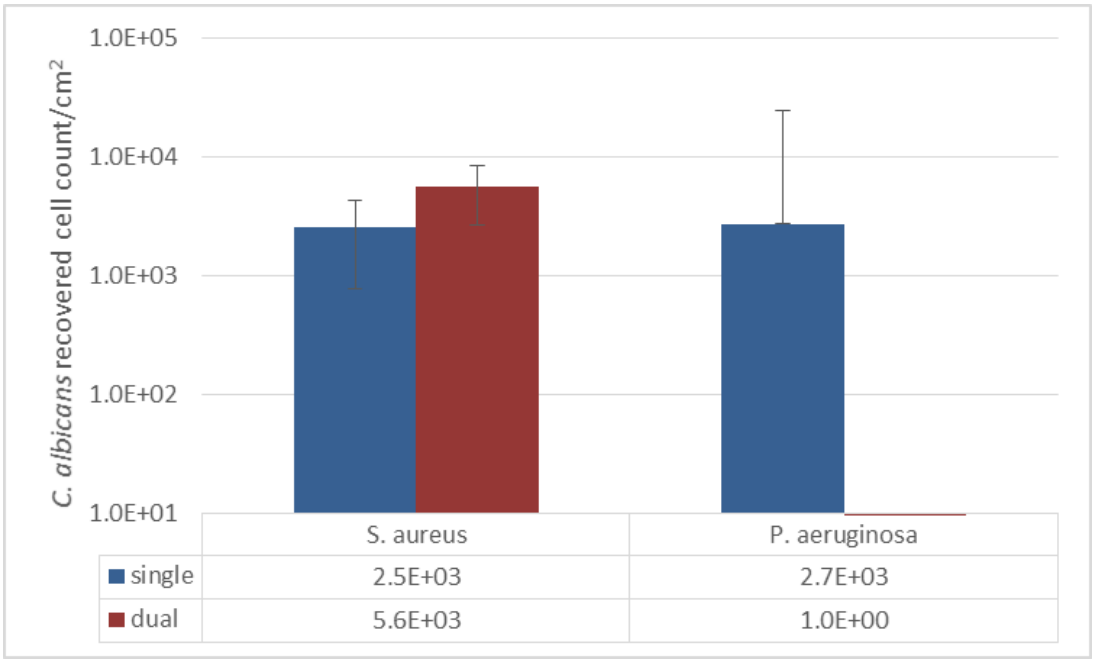

Figure 4.7 Recovery of *Candida albicans* from dual species in vitro biofilms with *Staphylococcus aureus* or *Pseudomonas aeruginosa*.

Mean results shown for estimated number of recovered viable cells per cm^2 of biofilm grown in endotracheal tube sections. Bars represent standard deviation.

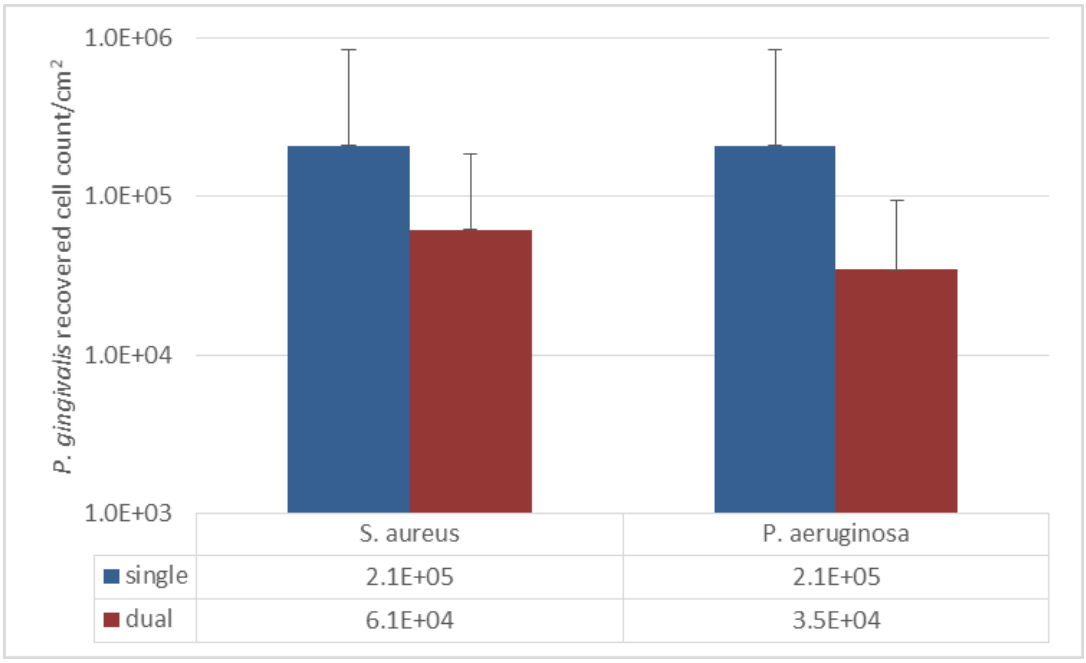

Figure 4. 8 Recovery of *Porphyromonas gingivalis* from dual species in vitro biofilms with *Staphylococcus aureus* or *Pseudomonas aeruginosa*.

Mean results shown for estimated number of recovered viable cells per cm^2 of biofilm grown in endotracheal tube sections. Bars represent standard deviation.

4.4.2 FISH, Gram staining and SEM imaging of biofilms

All PNA probes were validated using planktonic cultures of the target species (Figure 4.9). Mixed planktonic cultures were used to validate probe specificity and each probe served as a negative control to each other to discard autofluorescence or background fluorescence. Using CLSM, up to 3 probes could be exited simultaneously and an overlay with all probes subsequently generated (Figure 4.10).

FISH using PNA probes applied to ETT sections, and these *in vitro* biofilms showed multiple cell layers and channels appropriate of mature biofilms (Figure 4.11). However, it was not possible to obtain multiplex images involving all the different probes. Gram staining also showed a multiple cell layered biofilm (Figure 4.12), whilst SEM showed high density biofilms embedded in a matrix with multiple cell layers and possibly water/nutrition channels (Figure 4.13).

4.4.3 RNA Extraction

RNA extraction from biofilms created in ETT sections failed to yield adequate RNA quantities with all protocols. Successful RNA extractions were only achieved from biofilms developed in 6-well plates using the combined phenol/Qiagen method with satisfactory A260/A280 ratios evident between 1.8–2.2. RNA gels confirmed the presence of the 16S and 23S bands (Figure 4.14).

Elimination of gDNA using DNase I treatment during column RNA recovery (as per manufacturer's instructions) was insufficient, as gDNA band was evident prior to cDNA preparation using gel electrophoresis. Longer incubation periods for the DNase I treatment, or repeating the treatment still did not eliminate gDNA. The additional step of treating RNA extracts with RQ1 RNase-Free DNase (Promega) was however successful in completely removing gDNA remnants, as evident by the absence of visible products from non-RT controls (Figure 4.14).

4.4.4 Gene expression

Figure 4.15 shows relative gene expression results for *P. aeruginosa* biofilms. Biofilms combining *P. aeruginosa* with *S. mutans* did not lead to different gene expression for any of the studied genes (p= >0.05), whereas dual preparations involving *C. albicans* showed upregulation of the *algD* and *cupA* genes (p= <0.05). Mixed species biofilms increased expression of the *alg D*, *mucA, muc B, cupA* and 5033 (p= <0.05) In the case of the *P. aeruginosa* 2070 gene, no difference in expression for single and dual/mixed species biofilms were encountered (p= >0.05).

Figure 4.16 shows relative *S. aureus* gene expression in different species combination biofilms. The combination of *S. aureus* with *S. mutans* upregulated expression of the *S. aureus* genes *agrI, icaC, icaAb, icaBa, icaRa* (p= <0.05). However, these genes were not altered when *S. aureus* was cultured in biofilms with *C. albicans* (p= >0.05). Mixed species biofilms also exhibited upregulation of *agrI, icaC, icaAb, icaRa.* Expression of the *S. aureus fnbA* gene did not significantly alter between single, dual or mixed biofilm combinations.

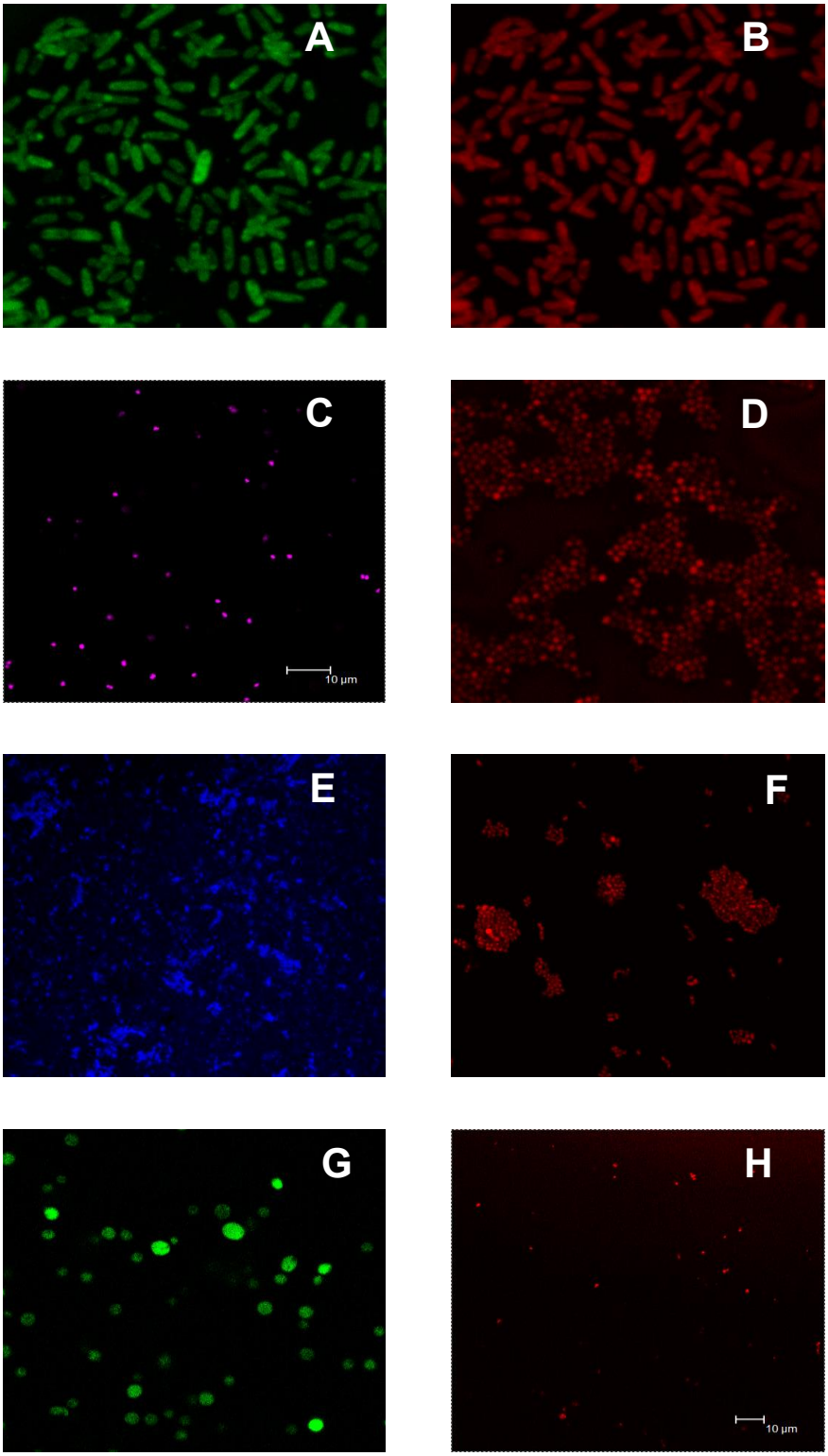

Figure 4.4.9 PNA-FISH targeting single species of planktonic bacteria. **A:** *P. aeruginosa* specific PNA probe, **B:** *P. aeruginosa* with the universal bacterial PNA probe. **C:** *S. aureus* specific PNA probe, **D:** *S. aureus* universal bacterial PNA probe, **E:** *S. mutans* specific PNA probe, **F:** *S. mutans* with universal PNA probe, **G:** *C. albicans* specific PNA probe, **H:** *P. gingivalis* with the universal bacterial PNA probe.

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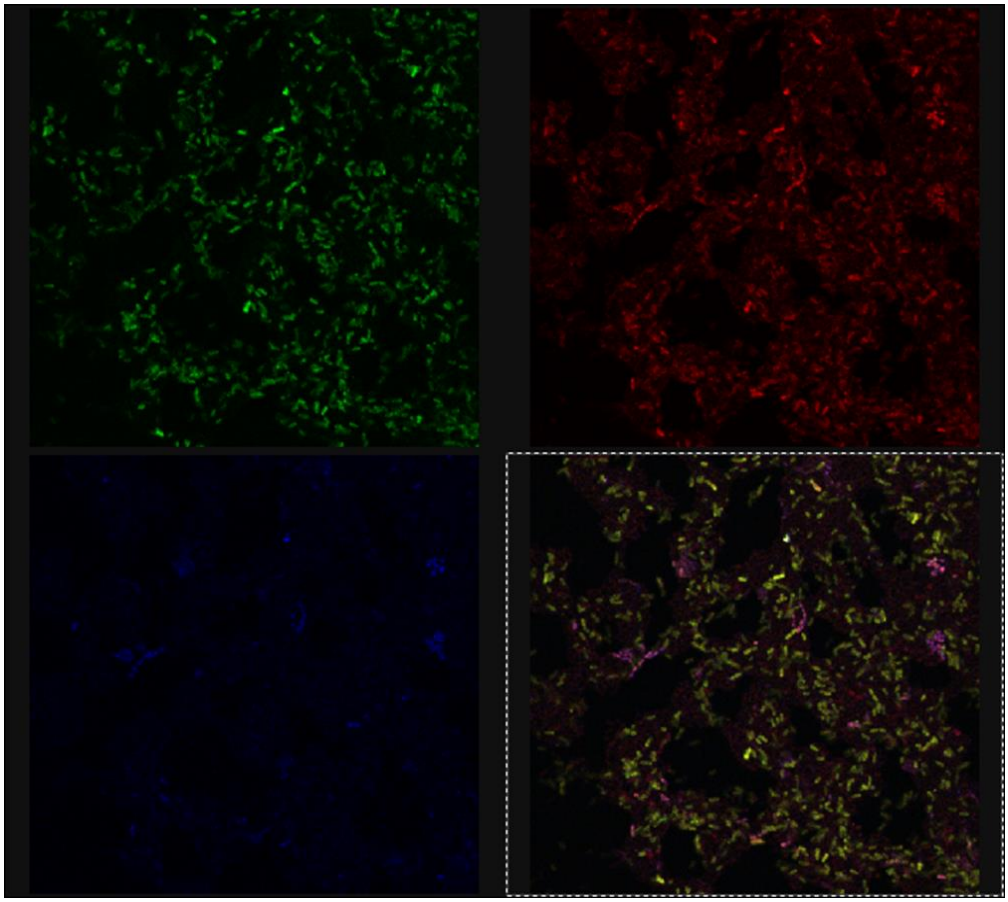

Figure 4.10 Multiplex PNA-FISH targeting mixed species of planktonic bacteria.

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Top left: *P. aeruginosa* specific PNA probe. **Top right:** Bacteria stained with a universal bacterial PNA probe. **Bottom left:** *S. aureus* specific PNA probe. **Bottom right:** overlay of the three different channels, *S. aureus* depicted as purple as hybridises with both the universal (red) and specific probe (blue), similarly, *P.aeruginosa* depicted as light green/yellow showing hybridisation of both specific and universal PNA probes.

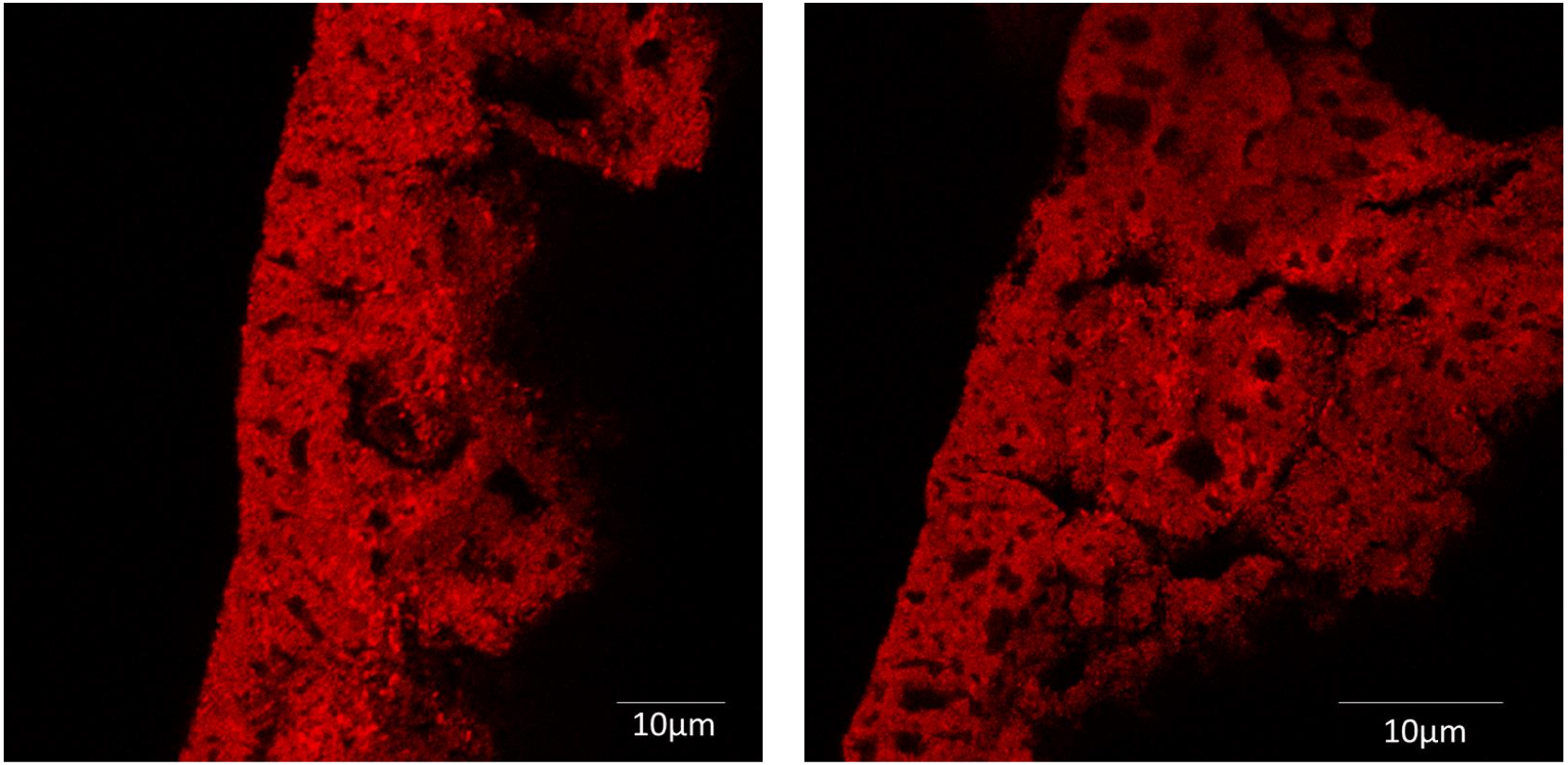

Figure 4.11 Confocal laser scanning microscopy of a *Streptococcus mutans* biofilm grown on an endotracheal tube section and stained with a universal bacterial PNA probe.

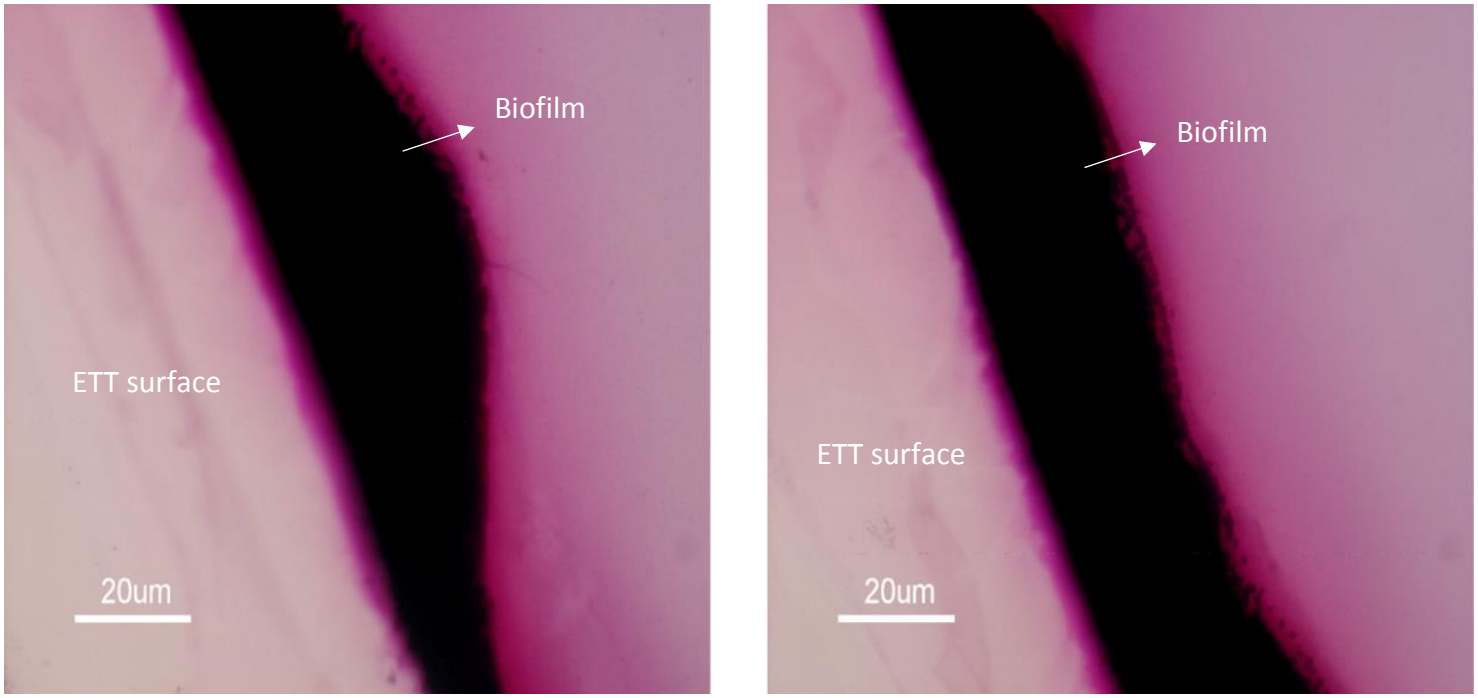

Figure.4.12 Gram staining of a mixed species *in vitro* biofilm.

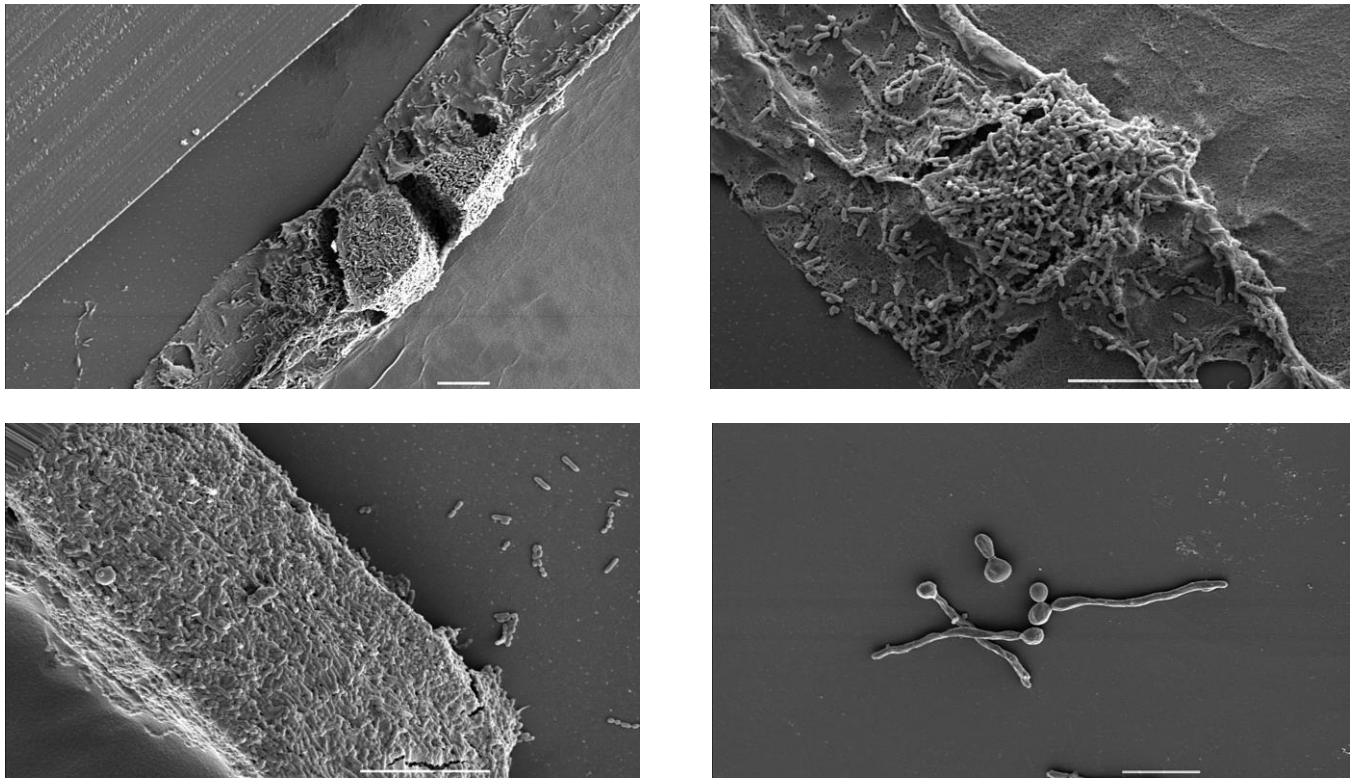

Figure 4.13 : Scanning electron microscopy of in vitro biofilms grown on endotracheal tube sections.

Top left and right are *S mutans* and *P. aeruginosa* dual species biofilms. Bottom left: Mixed species biofilm. Bottom right: image from a *C. albicans* single species biofilm illustrating hyphal formation by adherent *Candida*. Bar marker is 10 µm.

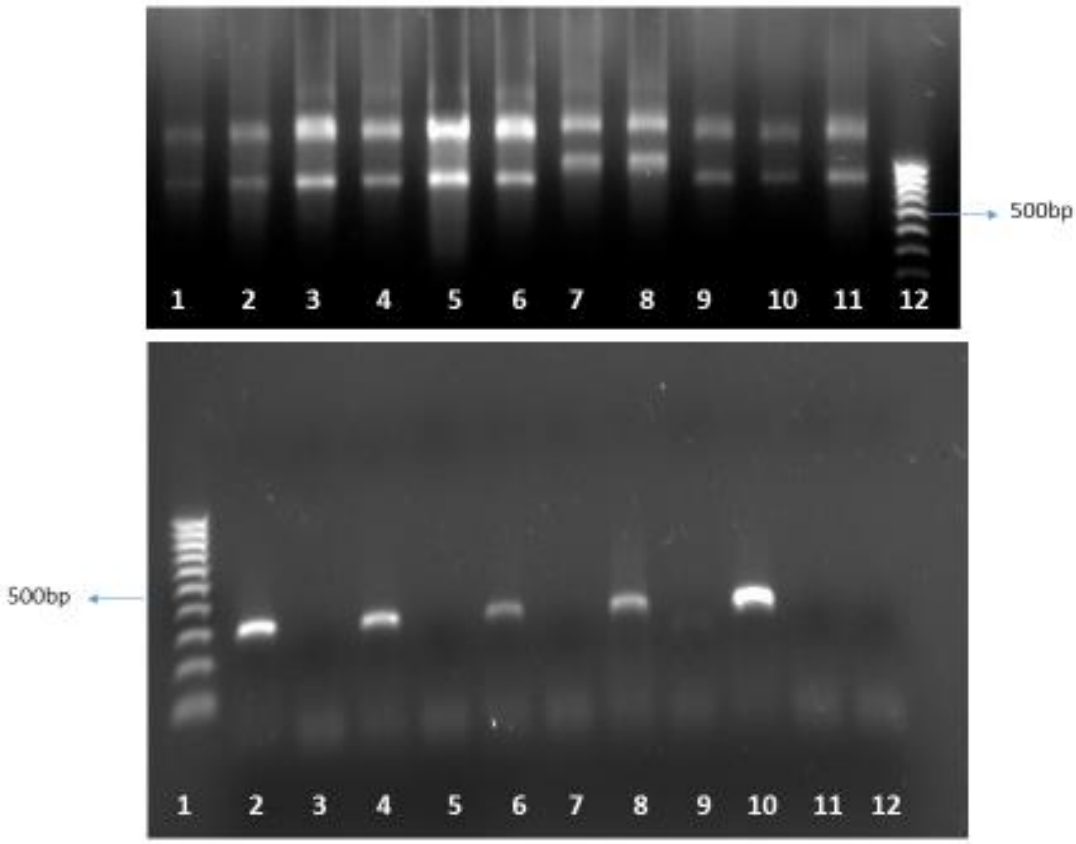

Figure 4.14 Agarose gels showing RNA extracts and qPCR products from in vitro biofilms.

Top: 1% agarose gel showing RNA extracts from *in vitro* biofilms *P. aeruginosa* single, 2. *P. aeruginosa* + *S. mutans*, 3. *P. aeruginosa* + *C. albicans*, 4, 5, 6 mixed species, 7. *S. aureus* single, 8, 9. *S. aureus* +*S. mutans*, 10, 11. *S. aureus* + *C. albicans*, 12. 100bp ladder.

Bottom: 1.5% agarose gel showing qPCR products using primer pair for the housekeeping gene (*rplU*) of *P. aeruginosa.* Lanes 2, 4, 6, 8 and 10 are PCRs derived from *P. aeruginosa* single dual and mixed biofilms. Lanes 3, 5, 7, 9 are negative controls (no reverse transcriptase). Lanes 11 and 12 are water controls. The absence of bands in controls demonstrated complete removal of gDNA in the RNA template.

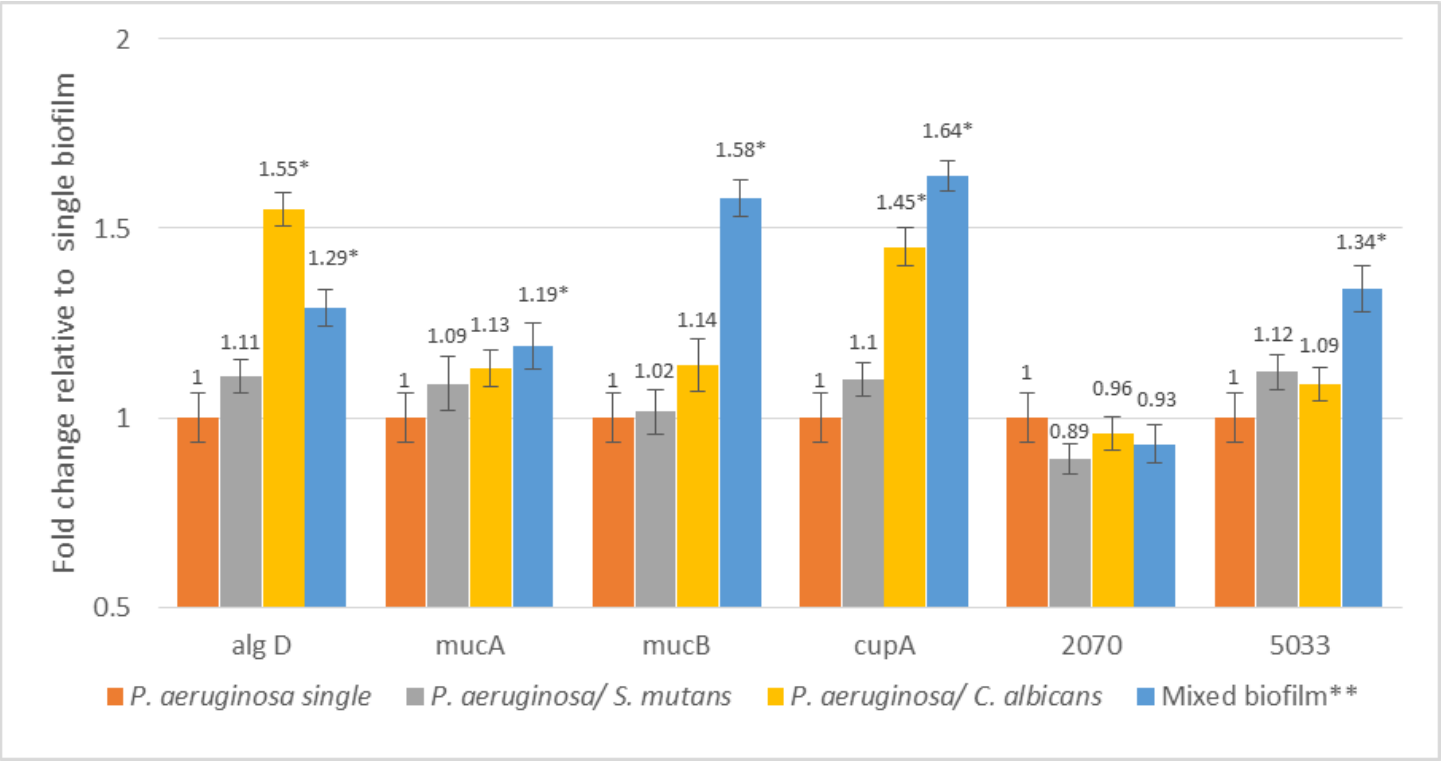

Figure 4.15 Relative expression of *P. aeruginosa* biofilm production genes.

Comparisons were made between *P. aeruginosa* only and dual species biofilms with *S. mutans* and *C. albicans*, as well as mixed biofilms (**) containing *P. aeruginosa, S. mutans, C. albicans* and *S. aureus*. Analysis of quantitative RT-PCR was made by the $^{\Delta\Delta}$Ct method. *P. aeruginosa* single biofilms were used as reference biofilms for relative expression of other groups. * p= <0.05

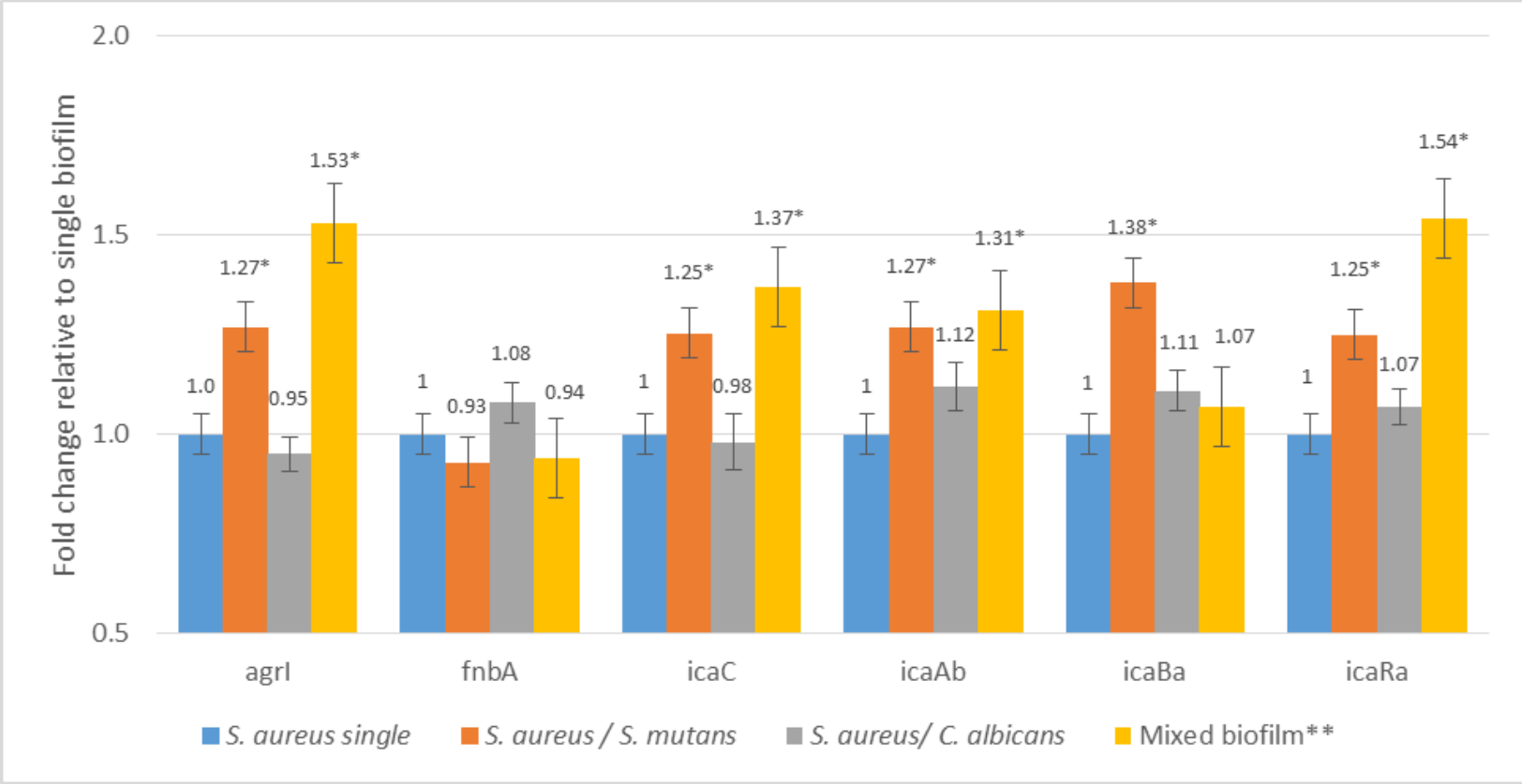

Figure 4.16 Relative expression of *S. aureus* biofilm production genes.

Comparisons were made between *S. aureus* only and dual species biofilms with *S. mutans* and *C. albicans*, as well as mixed biofilms (**) which contained *P. aeruginosa*, *S. mutans*, *C. albicans* and *S. aureus*. Analysis of quantitative RT-PCR was made by the $^{\Delta\Delta}$Ct method. *Staphylococcus aureus* single biofilms were used as reference biofilms for relative expression of other groups. * p= <0.05

4.5 Discussion

Respiratory pathogens frequently colonise the dental plaque of mechanically ventilated patients (Munro *et al.* 2006) who are at a high risk of developing VAP, a difficult to treat nosocomial infection that increases both mortality and hospital stay (Kollef *et al.* 2012). Currently there is very limited evidence of the effects that oral microorganisms may exert on the behaviour respiratory pathogens. The aim for this work was therefore to characterise *in vitro* biofilms formed by oral and respiratory pathogens and to assess the possible synergistic or antagonistic effects that could occur between the two groups of microorganisms in terms of biofilm formation and gene expression as well as visualise the spatial location of each microorganism within a mixed species biofilm.

For clarity, interpretation and reproducibility these experiments utilised simple combinations of microorganisms rather than trying to generate complex *in vitro* oral biofilms. Selected key target microorganisms were included. For the respiratory pathogen group, the two main VAP pathogens *P. aeruginosa* and *S. aureus* (Chastre and Fagon 2002) were used. For the oral microorganisms group, *S. mutans*, *P. gingivalis* respectively represented supra and sub gingival plaque bacteria. Both of these oral species are well-recognised for their biofilm production and capacity to attach to the tooth surface (Kuramitsu *et al*. 2007; Yamada *et al*. 2005) and have both previously been detected in ETT biofilms (Cairns *et al.* 2011). Additionally, the yeast *C. albicans* was also included as an 'oral' microorganism since carriage in the oral mucosa is frequently encountered and it is also an organism adept at biofilm formation (Chapter 1, section 1.5.3).

Importantly, all selected species formed single species biofilms in ETT sections, with *P. aeruginosa* and *P. gingivalis* creating the most abundant biofilms (Figure 4.3). It was clear that the PVC material surface of ETT sections provided a suitable substrata for biofilms development. *In vivo*, indwelling medical devices including ETTs are colonised in a matter of

hours after insertion into a patient (Adair *et al*. 1999; Donlan 2001) and the microbial biofilms that form on these devices have been identified as the cause of serious infections like VAP, urinary tract infections, infective endocarditis and sepsis (Donlan 2001; Vaudaux *et al.* 1994).

One important observation from the results of this study in terms of biofilm formation was that *S. mutans* appeared to enhance *S. aureus* growth. There are multiple reasons why this might have occurred but it is tempting to speculate that *S. mutans'* capacity to adhere to hard surfaces using sucrose dependent and independent mechanisms (Cvitkovitch *et al.* 2003; Koga *et al.* 1986) could have conditioned the surface for *S. aureus* colonisation. *Streptococcus mutans* produces several exopolysaccharides, which are predominantly glucans, through glucosyltransferases and fructosyltransferases. These polysaccharides will adsorb to surfaces, and facilitate adherence of bacteria to the surface (Koo *et al.* 2010; Kuramitsu *et al*. 2007). The initial colonisation of surfaces by *S. mutans* may promote aggregation by species that may have not been able to colonise surfaces on their own. In addition to glucans, *S. mutans* synthesises Autoinducer-2 (AI-2), a furarone borate diester which has been proposed to act as a universal quorum sensing signal molecule (Shemesh *et al.* 2010). AI-2 synthesis and detection is widespread in bacteria (Gamma-, Beta-, Epsilonproteobacteria, Firmicutes, Archaea, Eukarya, Alphaproteobacteria, Actinobacteria and Cyanobacteria) with the exception of *Vibrio* species (Sun *et al.* 2004). AI-2 has been found to contribute to interaction between bacterial species and contribute to cell-cell aggregation. It was described that dual biofilms formed by *Streptococcus oralis* and *Actinomyces naeslundii* were dependent upon production of AI-2 as biofilms formed by a *S. oralis* mutant lacking AI-2 production were 10-fold lower in biomass for each species (Rickard *et al.* 2006). Even when AI-2 is not produced, it can be detected by other species, *P. aeruginosa* does not synthesise AI-2 but upregulation of virulence gene promoters has been

reported in the presence of AI2.synthesized enzymatically and in co-culture with a AI-2 producer *Streptococcus* strain (Duan *et al.* 2003).

In contrast, dual species biofilms of *P. aeruginosa* with an oral species had no effect on the quantity of *P. aeruginosa* cells recovered. The reason for this could be the already high capacity of *P. aeruginosa* to adhere and form biofilms on the PVC surface (Triandafillu *et al.* 2003). However, compared with single species biofilms, increased numbers of *P. aeruginosa* occurred in the mixed species biofilms. Although not statistically significant, it is possible that the added presence of *S. aureus* in these mixed species biofilms was responsible for this synergistic effect. Dual species biofilms of *P. aeruginosa* and *S. aureus* were not examined in these studies and this represents an area for future investigations to establish this effect. Interaction between *S. aureus* and *P. aeruginosa* have, however, previously been found to be mutually beneficial for biofilm formation as well as increasing resistance to antibiotic treatment within *in vitro* wound models (DeLeon *et al.* 2014). Atomic force microscopy of *P. aeruginosa* and *S. aureus* (isolated from ETT biofilms) *in vitro* biofilms (4 h, 24 h, 48 h and 5 d) on PVC discs revealed progressive accumulation of bacterial EPS. Importantly these biofilms demonstrated increased resistance to two antimicrobials (ceftazidime and hexetidine) (Gorman *et al.* 2001). However, in a cystic fibrosis model it was reported that although these two species initially appear to co-exist, *P. aeruginosa* drives the *S. aureus* expression profile from aerobic respiration to fermentation, affecting viability in thelonger term, but equally increasing *S. aureus* lactate production which *P. aeruginosa* consumes. The authors proposed that eventually the combination results in lysis of *S. aureus* which further benefits *P. aeruginosa* through provision of an additional iron source (Filkins *et al.* 2015). *Staphylococcus aureus* and *P. aeruginosa* are frequently pathogens in the same infections, as seen in VAP, chronic wounds and cystic fibrosis (Fazli *et al.* 2009; Sands *et al.* 2016a;

Trivedi *et al.* 2014; Zemanick *et al.* 2015), and their interactions are thus clearly important and worthy for future research.

Another significant finding was the inhibition of *C. albicans* in dual species biofilms with *P. aeruginosa* (Figure 4.7). An earlier *in vitro* study using clinical isolates from sputum, found complete inhibition of *C. albicans* by *P. aeruginosa* and it was suggested that pyrrolnitrin (a *Pseudomonas* metabolite with antifungal effects) was responsible for this effect (Kerr 1994). It has also been reported that *P. aeruginosa* can selectively kill hyphae of *C. albicans* with blastopores remaining viable (Hogan and Kolter 2002). It appears that *C. albicans* hyphae stimulate the release of phenazines by *P. aeruginosa*, which in turn uses *C. albicans* hyphae as a growth substrate (Harriott and Noverr, 2011). Phenazines are heterocyclic, redox-active compounds that can be highly toxic to competing microorganisms and are also a terminal signalling factor in the quorum sensing network of *P. aeruginosa* (Dietrich *et al.* 2006). Additionally, the cell-cell signalling molecule, 3-oxo C12 homoserine lactone, has been found to inhibit *C. albicans* filamentation (Hogan *et al.* 2004). This inhibition has also been observed using SEM (Thein *et al.* 2006). In the current study there was little or no recovery of *C. albicans* from ETT sections also colonised with *P. aeruginosa*. Initially for *C. albicans* enumeration, the dual species biofilm cells were plated on to SDA as this is the routine agar for yeast, however *P. aeruginosa* grows also well and swarms on this agar. It was thought that *P. aeruginosa* may have been overgrowing the *C. albicans* on SDA, however, changing the agar to the more selective CHROMagar® *Candida* did not yield significantly higher candidal counts. Additionally, the apparent inhibitory effect on *C. albicans* biofilm cells was also seen in mixed species biofilms (Figure 4.3) indicating the presence of the other species offered little or no protection to *C. albicans* from *P. aeruginosa.* The relationship between these two microorganisms *in vivo* conflicts with *in vitro* findings. In a mouse lung infection model short term *Candida* colonisation lowered *P. aeruginosa* load as well as severity of lung

injury, importantly, the administration of antifungal treatment resulted in the opposite (Ader *et al.* 2011). Another murine model study reported that *C. albicans* colonisation triggers the recruitment of white blood cells which results in secretion of interleukins (IL-17 and IL-22) and the production of antimicrobial peptides and which has a protective effect against *P. aeruginosa* infection (Mear *et al.* 2014). Interestingly, *P. aeruginosa* infection followed by *C. albicans* colonisation resulted in increased mortality in a burned mice model (Neely *et al.* 1986). In an acute lung injury murine model It was reported that *P. aeruginosa* type III secretion system diminishes the host response by cell damage and decreased production secretion of IL-17 (Faure *et al.* 2014). In contrast to findings in *in vitro* models, pre-existing colonisation by *C. albicans* has been suggested to be a risk factor for VAP caused by *P. aeruginosa* with poorer clinical outcomes including higher mortality and longer hospital stay in two studies including 803 and 639 patients (Azoulay *et al.* 2006; Delisle *et al.* 2011; Delisle *et al.* 2008).

Candida albicans and *Staphylococcus aureus* are commonly co-isolated in a range of infections including denture stomatitis, angular cheilitis and infective endocarditis (Baena-Monroy *et al*. 2005*;* Peters *et al*. 2012a*;* Shirtliff *et al*. 2009*)*. Although in this study *C albicans* cause no significant increase on biofilm production or gene expression of *S. aureus*, *C. albicans* hyphae have been shown to aid *S. aureus* invasion to mucosal tissue and induce amplified virulence during coinfection (Schlecht *et al.* 2015). Additionally, enhanced tolerance to vancomycin has been observed in *S. aureus* when cultured *with C. albicans* mediated by polysaccharides secreted into the environment (Kong *et al.* 2016).

An interesting finding from these *in vitro* biofilm experiments was that *P. gingivalis*, an obligated anaerobic bacterium, could survive in the aerobic biofilm model in both the mixed and dual species biofilms. Previous studies have observed that when *P. gingivalis* and *P. nigrescens* were combined with the bridging bacterium *Fusobacterium nucleatum*, the black

pigmented anaerobes survived the aerated conditions (Bradshaw *et al.* 1998). This effect occurred both with biofilms and planktonic cultures, and the authors proposed that metabolically organised aggregates facilitated persistence of the anaerobes (Bradshaw *et al*. 1998). More recently, it was found that *P. gingivalis* could thrive in the presence of low concentrations of oxygen (6%) which was explained by the presence of encoding components of aerobic respiration in the *P. gingivalis* genome. The finding implicated this species with the potential ability to use formate and lactate for nutrition, which are secondary metabolic products of other bacteria including *Streptococcus* species and *S. aureus* (Filkins *et al*. 2015; Lewis *et al.* 2009).

Survival of anaerobic bacteria in the presence of high concentrations of oxygen is obviously evident in the oral cavity and chronic wounds. Interestingly, in Chapter 2 and 3 of this thesis *P. gingivalis* was detected using molecular methods in ETT biofilms of mechanically ventilated patients, and although the species was not cultured, its potential presence raises the question of what effect it could have in the ETT biofilm microbiota or indeed the lower airways. However, in the dual species biofilm combinations, there was no significant changes in the number of *S. aureus* or *P. aeruginosa* biofilm cells recovered. This could possibly relate to the inconsistent *P. gingivalis* growth in these biofilms.

A limitation to this study is the high level of variation in microbial growth between experiments which is evident in the standard variation values (Figures 4.3 to 4.8).It has to be noted that for the dual species biofilms, a monospecies biofilm (*S. mutans, C. albicans or P. gingivalis*) was created prior to inoculation with one of the respiratory pathogens. This would eliminate initial competition by the respiratory pathogen for attachment to the ETT. However, the rationale for this approach was that *in vivo Streptococcus* species would be present in the patient's dental plaque prior to potential colonisation by respiratory pathogens (Dewhirst *et al.* 2010; Sands *et al*. 2016a).

Gene expression studies showed similar results to *in vitro* culture analyses, with enhanced expression of the *S. aureus* biofilm genes *agrI, icaC, icaAb, icaRa*, when *S. aureus* was present in combination with *S. mutans* and mixed species biofilms. Up regulation of *P. aeruginosa* biofilm genes (*algD, mucA, mucB, cupA*, 5033) was also observed and this was primarily in the mixed biofilms and in the case of *alg D and cupA*, the presence of a pre-existing *C. albicans* biofilm. Although the change in gene expression (<2 fold) was not extensive for all genes and in both respiratory pathogens, such changes may be sufficient to promote significant alterations in behaviour and biofilm development. Similar levels of upregulated gene expression from *C. albicans* combined with *Streptococcus* species was seen with biofilms grown in titanium surfaces, and resulted in significantly higher hyphal formation from *Candida* cells and virulence in an *in vitro* tissue model (Cavalcanti *et al.* 2016).

The *in vitro* models used in this chapter where selected because of ease of reproducibility and with identical conditions and it allowed an adequate number of experiments to be performed for each microbial species combination in a reasonable time frame and cost. An alternative option could have been to add a "substrate conditioning step" by incubating the materials with for example artificial saliva to mimic this biofilm forming step. Furthermore future studies should consider the use of clinical isolates instead of reference strains, both of this changes could improve the similarties to the *in vivo* situation.

An alternative *in vitro* model could have been to manufacture an artificial mouth to lung model that allowed the insertion of an ETT, mechanical ventilation and microbial infection. Such model was considered but it would have made difficult to perform experiments and controls simultaneously for the number of combinations.

One aim of this chapter, was to use PNA-FISH and CSLM to determine the spatial location of the different species within mixed and dual species biofilms grown in ETT sections. This could in turn, further inform on potential inter-species interaction. Whilst the probes were

validated for specificity and function against all tested strains in planktonic state, hybridisation of biofilm cells with the PNA probes proved difficult. This could have related to impaired access of the probes to biofilm embedded cells, possibly due to the presence of the biofilm matrix. Nevertheless, multi-layered well-structured biofilms could be detected with CSLM and SEM (Figures 4.11 and 4.13).

There is limited evidence on the interaction between oral microorganism and systemic pathogens such as *S. aureus* and *P. aeruginosa*. Recently, Peters *et al*., revealed a physical interaction between *S. aureus* and *C. albicans* that was associated with altered gene expression by *S. aureus* and an enhanced ability of the bacterium to invade epithelial cells (Peters *et al*. 2010; Peters *et al*. 2012b; Schlecht *et al*. 2015). However, more research in this subject would enhance our knowledge of how these bacterial interactions affect the clinical outcomes and may offer light in the search for novel and effective antimicrobials.

4.6 Conclusions:

- *Streptococcus mutans, P. gingivalis, C. albicans, P. aeruginosa* and *S. aureus* formed biofilms on the PVC surfaces of ETT, with the most extensive single species biofilms formed by *P. aeruginosa* and *P. gingivalis*.
- *Staphylococcus aureus* and *S. mutans* exhibited enhanced growth as part of mixed species biofilms.
- *Staphylococcus aureus* colonised biofilms to a greater extent in the presence of pre-existing *S. mutans*.
- *Pseudomonas aeruginosa* inhibited *C. albicans* growth in dual species biofilms.
- Enhanced biofilm gene expression occurred with the respiratory pathogens *S. aureus* and *P. aeruginosa* as part of mixed species biofilms and in the presence of *S. mutans* for *S. aureus,* and *C. albicans* for *P. aeruginosa.*

5. Evaluation of strategies to combat biofilms associated with ventilator associated pneumonia

5.1 Introduction

Ventilator-associated pneumonia (VAP) is a common nosocomial infection in mechanically ventilated patients. VAP is associated with high patient morbidity and mortality, significant cost to healthcare providers and the need for high levels of antibiotic use (Chastre and Fagon 2002; Melsen *et al.* 2013). Consequently, preventive measures that reduce VAP incidence are essential. There are a number of strategies that can be used to reduce the risk of VAP occurrence and some of these are outlined below.

Aspiration of oropharyngeal secretions has been identified as an important source of pathogens involved in the development of VAP (Brennan *et al.* 2004; Rodrigues *et al.* 2009; Rumbak 2005). Not only can aspiration lead to direct contamination of the lungs but it can also be the source of microorganisms for biofilm development within the endotracheal tube (ETT).

Dental plaque is an 'archetypal biofilm' and during critical illness, it can rapidly become colonised by potential respiratory pathogens. In these cases, dental plaque acts as a reservoir of VAP pathogens that can translocate to the lungs following aspiration (Fourrier *et al.* 1998; Sachdev *et al.* 2013; Sands *et al.* 2016; Scannapieco *et al.* 1992). Clearly, reducing the microbial bio-load of dental plaque in mechanically ventilated patients is an approach that could lower the risk of VAP occurrence. Indeed, a number of interventions aimed at improving oral cleanliness with oral care protocols including use of antimicrobials, mainly chlorhexidine (CHX) have demonstrated a reduction in VAP or improved mortality rates in randomised clinical trials (Chan *et al.* 2007; Shi *et al.* 2013; Snyders *et al.* 2011). However, these studies did not evaluate the efficacy of the applied cleaning methods and there remains a paucity of research conducted in mechanically-ventilated patients on what the optimal methods for improving oral hygiene are (Wise and Williams 2013). Indeed the majority of observational studies in the general critical care population demonstrate an

increase in plaque scores over time, despite receipt of 'routine' oral care (Fourrier *et al*. 1998; Sachdev *et al*. 2013; Scannapieco *et al*. 1992).

It has long been established that the best approach for removal of dental plaque is by its mechanical disruption (Silness and Loe 1964). Brushing of teeth can be undertaken using a number of devices including manual brushes, powered brushes, swabs and interdental brushes. The use of manual toothbrushes has been found to be superior than foam swabs (Pearson 1996), however this trial included healthy volunteers and not mechanically ventilated patients. Similarly in the general population powered toothbrushes have been shown to reduce more plaque and gingival inflammation than manual toothbrushes (Yaacob *et al.* 2014). This finding was also evident in mechanically- ventilated patients in a clinical trial (Needleman *et al.* 2011), however, this remains the only study evaluating powered toothbrushes in this patient group and the use of electric toothbrushes remains in ICUs remains low.

Within critical care, oral care has historically been viewed as a comfort measure, rather than an infection control strategy. It has also been noted that nursing staff attitudes towards oral care delivery, varies on how well informed staff members are. More positive attitudes to oral care is evident with registered nurses compared to nursing assistants (Wardh *et al.* 1997). Unfortunately, the problem of poor knowledge over the benefits of maintaining oral hygiene in the critically ill continues to be an issue. Some nursing staff continue to acknowledge a feeling of disgust and anxiety towards delivering mouth care and admit to deviating from oral care protocols (Johnson 2013). It is therefore not surprising that considerable variability exists in oral hygiene practices amongst critical care nurses (Dale *et al.* 2013; Rello *et al.* 2007). Currently, the most common methods of delivering oral care in the critically ill are through the use of foam brushes or toothbrushes, and with or without antiseptic solutions (Binkley *et al.* 2004; Rello *et al*. 2007).

Antimicrobial mouthwashes are frequently used as coadjuvants in the delivery of oral care, primarily to assist in the prevention of caries and periodontal disease, where their antimicrobial effectiveness has been established (Bonez *et al.* 2013; Hendry *et al.* 2009; Hooper *et al.* 2011; Malic *et al.* 2013; Masadeh *et al.* 2013). A number of randomised clinical trials have provided evidence of a reduced VAP incidence when CHX is incorporated as part of oral care (Garcia *et al.* 2009; Grap *et al.* 2011; Koeman *et al.* 2006). However, CHX use in critical care has not been found to reduce patient mortality rates or length of hospital stay. This finding has been determined by recent meta-analyses including a Cochrane review (Hoshijima *et al.* 2013; Hua *et al.* 2016). However there has been some limited evidence of the efficacy of antimicrobial mouthwashes in mechanically-ventilated patients, where CHX is the principle agent used. Other antibacterial mouthwashes available include essential oils, cetyl pyridinium chloride, tricolsan, octeneidine, delmopinol, polyvinylpyrrolidone, hyaluronic acid and Citroxx® (Tartaglia *et al.* 2016). The most widely used of these are CHX (Corsodyl®), "essential oils" (Listerine®) and cetyl pyridinium chloride (Colgate® Plax, Oral B™ Antiplaque).

CHX gluconate is a water-soluble, cationic biguanide that binds to the negatively charged bacterial cell wall, and by doing so alters the bacterial cell osmotic equilibrium. CHX has bactericidal and bacteriostatic effects and has been shown to prevent spore formation in *Clostridium difficile* and *Bacillus subtilis* high (Jones *et al.* 1995; Nerandzic and Donskey 2015; Tartaglia *et al.* 2016). As an antiseptic, CHX is commonly used in hospitals to disinfect skin, wounds and surfaces (Weinstein *et al.* 2008). As a mouthwash, CHX is used in the management of periodontal disease, and as a 0.12-0.2% solution in perioperative prophylaxis prior to dental surgical procedures (Jose *et al.* 2015). CHX has been shown to reduce bacterial load and prevent plaque re-accumulation on clean oral surfaces (Bonez *et al*. 2013; Lucchese *et al.* 2012).

One highly used essential oil mouthwash is Listerine® (Johnson & Johnson Ltd), based on essential oils (eucalyptol, menthol, methyl salicylate and thymol). Listerine® Total Care Zero, unlike other Listerine® formulations it has no alcohol content. Alcohol in mouthwashes has been a cause of concern, with reported side effects including dental erosion (Pontefract *et al.* 2001), links to oropharyngeal cancer (Ahrens *et al.* 2014), and alcohol poisoning (Kolikonda *et al.* 2014). Additionally, a mouthwash with alcohol may be unsuitable for hospital use as it may lead to multiple drug interactions and promote dryness of the oral mucosa (Werner and Seymour 2009).

In recent years a number of other mouthwashes based on plant extracts, and polyphenolic plant derivatives have been shown to have antimicrobial activity against oral bacteria and yeast (Hooper *et al.* 2011; Malic *et al*. 2013). Such natural products may offer the added advantage of improved safety profiles and better acceptance from patients. Citroxx® is based on a blend of soluble bioflavonoids derived from orange pith, with small amounts of malic and citric acids. Two Citroxx® formulations, referred to as BD and MDC (Oraldent) exist. BD is present in Oralclens® mouthrinse and toothpaste, and MDC is a surface disinfectant in Citroxx Bio™; both formulations are alcohol free (OralDent 2008).

Multiple studies have shown that the ETT develops a biofilm in its lumen during use (De Souza *et al.* 2014; Inglis *et al.* 1989; Perkins *et al.* 2010). This biofilm has been shown to be an independent risk factor in the occurrence of VAP (Danin *et al.* 2015; Wilson *et al.* 2012). Previous studies have reported that oropharyngeal secretions accumulate above the inflated cuff of ETTs and then leak passed the cuff (Chapter 1, section 1.3.5.1). After this leakage occurs and aided by the forces of mechanical ventilation, microorganisms can then contaminate the lumen of the ETT where they grow as a biofilm (Chapter 2, Section 2.1.1). ETT biofilm has been detected after only a few hours of intubation (Adair *et al.* 1999; Inglis *et al.* 1989). This biofilm will be protected from the host's immune system and any

administered systemic antibiotics (Bauer *et al.* 2002). The biofilm threfore provides a refuge of microorganisms that have unrestricted direct access to the lungs. Inglis *et al*., (1989) found that by the forces of mechanical ventilation, ETT biofilm particles could be displaced up to 45 cm away from the tip of the ETT. Therefore microorganisms from ETT biofilms can readily be expelled into the lower airways and potentially cause infection.

Given that aspiration from the oral cavity occurs as outlined above, it is perhaps not surprising, that oral microbial species have been detected in ETT biofilms (Cairns *et al.* 2011; Perkins *et al*. 2010). As previously shown in this Thesis (Chapters 2 and 3), along with oral species, *Staphylococcus aureus* and *Pseudomonas aeruginosa* as well as other respiratory pathogens can also be isolated and detected in ETT biofilms. Since biofilm microorganisms display greater tolerance to antimicrobials than planktonic equivalents (Luppens *et al.* 2002), it would be likely that this property would remain evident when aggregates of ETT biofilm are displaced to the lower airway.

Given the risk that the ETT biofilm presents for the mechanically ventilated patients, changes to the ETT have been proposed to eliminate or minimise the leakage of subglottic secretions past the cuff and to impede biofilm formation. Most of these changes have being in the design of the ETT, including the use of tapered cuffs, double cuffs and incorporation of ports for removal of pooling subglottic fluids (Doyle *et al.* 2011; Hwang *et al.* 2013; Muscedere *et al.* 2008) (Chapter 1, Section 1.3.5.1).

Changes to the ETT biomaterial include incorporation of silver coatings on the inner surface of the ETT as an alternative to the standard polyvinyl chloride (PVC) or polyurethane (Berra *et al.* 2008; Kollef *et al.* 2008). Silver has been used as a prophylactic antimicrobial or as a treatment agent since the times of Hippocrates (ca 400 BC) and was possibly the most important antimicrobial agent prior to antibiotics (Alexander 2009). Silver coated medical devices have previously shown limited success in urinary catheters (Bologna *et al.* 1999).

Therefore some expectation that silver coated ETTs could reduce VAP might be anticipated. Clinical evaluation have found a reduction in VAP incidence, although no impact in improving patient mortality rates have been reported (Berra *et al.* 2008; Kollef *et al*. 2008). Despite large randomised control trials involving over 2000 patients, the results have not been sufficiently robust for use of silver coated ETTs to be widely adopted, as reported recently in a Cochrane review (Tokmaji *et al.* 2015).

The PneuX™ (Formerly known as LoTrach™) ETT is constructed from silicone and includes wire re-enforcement. The PneuX™ ETT exhibits several features designed to minimise aspiration of oropharyngeal secretions with minimal trauma to the surrounding structures. The tube features three subglottic ports, a soft tip, a low pressure and low volume cuff, and a 'coated lumen' to prevent microbial colonisation (Fletcher *et al.* 2008). This ETT has been designed to be used as in conjunction with a tracheal seal monitor to maintain a constant cuff pressure. In a retrospective study of 53 patients using the PneuX™ system for a mean duration of 5.3 days, there were no VAP episodes while the tube was *in situ* and there was only one incident of respiratory failure. This followed after a planned extubation and re-intubation with a standard ETT where the patient subsequently developed VAP after 48 h. Based on an intention to treat basis, the incidence of VAP was calculated as 1.8% (Doyle *et al*. 2011) and this study therefore showed promising results for use of the PneuX™ system. However it remains unclear which of the design features contributed to this reported low VAP incidence.

Future, alternative therapies for management of ETT biofilms include photodynamic exposure. Indeed, one *in vitro* study reported the reduction of *P. aeruginosa* and MRSA ETT biofilms after a single treatment with a methylene blue (MB) photosensitizer and exposure to 664nm non-thermal activating light (Biel *et al.* 2011). Recently a multicentre randomised clinical trial involving 70 patients studied the effect of nebulised eucalyptus on biofilm

formation on ETT surfaces and found lower levels of *K. pneumoniae*, however this effect was not seen in other species (Amini *et al.* 2016).

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5.2 Objectives

The aims of this chapter were to evaluate different VAP prevention strategies that were aimed at reducing biofilm. These included an evaluation of oral hygiene approaches to mechanically disrupt dental plaque during mechanical ventilation, and *in vitro* studies assessing the relative efficacy of antimicrobial mouthwashes against ETT biofilms, as well as the ability of biofilms to develop on different ETT biomaterials. Specific this study aimed to:

- Compare the efficacy of foam swabs and toothbrushes at removing dental plaque in mechanically ventilated patients with outcomes measured based on plaque accumulation and gingival inflammation indexes, as well as total bacterial load.
- Compare biofilm growth on biomaterial sections of four different ETTs.
- Determine the antimicrobial activity of Citroxx®, Listerine® Total Care Zero and CHX against planktonic and biofilm constructs of oral and respiratory pathogen microorganisms.

5.3 Materials and Methods

5.3.1 Comparison of oral hygiene delivery in mechanically ventilated patients using swabs and toothbrushes

5.3.1.1 Study design

This study employed a 'split-mouth' design in which two oral hygiene methods *i.e.* use of a foam swab or a 'small-headed' toothbrush (Figure 5.1) were used to clean the teeth on different sides of a patient's mouth. The advantage of this split-mouth design over randomising individual patients was the reduction in inter-subject variability (Lesaffre *et al.* 2009). The side to which the cleaning method was allocated was determined by computer-generated randomisation. All researchers involved in this study (see Thesis acknowledgements) were blinded to the assigned hygiene method until the statistical analysis had been completed. Assistance in this study was received from Dr Matt Wise, Mrs Niki Palmer, Ms Jade Cole, Mr. Sean Haywood, Ms Tracey Kinsella and Dr Charlotte Emanuel who were responsible for taking patient consent, delivery of oral care and assessment of oral interventions. All laboratory aspects of this study and interpretation of the data was undertaken by the PhD candidate (Ms Paola Marino). Statistical advice was provided by Professor Ailish Hannigan (University of Limerick, Limerick, Ireland).

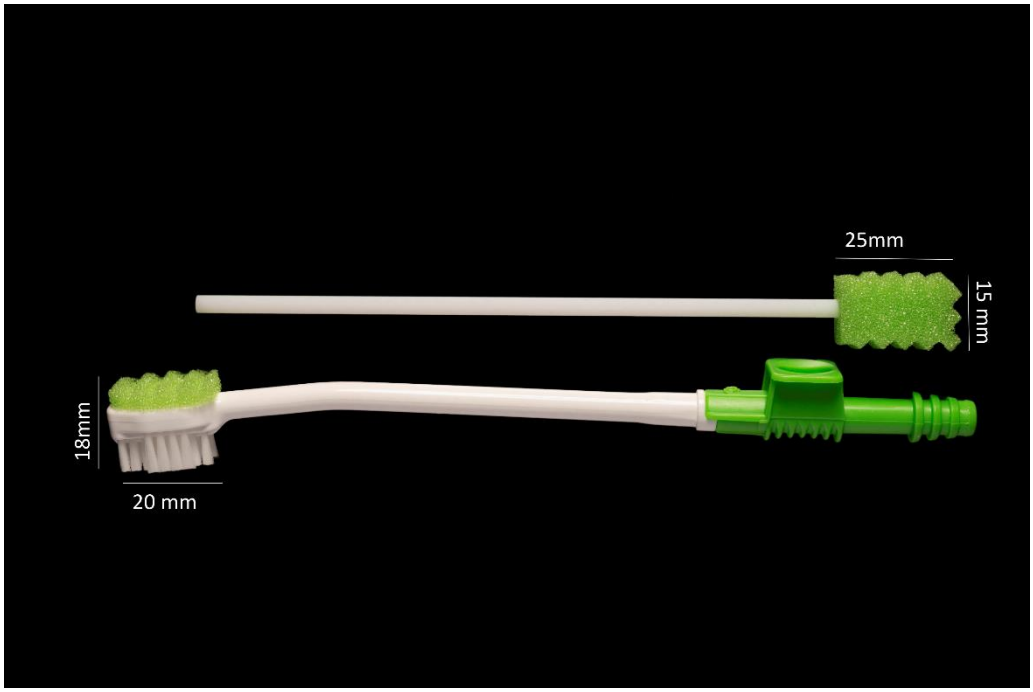

Figure 5.1 Toothbrush and foam swab (Sage products, Illinois, USA) evaluated for delivery of oral care in the studied patients.

5.3.1.2 **Patient recruitment**

Ethical approval was obtained from the Research Ethics Committee for Wales (09/MRE09/44); Trial registration: Clinical Trials.Gov NCT01154257 14th June 2010. Written informed consent was obtained in accordance with ethical approval (Appendix I). Mechanically-ventilated patients admitted to the adult intensive care unit (ICU) at the University Hospital of Wales, Cardiff, UK were eligible for the study. Patients were eligible if they were aged >18 years, were mechanically-ventilated with an ETT placed via the oral route, and had >20 teeth of broadly symmetrical (left and right) distribution. Patients that did not meet the inclusion criteria, or those who had thrombocytopenia (platelet count <30), uncontrolled coagulopathy, facial or oral trauma, or were expected to be ventilated for <24 h, were excluded from the study. Patients were randomised using a computer-generated sequence, with the right side of the mouth allocated to either use of a toothbrush or foam swab. The intervention allocations were placed in sealed envelopes which were opened at patient recruitment. Recruitment of patients occurred between July 2010 and April 2011.

5.3.1.3 **Intervention**

Prior to oral hygiene intervention, Silness-Löe plaque (Table 5.1) and gingival indices (Table 5.2) were recorded. Using this scoring system, scores ranged between 0 to 3, with 0 being equal to health and a score of 3 meaning gross plaque deposits or marked gingival inflammation (Silness and Loe 1964). These scores were recorded on the upper and lower first molars, first bicuspid and central incisors on each side of the mouth from the buccal surface. Plaque and gingival index scores were an average of the six teeth on each side. For patients with missing index teeth, the remaining teeth in closest proximity were scored. A decayed, missing and filled teeth (DMFT) index (Larmas 2010) was used as an indicator of the patient's oral health on admission to critical care.

A qualified dental hygienist (Ms Tracey Kinsella) trained to the ICU research nursing staff in the provision of oral hygiene.. Cleaning was performed solely by the research staff every 12 h until extubation (if <7 days) or up to seven days after recruitment. The modified Bass technique (Poyato-Ferrera *et al.* 2003) for brushing was used with toothbrushes and foam swabs, that had been pre-moistened with sterile water. CHX was not used in the study, as it was not part of the routine oral care in the critical care unit. Each side of the mouth was cleaned for 1 min (30 s per quadrant) and compliance with the intervention recorded. Silness-Löe plaque and gingival indices were recorded daily and on each side of the mouth by a single, dentally trained operator (Mr Sean Haywood, 5th year dental student) who was also trained for the study by the dental hygienist and was blinded to treatment allocation . The scores were recorded at the start of the study (*i.e.* baseline), before randomisation and then each morning.

Dental plaque was collected using sterile endodontic paper points (size ISO45; QEP, Peterborough, UK), with one paper point used per tooth from each side of the mouth to determine bacterial load. Plaque samples were obtained at the same time of day, and by the same individual (Mr Sean Haywood) prior to cleaning. Sampling commenced at the distal part of the buccal aspect of the tooth with 1 mm of paper point placed into the gingival sulcus. Using a slow and continuous motion the paper point was drawn towards the operator to recover the plaque. Paper points were immediately immersed in 1 ml of Reduced Transport Fluid (RTF) for analysis of viable microorganisms by culture (Syed and Loesche 1972).

Each of the described interventions had a written standad operating procedure wich was readily available to the individuals performing them, compliance to adherence to the protocols was also recorded.

Table 5.1 Plaque index scoring used in this study.

Score	Criteria
0	No plaque
1	A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen *in situ* only after application of disclosing solution or by using the probe on the tooth surface.
2	Moderate accumulation of soft deposits within the gingival pocket, or the tooth and gingival margin, which can be seen with the naked eye.
3	Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.

Adapted from (Silness and Loe 1964)

Table 5.2 Gingival index assessment (severity of gingivitis) used in this study based on gingival colour, presence of oedema and bleeding.

Score	Criteria
0	Normal
1	Mild inflammation, slight colour change and oedema, no bleeding
2	Moderate inflammation, redness, oedema, bleeds on probing
3	Severe inflammation, marked redness and oedema, ulceration, spontaneous bleeding

Adapted from (Silness and Loe 1964)

5.3.1.4 **Processing of dental plaque samples**

Paper points were vortex mixed for 20 s and the resulting solution serial decimally diluted in phosphate buffered saline (PBS). Fifty-µl volumes of the dilutions were then inoculated onto blood agar using a spiral plating (Don Whitley Scientific, Shipley, UK) (Lab M, Heywood, UK) system and incubated aerobically at 37°C for 48 h for bacterial counts. After incubation, colony-forming units (CFUs) were enumerated.

5.3.1.5 **Statistical analysis**

It was estimated that 20 patients would be required to detect a 0.63 shift in plaque scores with a power of 80% at the conventional 0.05 alpha level. Initially, it was intended to analyse plaque scores after 72 h of cleaning and it was estimated that recruiting 50 patients would give at least 20 patients allowing for drop out of extubated patients or patient death before 72 h. However, as 24 h of cleaning should be sufficient to reduce plaque scores, it was subsequently decided, prior to patient recruitment to analyse data after a minimum of 24 h.

The distribution of numeric data was tested for normality and presented as mean (standard deviation) for normally distributed data, and median (range) for non-normally distributed data. The overall change (baseline to end of follow-up) in plaque index, gingival index and bacterial counts was calculated for each patient and for each mechanical method. The null hypothesis of no change in outcomes over time was tested using the paired samples t-test for normally distributed differences, or a Wilcoxon-signed-rank test for skewed distributions. McNemar's test was used for paired proportions.

For normally distributed differences, repeated measures analysis of variance (AVOVA) was used to test for statistically significant differences in outcomes between methods within patients after adjusting for baseline DMFT, number of days follow-up and side of the mouth.

A 5% level of significance was used for all statistical tests. The statistical software package IBM SPSS for Windows Version 21 was used for analyses.

5.3.2 **Comparison of biofilm development of different endotracheal tube biomaterial surfaces**

Mixed species biofilms were aerobically cultured at 37°C for 5 days and recovered using the same method described in Chapter 4, sections 4.3.1 and 4.3.2. The ETT (Figure 5.2) surfaces tested included the standard PVC Portex® (Smiths medical, Kent, UK), the silicone PneuX™ (Venner medical, Jersey, UK), an experimental PVC silver coated tube (Mallinckrodt, Covidien, MA, USA) and non-silver-coated PVC tube (Taperguard Evac™, Mallinckrodt). The number of bacteria present in the developed biofilms was then assessed by quantitative microbial culture. Four replicates experiments for each surface were performed and were repeated on three separate occasions. Statistical analysis using the Wilcoxon Rank tests and were calculated using Graph Pad Prism®.

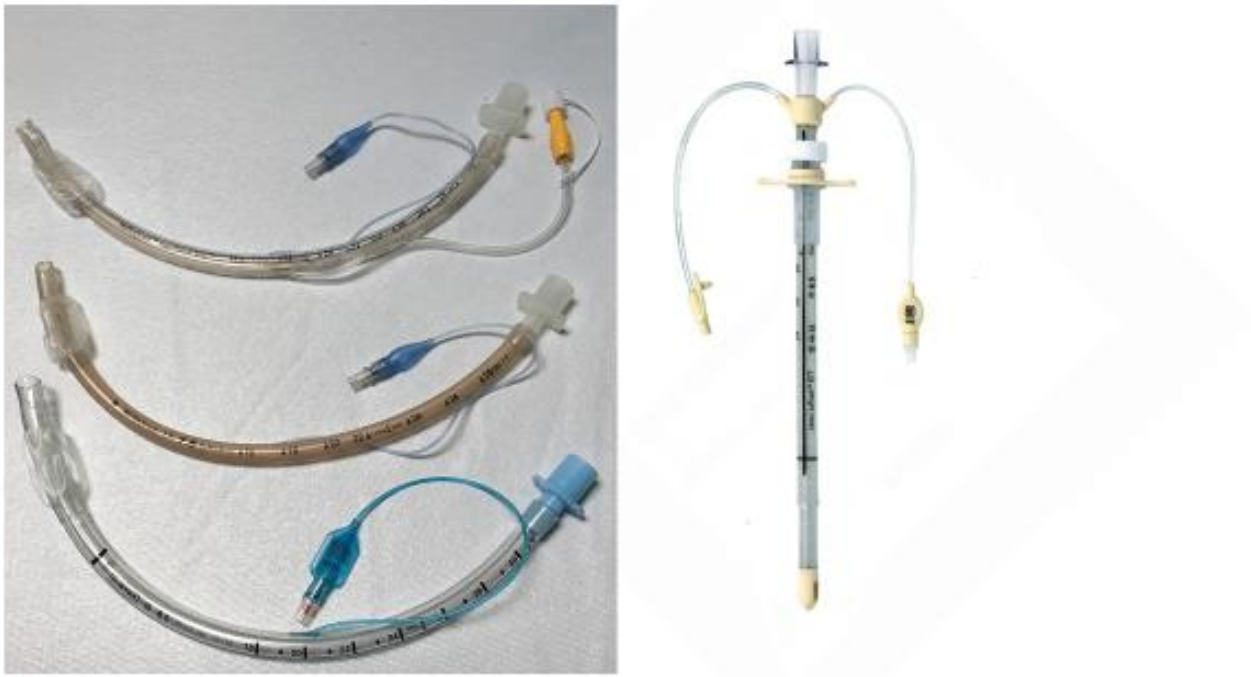

Figure 5.2 Endotracheal tube types from which a comparison of biofilm growth on tube sections was undertaken. Left: from top to bottom Evac®, silver coted and Portex®, Right: PneuX™.

5.3.3 Susceptibility of planktonically cultured microorganisms and biofilms to antimicrobial mouthwashes

5.3.3.1 Microorganisms

Streptococcus mutans DSM 20523^T and *Candida albicans* ATTC 90028 were used in this study as representative oral species with known biofilm forming capacity. In addition, the respiratory pathogens *Staphylococcus aureus* NCIB 9518 and *Pseudomonas aeruginosa* ATTC 5682 were also tested.

Streptococcus mutans, C. albicans, *P aeruginosa* and *S. aureus* were all initially cultured overnight at 37˚C using the following respective agar media (from Lab M unless otherwise stated), Mitis Salivarius Bacitracin agar (MSB; Difco), Sabouraud Dextrose Agar (SDA), Pseudomonas agar (PsA) and Manitol Salt Agar (MSA). For minimum inhibitory concentrations (MIC) assays, static overnight culture in Brain Heart Infusion (BHI) liquid medium at 37°C was used for all test isolates.

5.3.3.2 Planktonic cell susceptibility to antimicrobial mouthwashes

Overnight cultures of each isolate in BHI were standardised to a turbidity equivalent to a 0.5 McFarland standard (approximately 10^8 colony forming units/ ml). To achieve this, turbidity was measured at 600_{nm} using a spectrophotometer (Geneflow, Linchfield, UK) and the culture adjusted to an optical density between 0.08 and 0.10. The standardised culture was then further diluted 100-fold in BHI to obtain approximately 10^6 cells /ml.

Serial dilutions of Citroxx® BD (Oraldent, Kimbolton, UK), Listerine Total Care ZERO® (Johnson & Johnson, UK) and chlorhexidine (20% solution in water, Sigma, Poole, UK) (Table 5.3) were prepared in BHI. Double strength BHI was initially prepared and to this an equal volume of antimicrobial was added

Table 5.3 Concentrations of mouthwashes used for planktonic and biofilm assays.

Mouthwash	**Planktonic assay**	**Biofilm assay**
Citroxx®	0.008% to 2%	0.25 to 8%
Listerine Total Care Zero®	0.95% to 50%	3% to 100%
Chlorhexidine	0.000195% to 0.2%	0.000195% to 1.92%

Table 5.4 Microbial species used for planktonic and biofilm assays.

Single species biofilm preparations	**Dual species biofilm preparations**
Streptococcus mutans	*Streptococcus mutans* and *Staphylococcus aureus*
Candida albicans	*Streptococcus mutans* and *Pseudomonas aeruginosa*
Staphylococcus aureus	*Candida albicans* and *Staphylococcus aureus*
Pseudomonas aeruginosa	*Candida albicans* and *Pseudomonas aeruginosa*

One hundred-µl volumes of microbial broth, prepared as describe above was loaded into the wells of a 96-well tissue culture plate (Starstedt, USA), and 100-µl of each diluted test mouthwash in BHI was added to the microbial suspensions. Un-inoculated BHI was used as a negative control, and a bacterial suspension with no mouthwash used as a positive growth control.

Microtitre plates were incubated aerobically for 24 h at 37˚C, after which time the relative growth of each microbial species was determined by measuring the turbidity of each well by spectrophotometric absorbance at 600_{nm} (FLUOstar® Omega; BMG Labtech, Aylesbury, UK). Absorbance readings were then adjusted using wells containing un-inoculated BHI to provide a baseline. Each preparation was done in triplicate and on three separate occasions and the MIC was determined as the concentration that caused ≥80% reduction absorbance reading compared to the positive control.

5.3.3.3 **Biofilm susceptibility to antimicrobial mouthwashes**

Single and dual species biofilms (Table 5.4) were prepared by inoculating a 100-µl volume of microbial suspensions of approximately 10^6 CFU/ml (prepared as described above) in to the wells of 96-well tissue culture plates and these were incubated without shaking at 37°C for 24 h. For dual species biofilm preparations, 100 µl of each species was used. After 24 h incubation, the growth medium was carefully aspirated with a sterile pipette. The biofilm was then gently washed by addition of an equal volume of phosphate buffered saline (PBS) to each well to remove planktonic cells. Biofilms were then re-suspended in serial dilutions of fresh medium containing test mouthwash or no mouthwash for a positive control by mechanical disruption using vigorous pipetting (for 10 s). The re-suspended biofilms were then transferred to the wells of a new 96-well plate. The turbidity of the re-suspended biofilm was immediately measured at an absorbance of 600_{nm} for a baseline measure and repeated after a 24h incubation at 37°C. The antimicrobial effect of the diluted mouthwash

concentrations was evaluated as described in the planktonic assay with the difference that *S. mutans* dual cultures were incubated anaerobically at 37°C as *P. aeruginosa* growth was detected on MSB.

Mean values were determined from triplicate experiments and the antibiofilm effect recorded as the lowest concentration of the antimicrobial agent that demonstrated a ≥80% reduction in absorbance compared with the positive control of untreated biofilm suspensions. All experiments were performed in triplicate and on three separate occasions.

The minimum biofilm eradication concentration (MBEC) was defined as the lowest concentration of antimicrobial agent that showed ≥80% reduction in absorbance compared to the positive control.

5.3.3.4 **Assessment of microbial viability after antimicrobial exposure**

To evaluate whether the antimicrobial effect was bacteriostatic or bactericidal, a portion of culture from the test wells (including at the optically determined MIC) was plated on appropriate agars *i.e.* MSB for *S. mutans*, SAB for *C. albicans*, MSA for *S. aureus* and Pseudomonas agar (PsA) for *P. aeruginosa.* Inoculated plates were incubated for 48 h to assess viability of isolates, with the exception of *S. mutans,* which was incubated for 5 days, as the growth of this organism was slower on the selected agar.

5.4 Results

5.4.1 Comparison of oral hygiene delivery in mechanically ventilated patients using swabs and toothbrushes

Informed consent for 28 patients was obtained by a consultant (Dr Matt Wise). After inspection by the dental professional (Mr Sean Haywood, Ms Tracey Kinsella or Dr Charlotte Emanuel), 6 consented patients could not enter the study as they had insufficient or

asymmetric teeth and one further patient died within 24 h, leaving 21 patients to complete investigations. Following a safety alert from the UK medicines and healthcare products regulatory agency (MHRA 2012)(not arising from this study), foam swabs were subsequently withdrawn from clinical use in Wales (but not elsewhere in the UK) and no further recruitment was allowed.

The 21 patients comprised of 10 males and 11 females, aged between 23 and 70 years (mean age 49 years). Recorded patients'demographic characteristics on admission, diagnosis, and DMFT index (Table 5.5). The length of hospitalisation and intubation at time of recruitment was presented in Chapter 2 (Table 2.7). On average, days of hospitalisation prior to admission in intensive care were 3.4 (4.5 SD), and ventilator days prior to randomisation was an average 3.4 (3.2 SD) days. The majority of patients (16/21) were in receipt of antibiotic therapy at the start of the study. The median number of follow-up days was 4 days (range 2 to 7 days) and mean DMFT score was 10.7 (SD 5.2, range 3 to 23). Compliance with the oral interventions was 100% and there were no reports of harm or unintended effects for any of the participants.

Baseline scores together with changes in each outcome over time for plaque index, gingival index and bacterial counts for each treatment are presented in Table 5.6 and Figures 5.3 - 5.5.

There was a significant reduction in plaque index over time for use of toothbrushes (mean change=-1.26; 95% confidence interval=-1.57, -0.95; p=< 0.001) and foam swabs (mean change=-1.28; 95% confidence interval=-1.54, -1.01; p=< 0.001). There was also a significant reduction in gingival index over time using toothbrushes (mean change= -0.92; 95% confidence interval= -1.19, -0.64; p=<0.001) and foam swabs (mean change= -0.85; 95% confidence interval= -1.10, -0.61; p=<0.001). There was no significant difference in reduction of plaque index between the two interventions (p=0.24). Greater reduction in gingival index

was observed for toothbrushes compared to foam swabs (Figure 5.6), although this was not statistically significant (p=0.12). The number of days of cleaning was a significant covariate in the analysis of change in gingival index (p=0.003) and plaque index (p=0.05). There was some evidence of an interaction between treatment and baseline DMFT score for both change in gingival index (p=0.07) and plaque index (p=0.06), suggesting that the impact of toothbrushes and foam swabs may be highest in those with poorest oral health.

An overall analysis of patients' oral health status was compared to results from the 2009 Adult Oral Health survey (Health and social care information centre 2011) undertaken by the National Health Service (NHS) Information Centre for health and social care (Table 5.7). Based on this previous survey, it was apparent that the patient cohort examined in this study had a similar number of teeth present and less obvious caries than the general population. However, periodontal health was worse, impacting on overall oral health (Table 5.7). The 2009 survey reported that 10% of the population had excellent oral health, but if similar parameters of excellence were applied to the participants in this current study, only one patient (4%) met these criteria. For patients that were not randomised on the same day of hospital admission, it was possible that their periodontal health had progressively deteriorated due to inability to perform their normal oral hygiene routine or reliance on healthcare workers to perform oral hygiene tasks.

No significant change in bacterial counts occurred with toothbrushes or foam swabs (Table 5.6). Ten patients had high baseline bacterial counts ($\geq 10^6$ colony forming units), with 7 and 4 patients showing a reduction in these counts when toothbrushes and foam swabs were respectively used.

Table 5.5 Characteristics of the 21 mechanically ventilated patients participating in the study assessing oral care delivery.

Patient	Sex	Age	Diagnosis	DMFT
P01	M	53	Sepsis	7
P02	F	61	Urosepsis	21
P03	M	70	Pneumonia/ Pneumocystis Pneumonia (PCP)	14
P05	M	43	Substance overdose/ Aspiration pneumonia	7
P06	F	55	Respiratory Failure	14
P07	F	37	Aneurysm	3
P08	F	26	Ventricular fibrillation arrest postpartum	7
P09	F	64	Respiratory failure	12
P10	F	68	Head injury	17
P11	F	55	Urinary sepsis	10
P13	M	52	Respiratory/renal failure	15
P14	M	29	Head injury	6
P16	F	64	Type 2 respiratory failure	13
P17	M	55	Respiratory failure	11
P19	M	45	Sepsis and respiratory failure	17
P20	F	23	Alcoholic liver disease and pneumonia	4
P21	M	32	Cardiac arrest	10
P24	F	44	Sepsis	4
P25	F	49	Cardiogenic shock	4
P27	M	39	Type 1 respiratory failure	9
P28	M	52	Septic shock	9

Table 5.6 Baseline scores and change in outcomes (plaque index, gingival index and bacterial counts) by mechanical method (n=21 patients).

Outcome	**Method**	
	Toothbrush	**Foam Swab**
Mean baseline plaque index (SD)	2.1 (0.49)	2.1 (0.42)
Mean baseline gingival index (SD)	2.1 (0.57)	2.0 (0.53)
Median baseline bacterial count (min, max)	4.6×10^5 (2.4×10^3, 2.5×10^{10})	5.9×10^5 (4.0×10^2, 3.1×10^{10})
Mean change in plaque index (SD)	-1.26 (0.68)[a]	-1.28 (0.59)[a]
Mean change in gingival index (SD)	-0.92 (0.61)[a]	-0.85 (0.54)[a]
Median change in bacterial counts (CFUs/sample[b] min) max)	-3.7×10^4 (-2.5×10^{10}, 8.7×10^7)	-9×10^4 (-3.1×10^{10}, 3.0×10^7)

[a]Significantly different from baseline (P=<0.001); [b]Each sample consisted of plaque obtained from 6 teeth resuspended in 1 ml of transport medium.

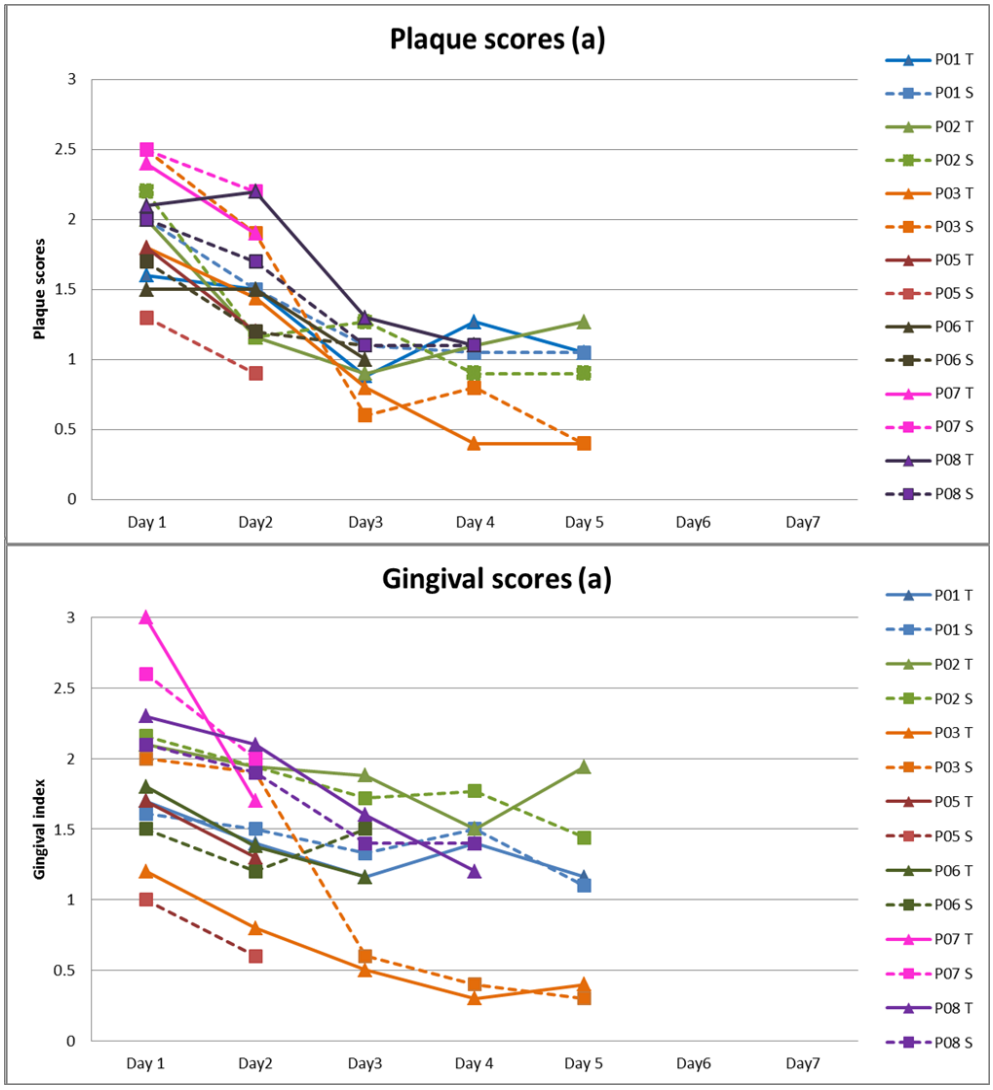

Figure 5.4 Changes in plaque and gingival index patients 9 to 17.

Changes in Silness and Löe (1964) plaque and gingival indices over a 7-day period using oral hygiene intervention either with a toothbrush (solid line) or foam swab (broken line).

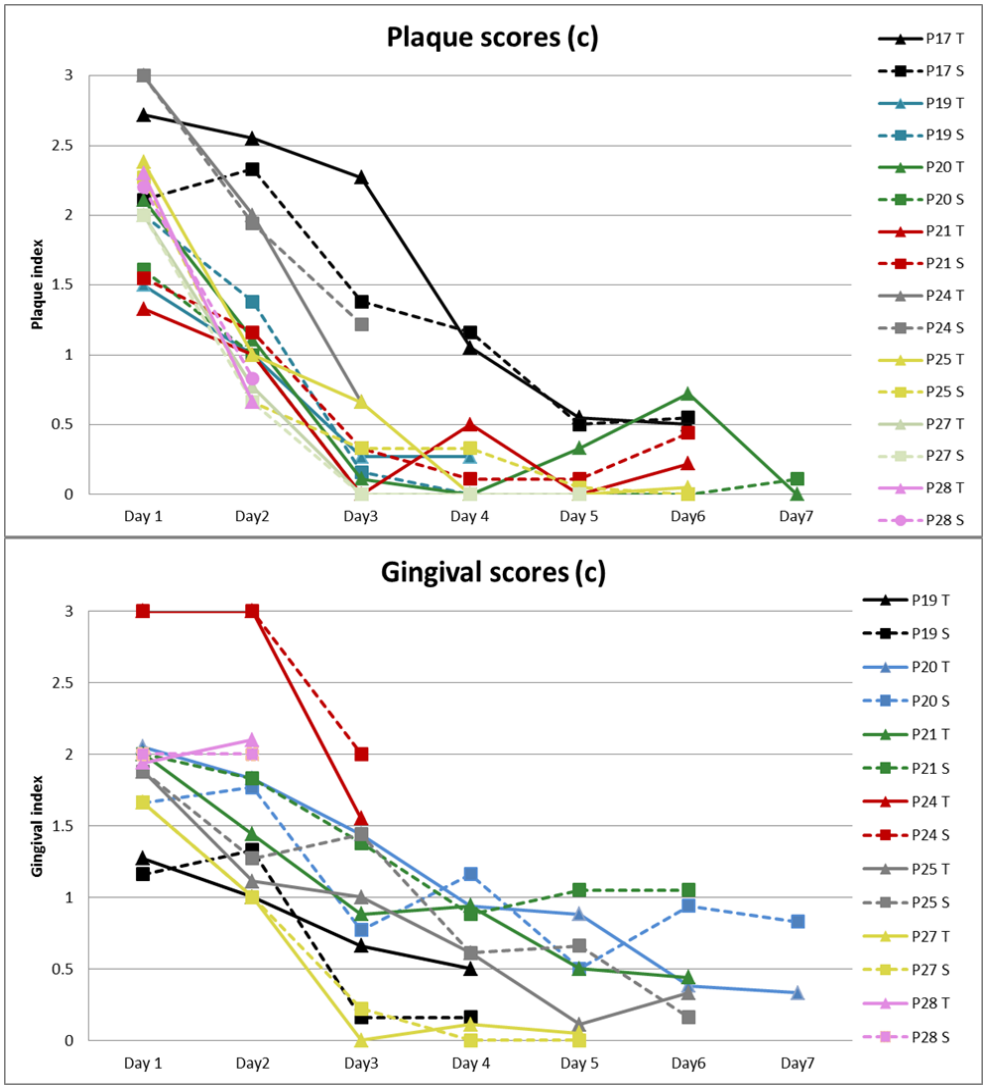

Figure 5.5 Changes in plaque and gingival index patients 19 to 28.

Changes in Silness and Löe (1964) plaque and gingival indices over a 7-day period using oral hygiene intervention either with a toothbrush (solid line) or foam swab (broken line).

Table 5.7 Comparison of oral health parameters of this study's patients and the general population

Parameter	**ADH Survey**[a]	**Study**
Overall number of teeth	25.7	24.4
Healthy periodontal tissues[b]	17%	10%
Obvious tooth caries	31%	23%
Excellent oral health[c]	10%	4%

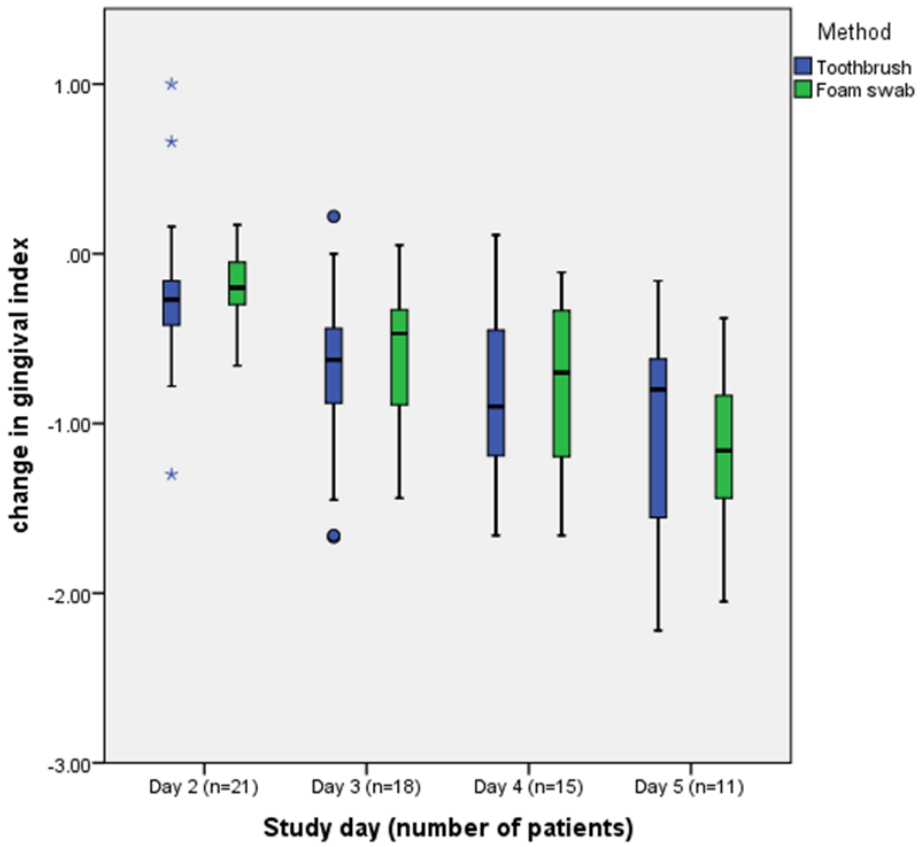

Figure 5.6 Boxplots of change in gingival index (from baseline) for each study day. Asterisks represent extreme outliers > 3 IQR; circles represent outliers > 1.5 IQR.

5.4.2 **Comparison of biofilm development of different endotracheal tube biomaterial surfaces**

Figure 5.7 presents the relative number of colonising microorganisms on the PneuX™ and Portex®, Evac® and silver coted ETTs. In terms of total microbial counts, there was no significant difference for the tested ETTs (p=>0.05). It was evident from this data that numbers of *C. albicans* were consistently low on all of the surfaces, whilst similar levels of *S. aureus*, *P. aeruginosa*, *S. mutans* and *P. gingivalis* were encountered on all ETT types. It would appear that all the ETTs tested were equivalent in terms of *in vitro* biofilm generation.

5.4.3 **Susceptibility of planktonically cultured microorganisms and biofilms to antimicrobial mouthwashes**

The susceptibility of planktonic, single species and dual species biofilms to three antimicrobial mouthwash preparations, Citroxx®, Listerine® and CHX was determined.

A summary of the results are presented in Table 5.8 (average results per tested concentration in Appendix II). All microorganisms tested as planktonic cultures were susceptible to all the antimicrobial mouthwashes, except for *P. aeruginosa* which was resistant to 50% (v/v) Listerine.

In all experiments, the MBEC was higher for single species biofilms than the MIC for planktonic cultures. Increased tolerance of biofilm cells was determined to vary between 2 and >1200 fold depending on species. The exception to this was for *S. aureus* where equal susceptibility of planktonic and single species biofilm preparations to 2% Citroxx® BD was evident. Additionally, a number of dual biofilm preparations had higher resistance than the single species biofilms to the mouthwashes. For example, in the case of susceptibility to Citroxx® BD, the dual species biofilm combination of *S. aureus*/*S. mutans* and *S. aureus /C. albicans* exhibited MBEC of 8% (v/v) and 4% (v/v) respectively, compared with 2% (v/v) for

single species biofilms. In addition, some biofilms were not affected by exposure to the antimicrobial mouthwashes (Table 5.8). *Pseudomonas aeruginosa* biofilms exhibited resistance to Listerine®, CHX and, when cultured in combination with *C. albicans*, resistance to Citroxx® was also apparent.

Subsequent culture of mouthwash treated biofilms on to appropriate agars was used to assess whether viable cells still persisted at the MIC/MBEC of the mouthwashes (Table 5.8). For the majority of preparations exposed to CHX (10/12) and Citroxx® (8/12) viable microorganisms could be detected post treatment. Listerine® treatment also showed the persistence of viable cells (6/12), albeit at a reduced incidence. In dual species biofilms of *P. aeruginosa* or *S. aureus* with an oral species (*i.e. C. albicans* or *S. mutans*) only the respiratory pathogens retained viability at the apparent MBEC.

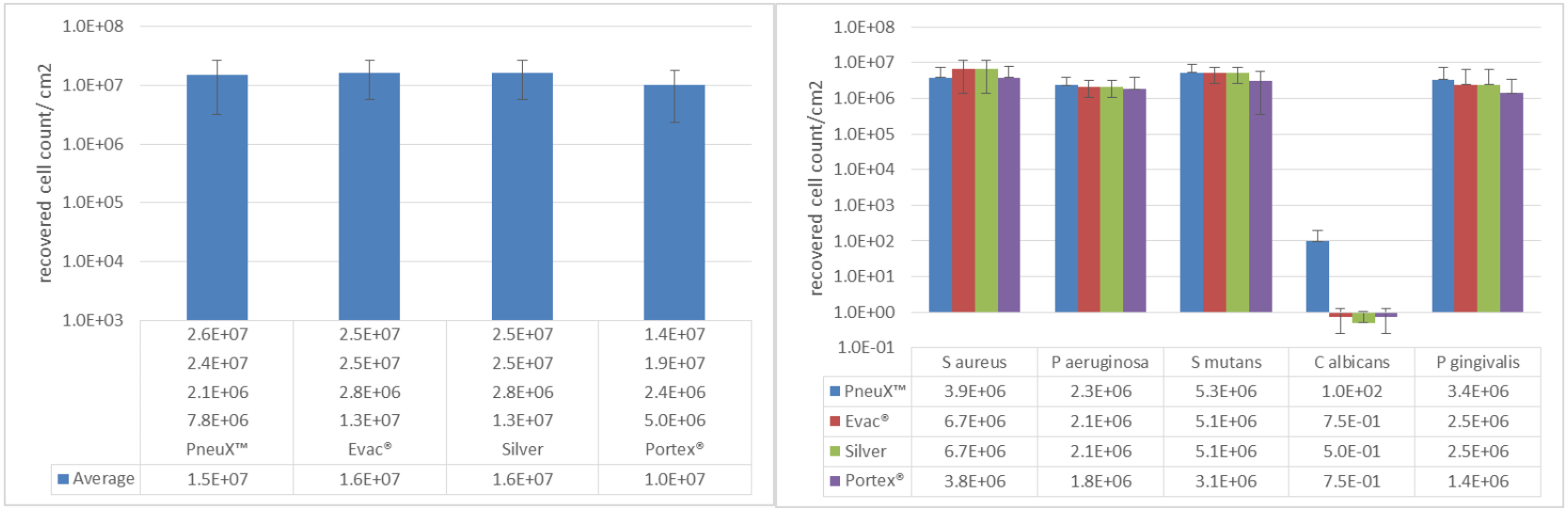

Figure 5.7 Total microbial counts per for four different endotracheal tubes PneuXTM, Evac, Silver coated and Portex (Right). Recovered counts per species, figures show average counts for 4 replicates (Left). Error bars show standard deviation.

Table 5.8 Summary table of minimum inhibition concentrations (MIC) for Listerine®, Citroxx® and Chlorhexidine (CHX). Growth from culture at the MIC are indicated as Yes or No; F represents number of fold change resistance of biofilms compared to planktonic.

	Listerine®	**F**	**Citroxx®**	**F**	**CHX**	**F**
S. aureus planktonic	12.5%		2%		0.0004%	
	No		Yes		Yes	
S. aureus single biofilm	>50%%	6	2%	0	0.008%	
	Yes		No		Yes	20
S. aureus (with *S. mutans*)	50%	4	8%	4	0.008%	20
	No		No		Yes	
S. aureus (with *C. albicans*)	>50%	>8	4%	2	0.03%	75
	Yes		No		Yes	
P. aeruginosa planktonic	>50%		2%		0.004%	
	Yes		Yes		Yes	
P. aeruginosa single biofilm	>50%		8%	4	>1.92%	>480
	Yes		Yes		Yes	
P. aeruginosa (with *S. mutans*)	>50%		8%	4	>1.92%	>480
	Yes		Yes		Yes	
P. aeruginosa (with *C. albicans*)	>50%		>8%	>4	>1.92%	>480
	Yes		Yes		Yes	
S. mutans planktonic	12.5%		4%		0.0002%	
	No		No		Yes	
S. mutans single biofilm	25%	2	>8%	>2	>0.24%	>1200
	No		Yes			
S. mutans (with *S. aureus*)	No		No		No	
S. mutans (with *P. aeruginosa*)	No		No		No	
C. albicans planktonic	25%		4%		0.002%	
	No		Yes		No	
C. albicans single biofilm	50%	2	>8%	>2	1.92%	960
	No		Yes		No	
C. albicans (with *S. aureus*)	No		No		Yes	
C. albicans (with *P. aeruginosa*)	No		No		No	

5.5 Discussion

The oral cavity has been shown to become colonised with respiratory pathogens associated with VAP in patients who are mechanically ventilated (Chapters 2 and 3). The co-existence of oral microorganisms and respiratory pathogens may also lead to symbiotic effects such as enhanced biofilm production and gene expression (Chapter 4) which could lead to more difficult to eradicate biofilms.

A logical approach aimed at preventing VAP would therefore be to reduce the bacterial load within the oral cavity of mechanically ventilated patients either through mechanical (toothbrushing) and/or chemical interventions (antimicrobial mouthwashes).

In addition, ETT biofilms have also been shown to be a source of respiratory pathogens alongside typical oral microorganisms. As this can also serve as a reservoir of infection approaches that limit such biofilm development (*e.g.* modification to the design of the ETT or its material) could also have benefit in reducing the risk of VAP. The primary aim of the research presented in this chapter was to evaluate different strategies that could potentially reduce the microbial load of both the oral cavity and biofilms in ETT lumens.

In health, the oral microflora is not traditionally considered a source of infection for the lower airways. This is despite recent research showing that the microbiome of lower airways include oral microorganisms (Beck *et al.* 2015; Cui *et al.* 2014). In health, the oral microbiome does not normally include respiratory pathogens such as *P. aeruginosa* and *S. aureus* (Dewhirst *et al.* 2010; Paster *et al.* 2001). In healthy adults oropharynx *P. aeruginosa* prevalence is estimated between 0 to 6.6% (Lister *et al.,* 2009, Morrison and Wenzel, 1984) and *S. aureus* between 3% to 46% (Eick *et al.* 2016; Ohara-Nemoto *et al.* 2008). However, in critically ill patients the occurrence of these pathogens in the mouth is much more prevalent and during mechanical ventilation a rapid change in the dental plaque microbial community

to include respiratory pathogens culture based studies have reported respiratory pathogens prevalence of 26% to 65% (Fourrier *et al.* 1998; Sachdev *et al.* 2013), however culture independent studies (Sands *et al.* 2016) and the work presented in Chapters 2 and 3 have found that up to 100% of mechanically ventilated patients are colonised. Molecular analysis of the oral microbiota and that of the lower airways of VAP patients show that genetically identical organisms are present at both sites, supporting the concept that potentially pathogenic bacteria colonising the oral cavity are also involved in pulmonary infection (Bahrani-Mougeot *et al.* 2007; Heo *et al.* 2008). Additionally, in Chapter 2, microbial species from ETT biofilms and non-directed bronchial lavages (NBLs) from mechanically ventilated patients had identical genetic profiles (electrophoretic karyotypes and RAPD fingerprints) to dental plaque microorganisms. These findings strongly link the mouth as a source of VAP causing pathogens.

Importantly, improvement in oral hygiene during mechanically ventilation has been identified as a VAP prevention strategy. Indeed, this approach is a component of current VAP prevention guidelines in the UK, Europe and USA (Masterton *et al.* 2008; Rello *et al.* 2010b; Speck *et al.* 2016). Importantly, most studies designed to improve oral care in mechanically ventilated patients tend to involve antiseptic or antibiotic strategies (Cutler and Sluman 2014; Hoshijima *et al.* 2013; Koeman *et al.* 2006). Frequently, CHX treatment is used unlike typical approaches employed in dentistry to prevent plaque mediated-disease such as dental caries and gingivitis. In dentistry most effort is directed to the mechanical removal of dental plaque by toothbrushing and flossing (Claydon 2008). The reason for this discrepancy in practice may be due to critical care healthcare professionals being more familiar with employing pharmacological interventions (Wise *et al.* 2008).

Almost all studies demonstrate that plaque scores increase in critically ill patients even when oral care programmes are in place (Fourrier *et al*. 1998; Sachdev *et al*. 2013; Scannapieco *et*

al. 1992). It should also be highlighted that there is considerable variability amongst critical care nurses on how they deliver oral care, with some opting for antiseptic solutions or gels, and when teeth are brushed, some use a foam swab in preference to a toothbrush (Feider *et al.* 2010; Grap *et al.* 2003; Rello *et al*. 2007). As such it is important to determine optimal oral care approaches to improve oral hygiene in mechanically ventilated patients (Wise and Williams 2013).

In this current research, an evaluation of two approaches to deliver oral care in mechanically ventilated patients was undertaken. These approaches were the use of a small headed toothbrush or a foam swab to brush patients' teeth. The study was a split-mouth design which was used as it reduces inter-individual variability. The results demonstrated that both plaque and gingival scores improved following these interventions and there was no significant difference between the approaches in improving oral cleanliness. Similar findings were reported in a recent study of 48 critically ill patients which compared toothbrushing against use of a gauze swab. Included with both interventions was administration of CHX at 12 h and 24 h intervals. The study showed a similar reduction in visible plaque and gingival bleeding with all interventions (Oliveira *et al.* 2014). This is in contrast with other studies where the use of a toothbrush for mechanical disruption of plaque has been found to be most effective (Addems *et al.* 1992; Pearson and Hutton 2002).

In this present investigation, the foam swab used had a coarse ridging (Figure 5.1), which may have assisted plaque removal, the head was of a similar size to that of the toothbrush used and both devices were applied using the same technique, which may account for the similarity in the results. Importantly, there was invariably some restricted access to dental surfaces given the presence of the ETT. A limitation to this study was its relatively short follow up period (2 to 7 days). However it would be expected that the changes in the level of dental plaque accumulation would be most noticeable in the first 48 h, and it was possible

that any improvement in plaque reduction would reach a plateau where no further significant improvement could be achieved. Nevertheless, lower levels of dental plaque would be expected when a strict protocol was followed. A similar dynamic was expected with gingival inflammation scores although the initial response was anticipated to be lagged compared with plaque scores, given that gingival responses to the new conditions would take time. A previous study compared use of an electric toothbrush with a foam swab in 46 individually randomised patients, with cleaning undertaken four times a day for 2 min in combination with 20 ml of 0.2% CHX (Needleman *et al.* 2011). This previous study found that a powered toothbrush was significantly better at reducing plaque compared with the foam swab. However, it was reported that the foam swabs also reduced plaque scores significantly from initial baseline scores. Powered toothbrushes have the advantage of a much smaller head than a manual brush and direct visualisation of brushing is not necessary, as the rotating head has only to be held on the tooth surface. In mechanically ventilated patients, a powered toothbrush may have an advantage over a manual toothbrush for these reasons and could be an area for future work.

No reduction in the number of bacteria that were isolated from plaque was seen with either method. This might reflect difficulties in plaque collection, or arise from the fact that plaque quantity is not just a reflection of bacterial number but also of extracellular polymeric substances (EPS) that are be present. For example, there could be instances where plaque indices differ not because of significant changes in bacterial number but due to removal of large quantities of EPS. This result is in contrast to the study of Needleman, where CHX was used in combination with a toothbrush or foam swab and led to significant reductions in the number of bacteria recovered from plaque.

It is important to clarify that this study focused on the efficacy of two oral hygiene practices rather than their effect on VAP incidence, ICU length of stay or mortality rates.

Toothbrushing has been used in a number of critical care studies as a means to reduce VAP, with mixed findings arising (Lorente *et al.* 2012; Munro *et al.* 2009; Pobo *et al.* 2009; Yao *et al.* 2011). Unfortunately, within these studies, compliance with the intervention was typically variable (Ames 2011) and none of the studies documented whether there was a reduction in dental plaque (which serves as the reservoir for respiratory pathogens). In this study, both foam swabs and toothbrushes were able to reduce plaque scores in mechanically-ventilated patients and were not significantly different in this regard. However, considerably more research is required to define the optimal method for mechanically removing plaque; a powered toothbrush, a manual brush with a smaller head, or increased frequency of cleaning may prove more effective and larger sample size will make findings more robust.

Since mechanical disruption appears to yield similar results regardless of the method used, other prevention strategies should be considered, including antimicrobial intervention. In a recent Cochrane review, the use of CHX and other oral mouthrinses to deliver oral care in the critically ill were analysed. The study concluded that there was evidence for a reduction in risk VAP from 25% to 19%, with a number of needed to treat of 17 (Hua *et al*. 2016).

In order to assess efficacy of oral mouthwashes against mixed species biofilms comprising of oral species and respiratory pathogens, an *in vitro* study was performed. The *in vitro* model is simple compared to the clinical scenario, however it allows control for environmental factors and avoids confounding variables such as the use of antibiotics and age and co-morbidities. The simplicity of the *in vitro* model also makes the study financially and logistically affordable. The results from these experiments showed high variability in microbial susceptibility to the test agents, depending on the microbial species/combination. Generally, CHX was the most effective mouthwash as it retained activity, below its therapeutic dose (0.12% -0.2%) and significantly inhibited all planktonic cultures and *S.*

aureus biofilms. However, *P. aeruginosa*, and *C. albicans* biofilms were resistant to CHX even at 1.92%, and *S. mutans* showed an increased resistance of >1200 fold compared to the planktonic growth. *Pseudomonas aeruginosa* biofilm resistance to CHX has previously been reported (Bonez *et al.* 2013). Elevated resistance (8-fold higher) of *C. albicans* biofilms on denture acrylic discs compared with planktonic cultures has also been found (Lamfon *et al.* 2004). Previously, *S. mutans* has been shown to be susceptible to CHX and there are no reports of resistance. However, most studies have been performed on isolates in planktonic state using disc diffusion assays and not against biofilms (Grönroos *et al.* 1995; Järvinen *et al.* 1993), although other *Streptococcus* species biofilms have however been shown to be susceptible to low CHX concentrations (Malic *et al*. 2013). CHX is less effective *in vitro* than other antiseptics, but *in vivo* the agent binds to clean tooth surfaces and is released over time, a property called substantivity. This property serves to slow plaque accumulation on 'clean' tooth surfaces (García-Caballero *et al.* 2013; Singh *et al.* 2011). It follows that CHX is considered to be most effective when the plaque has previously been disrupted (Shen *et al.* 2010).

The antimicrobial effectiveness of Citroxx® BC was initially encouraging, as all planktonic cultures and most biofilms (8/10) were susceptible to this agent, with MICs of between 2% and 8% (maximum concentration tested). However, the commercial preparation of Citroxx® BC is a 1% formulation, which was less effective at inhibiting planktonic and biofilms microorganisms. However, <1% Citroxx® concentrations have previously been reported to be effective against *Candida* and *Streptococcus* species in both planktonic and biofilm growth forms (Hooper *et al.* 2011; Malic *et al.* 2013). Listerine® Total Care Zero was the least effective mouthwash tested, with resistance shown by 5/8 of the biofilm preparations, as well as *P. aeruginosa* in planktonic state. In a clinical study involving 398 mechanically-ventilated patients, Listerine use led to no improvement in dental plaque colonisation by

respiratory pathogens, nor resulted in a reduced VAP incidence (Berry 2013). Despite these findings, the use of essential oils and plant extract-based agents should not be completely disregarded, as synergistic activity has been demonstrated between CHX and essential oils including 1,8-cineole against *S. aureus*, methicillin-resistant *S. aureus* (MRSA), *Escherichia coli* and *C. albicans*. Furthermore, combinations of CHX and Citroxx® BC have also been reported to have enhanced antimicrobial effects against biofilms of MRSA and *P. aeruginosa* (Hendry *et al*. 2009), and *Candida* and *Streptococcus* species (Malic *et al*. 2013).

In all dual species biofilms involving either *S. aureus* or *P. aeruginosa* combined with *C. albicans* or *S. mutans,* only *S. aureus* and *P. aeruginosa* were found to retain viability at the MIC concentration. One obvious reason for this finding would be the inherent difference in susceptibility between the combined species. Indeed, the previously determined MICs were lower for the species that had been eradicated. For example, the MBEC values of Listerine® were 25% and 50% for *S. mutans* and *C. albicans* single species biofilms, respectively. For *S. aureus* single species biofilms, the MIC was >50%. Another possible explanation was that one microorganism may have outcompeted the other in the biofilm prior to antimicrobial treatment as was observed in Chapter 4. It was found that *P. aeruginosa* inhibited the growth of *C. albicans* in dual biofilm preparations, this inhibition has previously attributed to the release of phenazines by *P. aeruginosa* (Hogan *et al.* 2004; Kerr 1994). Additionally, *P. aeruginosa* tends to grows rapidly *in vitro* which could have impacted in *S. mutans* growth (LaBauve and Wargo 2012).

An improved understanding of how antimicrobials interact with mechanical methods is required. In a recent meta-analysis, Klompas *et al.,* (2014) suggested that CHX could be ineffective or possibly harmful in general critical care units, as it may promote occurrence of multi-resistant species. Additionally, allergic reactions to CHX mouthwash, including

anaphylactic reactions appear to be more frequently reported (Pemberton and Gibson 2012).

The last prevention strategy studied in this Chapter was modification of the ETTs biomaterial. For this study, four different ETTs were evaluated including a silicone ETT with a 'coated lumen' referred to as PneuX™, an experimental silver coated ETT, a standard PVC Evac® ETT, and the Portex® ETT. Experiments consisted of developing *in vitro* biofilms on ETT sections over 5 days using oral microbial species (*S. mutans, C. albicans* and *P. gingivalis*) and known VAP pathogens (*S. aureus* and *P. aeruginosa*).

Results showed comparable levels (in terms of total microbial counts) of biofilm formation for all the microbial species for the tested ETTs. When analysed by species type, all tested ETT surfaces had lower numbers of *C. albicans* on the ETT surface compared with bacteria.

These findings would appear to suggest that the actual biomaterial surface of these ETTs, including the PneuX™ and silver coated surfaces, has a limited effect in preventing biofilm development. In the case of the PneuX™ ETT, observed *in vivo* biofilm reduction most likely reflect reduced subglottic leakage below the cuff, and the ability to obtain subglottic drainage above the cuff. One interesting observation for the PneuX™ tube was the apparent presence of 'ridges' on the inner luminal surface, which arose from the inner supporting wire of the PneuX™ tube. As part of future studies, it would be interesting to examine where microbial colonisation is occurring preferentially within these. There are no other *in vitro* studies available for the PneuX™ ETT.

The silver coating of the 'experimental' ETT tested did not appeared to have an impact on biofilm growth, this was an unexpected finding as *in vitro* studies including animal models with rabbits and dogs had reported less bacterial colonisation on silver coated ETTs (Olson *et al.* 2002; Rello *et al.* 2010a). Rello *et al*., (2010) reported a significant (>90%; $p<0.05$) reduction in microbial attachment for (12/21) isolates including *P. aeruginosa*, in the

colonisation of tracheal and lung tissue of rabbits when a silver coated ETT was compared to an uncoated control. Additionally, clinical trials have reported lower colonisation and a lower incidence of VAP when silver coated ETTs were used (Berra *et al*. 2008; Kollef *et al*. 2008; Tokmaji *et al*. 2015). It is possible that as the tubes used in this experiments were from a different manufacturer, the performance was not the same as the commercially available tubes. In addition it is conceivable that the silver coating could have dissipated over the 5 day study period used in these experiments.

5.6 Conclusions

- The oral health of patients admitted to the adult ICU was generally poor. Dental plaque and gingival inflammation indices were reduced when either a toothbrush or foam swabs were used in a strict oral care program. No differences were found in terms of bacterial load.
- Planktonic cultures were susceptible to antimicrobial mouthwashes although varied levels of susceptibility were seen for single and dual biofilms, with higher levels of resistance seen in biofilms compared to planktonic cultures.
- Microbial growth in biofilms was similar for four ETT surfaces, including a coated silicone ETT and an experimental silver coated ETT.

6. General Discussion

6.1. General discussion

The impact of poor oral health on the general wellbeing of a person has been acknowledged for a number of conditions (Linden *et al.* 2013), and it is no longer considered only in relation to dental caries and periodontal disease. Indeed, poor oral health has now been linked with cardiovascular disease (Niederman and Weyant 2012), diabetes (Winning *et al.* 2017), low birth weight babies (Saini *et al.* 2011), rheumatoid arthritis (de Pablo *et al.* 2009) and renal disease (Sharma *et al.* 2016a). Of specific focus to this thesis, is that poor oral hygiene has also been implicated with higher incidence of respiratory infections, including community-acquired pneumonia (CAP), health care-associated pneumonia (HCAP), hospital-acquired pneumonia (HAP) and ventilator-associated pneumonia (VAP) (Azarpazhooh and Leake 2006; Fourrier *et al.* 1998; Scannapieco *et al.* 1992).

Ventilator associated pneumonia (VAP) is the most common nosocomial infection in intensive care units (UCI), and defined as a pneumonia that occurs 48h after the start of mechanical ventilation. The condition affects between 8 and 28% of mechanically ventilated patients (Chastre and Fagon 2002) whose hospital stay is subsequently extended to an average of an extra 5 to 7 days (Safdar *et al.* 2005). Additionally, VAP has an attributable overall mortality rate estimated at 13% (Melsen *et al.* 2013) and results in increased treatment costs of up to £22.000 per case (Wagh and Acharya 2009). Clearly, VAP is a highly significant clinical problem and investigation into contributory factors are undoubtedly warranted.

One of the associated factors for VAP, is respiratory pathogen colonisation of dental plaque in mechanically ventilated patients, which has been estimated to occur in 40% to 65% of patients (Fourrier *et al.* 1998; Sachdev *et al.* 2013; Scannapieco *et al.* 1992). The movement of these pathogens from the mouth to the lower airways is likely assisted by the placement

of an endotracheal tube (ETT), which will impede normal host defence mechanisms in the trachea (ciliary movement and cough reflexes). Oropharyngeal secretions ultimately pool above the inflated ETT cuff and following micro leakage via channels formed in the cuff, microorganisms in these secretions can then colonise the lower airways, promoting VAP (Dave *et al.* 2010; Hamilton and Grap 2012). Furthermore, not only can these microorganisms directly colonise the lungs, but soon after commencement of mechanical ventilation, biofilms consisting of both respiratory pathogens and oral microorganisms can be detected within the ETT lumen (Cairns *et al.* 2011; Perkins *et al.* 2010; Vandecandelaere *et al.* 2012). As these biofilm have unrestricted and direct access to the lower airways they serve as likely reservoirs of infectious agents. Since the ETT lumen is not accessible to either administered antibiotic therapies or host defence factors, eradication of these biofilms is extremely problematic and currently not achievable without ETT replacement. Furthermore, the replacement of ETT is considered an independent risk factor for VAP incidence (de Lassence *et al.* 2002; Torres *et al.* 1995).

It is important to mention that throughout this research project key microorganisms that were representative of both the normal oral microflora (*Streptococcus mutans*, *Porphyromonas gingivalis* and *Candida albicans*) and respiratory pathogens associated with VAP (*i.e. Staphylococcus aureus* and *Pseudomonas aeruginosa*) were selected as target species for the analysis of clinical samples and *in vitro* experiments.

Staphylococcus aureus and *P. aeruginosa* were selected as respiratory pathogens because they are deemed responsible for approximately 50% of VAPs (Chastre and Fagon 2002) and are not microorganisms that are normally considered inhabitants of the oral microflora in health. Indeed oral carriage in health has been estimated as < 7% for *P. aeruginosa* (Morrison and Wenzel 1984; Rivas Caldas *et al.* 2015) and between 3 to 50% for *S. aureus*, although when present, has been considered to be a transient coloniser rather than a permanent

resident (Eick *et al.* 2016; Ohara-Nemoto *et al.* 2008). *Streptococcus mutans* was as a typical oral microorganism given the high prevalence of the *Streptococcus* genus in dental plaque (Dewhirst *et al.* 2010). *Streptococcus mutans* itself has an incidence (72% to 95%) in the mouth and can be detected at levels of 10^6 colony forming units (CFU)/ml of saliva (Emilson and Thorselius 1988; Sharma *et al.* 2016b). *Streptococcus mutans* is also an adept biofilm former and readily attaches to the hard enamel surfaces of teeth (Lynch *et al.* 2013). *Porphyromonas gingivalis* was selected as a representative oral microorganism given its high incidence in subgingival plaque. Additionally, given the fact that critically ill patients were likely to have poor oral hygiene, it was anticipated that they would exhibit higher levels of gingivitis which is associated (50-79% of cases) with *P. gingivalis* (Ertugrul *et al.* 2013; Griffen *et al.* 1998; Ito *et al.* 2014). Furthermore, this anaerobic bacterium had previously been found in ETT biofilms (Cairns *et al.* 2011). The last oral species targeted was *C. albicans*, which, although far from being exclusive to the oral cavity, often colonises the oral mucosa with a prevalence estimated between 50 % to75% (Javed *et al.* 2013; Mayer *et al.* 2013) and it is also able to form biofilms in biomaterials including ETTs (Vandecandelaere *et al.,* 2012).

One of the underlying hypotheses of this thesis was that oral microorganisms would play a contributory role in the development of the ETT biofilm and facilitate colonisation by respiratory pathogens. To investigate this hypothesis it was firstly important to demonstrate the presence of typical oral microorganisms within ETT biofilms and in non-directed bronchial lavages (NBLs). These investigations were undertaken using traditional culture methods (Chapter 2) and contemporary molecular approaches (Chapter 3).

Initially, this research used culture and species-specific PCR to detect the key target microorganisms mentioned earlier. Often microorganisms are difficult or not possible to cultivate from the oral cavity with the currently available media and culture conditions, and it is considered that approximately half of the species are not yet cultivable (Dewhirst et al.

2010). PCR-based methods offer the advantage of rapid high sensitivity in detecting species without the need for culture, and current technologies like next generation sequencing (NGS) can also inform on the relative abundance of individual species and characterise whole microbiomes in a cost effective manner (Didelot *et al.* 2012).

The results of this research showed that oral species and respiratory pathogens were simultaneously present in the dental plaque, NBLs and ETT biofilms of the mechanically ventilated patients. Demonstrating the same species simultaneously at these sites within a given patient would imply a likely common origin of these microorganisms *i.e.* the oral cavity. However, this view would be reinforced if the organisms were shown to the same strain types. As a consequence, typing of isolates was undertaken by random amplification of polymorphic DNA (RAPD) and pulse field gel electrophoresis (PFGE) (Chapter 2). These results did indeed demonstrate that in many cases the isolated species from dental plaque were of the same genetic fingerprint as those recovered from NBLs and ETT biofilms within a given patient. These findings were similar to those reported by Heo *et al.,* who studied dental plaque and bronchoalveolar lavage fluid from a hundred mechanically ventilated patients and found identical strains of *S. aureus, P. aeruginosa, Escherichia coli* and *C. albicans* by means of PGFE and multilocus sequence typing (Heo *et al.* 2008; Heo *et al.* 2011). Importantly, no previous studies have undertaken equivalent typing studies for isolates from the ETT biofilm.

One limitation of the approach used in Chapter 2 was that only a few species were targeted. To address this, in Chapter 3, next generation sequencing (NGS) was used to characterise the microbiomes of dental plaque, NBL and ETT biofilm communities from mechanically ventilated patients. NGS theoretically allows all bacterial species in a sample to be detected as it is not reliant on culture or microbial viability (Didelot *et al.*, 2012). There are of course limitations associated with NGS, some of which relate to potential bias in regards to DNA

extraction and PCR (Brooks *et al.* 2015) and others relate to the platform used and the downstream analysis that can lead to an overestimation of Operational Taxonomic Units (OTU) (Quince *et al.* 2011). NGS typically targets the 16S rRNA gene of bacteria and as these genes are normally in high abundance within the bacterial genome, the approach is generally more efficient than species-specific PCR (Poretsky *et al.* 2014). However, molecular methods do not normally distinguish between live and dead microorganisms, and without culture, phenotypic characteristics such as antibiotic resistance and virulence are arguably more difficult to ascertain. From these experiments it was found that the diversity in the microbiomes of dental plaque, ETTs and NBLs were similar. However, the NBL microbial communities were less similar to dental plaque compared with the ETT biofilm. Nevertheless, as previously reported by culture and species-specific PCR, typical oral species and respiratory pathogens were found at all sites. It is conceivable that in the lower airway, the lack of a hard surface for microbial attachment (or indeed other environmental differences from the other sites), was instrumental in the loss of some species leading to the observed differences in NBL microbiomes. Importantly, all of the 12 patients whose samples were studied by NGS had respiratory pathogens in their dental plaque including *Streptococcus pneumoniae*, *Haemophilus influenza*, *S. aureus* and *P. aeruginosa.* These species were amongst the top 20 most abundant in the dental plaque, and represent species that do not normally reside in the plaque of healthy individuals (Dewhirst et al. 2010; Paster *et al.* 2006). Whist the microbiome of ETTs, NBLs and dental plaque were similar, the most diverse sample was dental plaque which supports the notion of dental plaque being a reservoir for the other sites. Previous studies have used NGS methods to describe dental plaque, ETT biofilms and bronchial lavages (BAL) (Beck *et al.* 2015; Sands *et al.* 2016a; Vandecandelaere et al. 2012; Yang *et al.* 2015), but none have studied all three sites simultaneously. The results were similar to other studies which have shown respiratory

pathogens were frequently and abundantly found in dental plaque (Sands *et al.* 2016a) and, that ETT biofilms were dominated by oral species like *Prevotella* and *Peptostreptococcus* (Vandecandelaere *et al.* 2012). The lung, is a site that has previously been considered a sterile one, however NGS technologies have shown that in the healthy adult, the microbiome of the lung is largely similar to the oropharynx. This was been shown in previous analyses of oral rinses and BALs in healthy subjects, where oral species including *Streptococcus*, *Fusobacterium* and *Neisseria* were found in relative high abundance (Beck *et al.* 2015). Moreover, a study that analysed oral washes, nasal swabs, gastric aspirates and BALs from 28 healthy subjects using pyrosequencing concluded that the lung microbial community was different from that of the mouth, nose and stomach. However, bacterial communities of lungs and mouth overlapped with lower concentrations found in the lungs. Interestingly in this study, the nasal microbiome was distinct to that of the mouth and lung suggesting little contribution to the lung microbiome in health (Bassis *et al.* 2015). In VAP patients, the *Streptococcus* genus has previously been reported to be the most abundant from BAL samples, although species level identification was not undertaken (Yang *et al.* 2015). Kelly *et al.* (2016) demonstrated that intubated patients had an abnormal BAL microbiome at the time of intubation, which progressed to a characteristic pattern of lower diversity in clinical VAP cases. The same study also found in pneumonia patients where positive cultures were available they correlated to the most dominant species found through NGS but when cultures were negative the most predominant bacteria were those not usually thought to cause pneumonia (*Enterococcus faecalis*) and not normally cultured (*Ureaplasma parum*) (Kelly *et al.* 2016).

A limitation to the work presented here was that the fungal microbiome or 'mycobiome' was not studied by NGS. A recent investigation of 202 patients divided in 6 sub-study groups (healthy, extra pulmonary infection, mechanically ventilated, mechanically ventilated

without pneumonia but on antibiotic therapy, mechanically ventilated with pneumonia and candidaemia) reported that in the critically ill, the mycobiome became rapidly predominated by *Candida* species. Interestingly, the critical illness and not antibiotic therapy was identified as the risk factor for *Candida* colonisation. Candidaemia was not associated with pulmonary colonisation and no association with bacteria was found in the pneumonia cases (Krause *et al.* 2016). This is in contrast to previous reports of *Candida* colonisation as a risk factor for *Pseudomonas* pneumonia (Azoulay *et al.* 2006; Roux *et al.* 2009).

A further limitation to this part of the study was the relatively small number of samples/patients analysed by NGS. It was not possible to recruit more patients to the study, as the patients were part of a clinical trial (Chapter 5) comparing toothbrushes and foam swabs as methods of oral hygiene, and after a separate clinical incident (not associated with this study) the Wales Government banned the use of foam swabs (Jewell *et al.* 2012). There were also some difficulties in extracting bacterial DNA from some NBLs and ETTs, which also served to limit the number of samples analysed. Another potential issue related to the technique used to obtain NBLs. This involved inserting a catheter into the ETT until resistance was felt by the operator. Once the catheter had reached the lower airway, a saline solution was then used as an irrigation fluid and immediately aspirated. This method could potentially lead to cross contamination from the ETT, as the operator is effectively blinded and the saline solution could contact the ETT. An alternative to minimise potential cross contamination would have been to use a bronchoscopic technique, such as a protected brush specimen, however this technique is considered more invasive and offers little clinical advantage over NLBs (Flanagan *et al.* 2000). Therefore, alternative approaches to the NBL may not have been appropriate for patients where VAP was not suspected. NBLs were also already part of the ICU's protocols for surveillance of VAP, and it is likely that a change of protocol would have not been ethically acceptable.

The primary aim of the research was to associate the microbiomes of dental plaque, ETT biofilms and the lower airways, and not to assess incidence of VAP to these microbiomes. VAP incidence was not recorded for all of the studied patients, partly due to the complexities of its diagnosis but also due to this information lacking from medical notes. Whilst this information could have been beneficial, it is likely that due to the relatively small patient cohort with wide variety of age and underlying medical conditions, a significant difference between VAP and non VAP patients would not have been achievable.

Whilst these results provide clear association between the studied sites in terms of microbial translocation, the actual impact of the oral microorganisms on respiratory pathogens has previously received limited attention.

Based on the findings from Chapters 2 and 3, it was evident that oral microbial species and respiratory pathogens co-existed in the dental plaque, the ETT biofilm and the lower airways. It was likely therefore that the oral cavity was a primary source of these species; however this finding on its own does not explain the potential interactions that may occur between these groups of microorganisms. The effect of oral bacteria on respiratory pathogens was the focus of the research presented in Chapter 4. In order to assess potential interactions, *in vitro* biofilms were generated and synergistic influences of the oral species (*S. mutans, P. gingivalis* and *C. albicans*) on biofilm formation and gene expression by targeted respiratory pathogens (*S. aureus* and *P. aeruginosa*) was assessed. The results showed that in dual biofilms, *S. mutans* increased biofilm formation by *S. aureus.* This increased biofilm development correlated with enhanced gene expression of genes associated with quorum sensing (*agrI;* Shopsin *et al*. 2003) and polysaccharide intercellular adhesin (PIA) production*, (icaC, icaAb, icaBA,* and *icaRa* genes; Malic, 2008). *Pseudomonas aeruginosa* was found to inhibit the growth of *C. albicans* in dual species biofilms, and this has previously been reported (Harriott and Noverr 2011; Hogan *et al.* 2004). In the presence of *C. albicans, P.*

aeruginosa releases toxic phenazines (Dietrich *et al.* 2006) which are considered cidal to *Candida* hyphae, thereby generating a growth substrate for *P. aeruginosa* (Harriott and Noverr 2011). The results showed that *C. albicans* presence led to upregulation of the *algD* and *cupA* genes of *P. aeruginosa* which encode for alginate production and adhesion to inert surfaces (Edwards and Saunders 2001; Mah *et al.* 2003). Importantly, gene expression and biofilm formation were enhanced for both respiratory pathogens as part of mixed species biofilms when all reference strains were included.

A limitation to the *in vitro* biofilm model was its simplicity compared with the *in vivo* situation. In these experiments, ETT sections were immersed in broth, whilst *in vivo*, the ETT lumen would be relatively dry with higher oxygen concentrations. Furthermore, biomaterials and hard surfaces are normally covered by a complex conditioning layer formed from the absorption of water, protein, lipids extracellular matrix molecules, complement, fibronectin and inorganic salts, as a precursor of biofilm formation (Chapter 1 Section 1.2.1.1).

Biofilms are associated with 'difficult to treat' and often recurrent infections due to their higher resistance to antibiotics (Luppens *et al.* 2002) and physical resilience (Normark and Normark 2002). It was therefore important to assess expression of biofilm related genes to further understand the infection process. Expression of a number of biofilm associated genes was therefore measured (Tables 4.4, 4.5). An alternative would have been to study genes associated with 'true' virulence factors including those involved in the invasion of host tissues or endotoxins linked with pneumonia. It has been reported that expression of type 3 secretion system, exotoxin secretion and elastase production were associated with increased lung injury in a *Pseudomonas* pneumonia murine model (Le Berre *et al.* 2011). Recently, the *S. aureus* serine protease-like (*spl*) operon which carries six genes (*slpA* to *splF*) has been associated with invasion of the host in a rabbit pneumonia model (Paharik *et al.* 2016). The research revealed more widespread lung damage in rabbits infected with the *slp*

mutant strain (Paharik et al. 2016). The same study also found that *splA* induced shedding of the mucin 16 glycoprotein from lung epithelial cells. Since mucin 16 is present at multiple body sites, including the airways, this function could facilitate infection (Paharik *et al.* 2016). Another study found that *S. aureus* strains exhibiting higher *in vitro* expression of *agr, saeRS, sarA, hla*, and Panton-Valentine leucocidin (*pvl*) genes were responsible for more severe cases of pneumonia, and higher bacterial counts in a rat pneumonia model (Montgomery *et al.* 2008).

For this *in vitro* studies reference strains were used, however an alternative option would have been to use the clinical isolates found in the clinical samples of intensive care patients (Chapter 2). It is possible that the use of clinical isolates would give a better representation of bacterial interactions between respiratory pathogens and oral microorganisms. This was not however possible as part of this thesis to overlap timings during the experimental phase of this doctoral studies.

As the research described in Chapters 2-4 had demonstrated both an involvement and influence of oral microorganisms on biofilms associated with VAP, it was a natural progression to consider potential interventions and strategies to disrupt and prevent these biofilms. These studies were undertaken in Chapter 5, which focused on evaluating two oral hygiene methods in mechanically ventilated patients, the effectiveness of mouthwash preparations for inhibiting *in vitro* biofilms and assessing different ETT surfaces in terms of biofilm formation.

It was noted that poor oral health indices were evident in the majority of patients recruited to the clinical study. Only 4% of patients were classed as having excellent oral health (Table 5.7) at the start of the study. The aim of this study was to determine the best method (of the two studied) for delivering oral hygiene in the mechanically ventilated patient. The normal practice in the ICU was to brush teeth using foam swabs with saline. It was presumed

that toothbrushes should be more efficient, which had been reported in a previous study comparing foam swabs and toothbrushes on healthy volunteers (Pearson and Hutton 2002). An alternative would have been to use an electric toothbrush as there is evidence of their superiority compared to manual toothbrushes in healthy individuals (Yaacob *et al.* 2014) and they are possibly easier to use.

Importantly, dental plaque accumulation and gingival inflammation improved when either toothbrush or foam swab cleaning was implemented. The study showed that both methods were equally effective in improving oral cleanliness, although no significant changes were seen in the bacterial load. The study demonstrated that well trained and motivated staff could provide satisfactory oral care in mechanically ventilated patients with either of these mechanical cleansing devices. In this study, no antimicrobials were used as adjuvants, as no such agents were part of the ITU protocols at the time of the study, and it was thought that the use of a mouthwash could interfere with dental plaque accumulation and create a potential bias on the evaluation of the effects of mechanical cleaning. For future studies, it may be of value to record baseline scores of the basic periodontal exam (BPE) (Matthews 2014). Despite its limitations in the diagnosis of periodontal disease, the BPE may offer a better indication of the periodontal condition pre- hospital admission. For example, the presence of dental calculus and deep periodontal pockets are unlikely to develop in just a few days of hospital stay and would not be expected to change despite an excellent toothbrushing technique or use of antimicrobials. A limitation of any toothbrushing technique, even in healthy adults is the presence of interproximal spaces that no toothbrush can effectively reach. It is because of such locations that daily use of dental floss or interproximal brushes is recommended to maintain good oral health (Claydon 2008). The problem of plaque removal from interproximal spaces has not been addressed in the mechanically ventilated patient. Routine oral care protocols do not address the problem,

probably as interproximal cleaning demands dexterity and good access to all sites in the mouth, which is a challenge in a heavily sedated patient with an ETT. Also, according to the oral health foundation, less than a quarter of people floss regularly (http://www.nationalsmilemonth.org/facts-figures/), and it is therefore not surprising that the practice is not at the forefront of most people's minds. However, in the hospitalised patient, when dental plaque becomes colonised by respiratory pathogens this is likely to include the interproximal spaces, as well as other oral sites *i.e.* cheeks, tongue, lip, palate and subgingival spaces (Paster *et al.* 2006). It is possible that these sites act as 'protected niches' for respiratory pathogen colonisation allowing them to persist despite toothbrushing and mechanical and chemical intervention. Therefore, complete plaque removal in the mechanically ventilated patient (as well as heathy adults) is not practically possible and only a decrease in bacterial load can be expected. It remains to be established if oral hygiene intervention in mechanically ventilated patients can effectively achieve this goal. A study in elderly patients, found that the prevalence of pathogens including *S. pneumoniae, S. aureus*, and *P. aeruginosa* was not significantly different when the patients had received professional assistance with their oral care (Abe *et al.* 2001). Needleman *et al.,* (2011) demonstrated improved dental plaque control and lower total bacterial counts in 46 mechanically ventilated patients when a powered toothbrush was used in comparison to a sponge toothette. This previous study also found no significant differences in the prevalence of respiratory pathogens in the dental plaque, although the incidence of respiratory pathogens was relatively low, making it difficult to assess if a more effective toothbrushing technique would lead to reduced colonisation. The study reported a decrease (from day 1 to 5) in *S. aureus* in the powered toothbrush group (26.1%-11%) that was not observed in the control group (33.3%-30%). The difference was not however statistically significant (p=0.3) (Needleman *et al.* 2011). This is in contrast to other studies that have reported an

increase in respiratory pathogens with increasing time of mechanical ventilation (Fourrier *et al.* 1998; Sands *et al.* 2016b). Additionally, Needleman's study used only culture-based methods to determine the presence of the respiratory pathogens, which is likely to have limited sensitivity. For example, as described in this thesis, *S. aureus* was detected in dental plaque by culture in 6/21 patients, but using species-specific PCR, the incidence increased to 13/21 (Chapter 2). Similarly, of the patients studied by NGS, 11 out of 12 plaque samples had *S. aureus* sequences detected (Chapter 3).

The impact of toothbrushing on the reduction of VAP incidence has not been determined. There are only five studies (Lorente *et al.* 2012; Munro *et al.* 2009; Pobo *et al.* 2009; Roca Biosca *et al.* 2011; Yao *et al.* 2011) that have evaluated effects of toothbrushing on VAP incidence. Only one study, involving 53 patients, reported a reduction in VAP following toothbrushing (17% powered toothbrush group, 71% control group; p=<0.05). This study did not include antimicrobials and also reported reduced plaque accumulation (Yao *et al.* 2011). Most often, other studies include chlorhexidine in both control and toothbrushing groups.

In Chapter 5, the efficacy of three antibacterial mouthwashes (Citroxx®, Listerine® Total Care Zero, and chlorhexidine) was tested against planktonic cultures and *in vitro* single and dual species biofilms developed by *P. aeruginosa, S. aureus, C. albicans* and *S. mutans*. It has been reported previously that biofilm cells are more resistant to treatment with mouthwashes than microorganisms in the planktonic state, with a degree of variability depending on the active ingredients (Hooper *et al.* 2011; Malic *et al.* 2013; Masadeh *et al.* 2013). Importantly, some bacterial species are more susceptible than others. It has been shown that whilst most *Streptococcus* species are susceptible to most available mouthwashes (Malhotra *et al.* 2011), *P. aeruginosa* and meticillin resistant *S. aureus* (MRSA) biofilms are reported as resistant to variety of mouthwashes, including CHX (Masadeh et al. 2013; Smith *et al.* 2013). This raises the concern that the use of antiseptic mouthwashes may favour the colonisation and

persistence of multidrug resistant species. Additionally there is a recent report that indicated that exposure to CHX resulted in *Klebsiella pneumoniae* developing resistance not just to CHX, but also to the colistin, which is considered a 'last resort' antibiotic (Wand *et al.* 2016). Despite these experiments not using a mouthwash preparation, it raises concerns over the potential promotion of antibiotic resistance.

It was decided that although *in vitro* studies have the limitation of simplicity compared to the clinical scenario, they avoid confounding factors such as different patient medical histories, and the additional use of antibiotics. Furthermore, the cost and logistics of undertaking further *in vivo* studies in the PhD were inhibitory. It would have been interesting to repeat these experiments with clinical isolates from mechanically ventilated patients to assess their innate antimicrobial resistance.

Importantly the results showed that biofilms were more resistant than planktonic cultures, and some dual species biofilms exhibited higher resistance than single species. These findings are in agreement with previous reports showing dual species biofilms having higher resistance, possibly due to clustering of the different species (Kara *et al.* 2007; Sanchez-Vizuete *et al.* 2015). Other authors have reported inefficacy of CHX, essential oils, cetylpyridinium chloride and isopropylmethylphenol mouthwashes in penetrating and disrupting *S. mutans* biofilms (Wakamatsu *et al.* 2014). The most effective agent was deemed to be chlorhexidine (CHX), although *P. aeruginosa* biofilms were resistant to CHX even at the highest concentration used (1.92%). Chlorhexidine is more frequently used at 0.12% or 0.2% formulations, although some investigations in mechanically ventilated patients have assessed 2% CHX solution and gels, and generally report a VAP reduction (Koeman *et al.* 2006; Tantipong *et al.* 2008). A recent Cochrane review reported that from 18 randomised clinical trials (RCTs), use of CHX led to a reduced VAP incidence from 25% to 19%, and no differences were found with CHX doses of 0.12%, 0.2% or 2%. However, no

difference was found in either mortality or hospital stay (Hua *et al.* 2016). Evaluation of other antimicrobial solutions in mechanically ventilated is very limited. A study evaluating Listerine® and sodium bicarbonate reported no reduction in VAP incidence (Berry 2013) Another RCT multicentre study on the effects of povidone-iodine also reported no improvement on VAP outcomes and a possible increased risk of acute respiratory distress syndrome (Seguin *et al.* 2014).

Finally, an evaluation of four different ETT materials including a silicone ETT with a 'coated lumen' (PneuX™), an experimental silver coated ETT and two standard PVC ETTs (Evac® and Portex®) for their ability to inhibit microbial colonisation was undertaken. The results showed that all test surfaces equally supported biofilm growth, and based on this mechanism were unlikely to provide clinical benefit. However, as previously mentioned, the model system used was considerably more simplistic than that encountered *in vivo*. The PneuX™ ETT also offers multiple mechanisms to inhibit biofilm development in its lumen and these were not assessed in this study.

6.2. Future work

The relationship between dental plaque, ETT biofilms and VAP is complicated and needs further research and a number of projects could arise from the current work.

Further phenotypic characteristics could be assessed from the isolates obtained from mechanically ventilated patients, including antibiotic resistance and the presence of genes previously described as being associated with resistance and virulence.

Since the microbiome of the dental plaque, ETT biofilm and NBLs appears to be similar and frequently shared the same species and strains, it is possible that sampling dental plaque may be a useful practice in the surveillance for VAP. Analysing dental plaque may be an alternative and less invasive approach than NBLs for detection of important respiratory

pathogens. Taking dental plaque samples would arguably be less demanding for medical staff as they could be taken by a single nurse, be better tolerated by the patient and could also be helpful in establishing likely antibiotic sensitivity.

Further studies of the oral/respiratory microbiome in mechanically ventilated patients should aim to recruit more patients for more robust results and evaluate the possibility of including the fungal microbiome in analyses for a more comprehensive study. For such a study, it would also be beneficial to develop improved DNA extraction techniques for NBLs and ETT biofilms.

To develop effective preventative strategies in this field, robust *in vitro* models, potentially displaying the complexities of the ventilator-mouth-trachea-lung interfaces are needed. The incorporation of a pre-conditioning layer to the ETTs *in vitro* biofilms and the use of a more diverse list of oral/respiratory microorganisms may offer further insight into bacterial interactions between oral species and respiratory pathogens. In this PhD study, a selection of biofilm–related genes were studied, and other genes related to virulence factor could be explored in future research.

Much variation is reported in the literature in terms of oral care protocols, and the frequency and use of antimicrobials (Hua *et al*. 2016), which makes policy making difficult, therefore ideally a large multicentre RCT that evaluates oral cleanliness, colonisation of respiratory pathogens and VAP incidence with and without the use of antimicrobials is required. Additionally the PneuX^{TM} ETT has showed promising results from *in vitro* research and in a retrospective study in preventing leakage of oropharyngeal secretions that pool above the ETT cuff (Fletcher *et al.* 2008; Young *et al.* 2006); an RCT study of this ETT is needed to demonstrate its impact on VAP prevalence.

6.3. Conclusions

Patients admitted to ICU have a poor oral hygiene status, and microbiological culture and molecular studies including next generation sequencing have revealed that the dental plaque of these patients becomes colonised by respiratory pathogens. Importantly, the same species were also found in the lower airways and ETT biofilms of these patients. Molecular genotyping showed that identical genetic profiles from isolates from dental plaque, ETTs and NBLs often occurred, and NGS showed that the microbiomes of these three sites were similar, thereby supporting the hypothesis that the microorganisms translocate from the mouth to the lower airway and ETT. Additionally, it was shown that oral microorganisms can enhance biofilm production and gene expression by targeted respiratory pathogens indicating a potential synergistic relationship.

Evaluation of oral hygiene methods, antimicrobial mouthwashes and different ETT biomaterials highlighted the challenges involved when attempting to control biofilms in mechanically ventilated patients. Of significance in these investigations was that mechanical toothbrushing had significant effect on improving oral hygiene indices. Given the *in vitro* research presented in this thesis which shows how oral microorganisms facilitate *in vitro* biofilms and gene expression by respiratory pathogens, such oral care interventions could be highly valuable in management and prevention of VAP. Nevertheless more research aimed at reducing respiratory pathogens colonisation of the dental plaque and preventing biofilm formation in the ETT lumen are needed, as this creates a risk of developing VAP.

6.4. Publications arising from the research presented in this thesis

- Marino P J, Wise M P, Smith A, Marchesi J R, Riggio M P Lewis M A O, Williams D W. Community analysis of dental plaque and endotracheal tube biofilms from mechanically ventilated patients. *Journal of Critical Care.* 2017 (*in press*).
- Marino P J, Hannigan A, Haywood S, Cole J M, Palmer N, Emanuel C, Kinsella T, Lewis M A O, Wise M P, Williams D W. Comparison of foam swabs and toothbrushes as oral hygiene interventions in mechanically ventilated patients: a randomised split mouth study. *BMJ Open Respiratory Research*. 2016; 3 (1):e000150. doi:10.1136/bmjresp-2016-000150.
- Marino PJ, Williams DW, Wise MP, Lewis MAO. Microbial Communities in the Dental Plaque of Mechanically Ventilated Patients. *Journal of Dental Research* 92 (B): 182216 (BSODR), 2013.
- Williams DW, Wise MP, Marino P, Lewis MAO. The oral cavity, Biofilms and Ventilator Associated Pneumonia. *Current Respiratory Medicine Reviews*. 8:163-167, (2012).
- Williams DW, Wise MP, Marino P, Thomas JG, Lewis MAO (2011). The role of human oral microflora in ventilator-associated pneumonia. *Annual Clinical Journal of Dental Health*. 1:16-21.
- Marino PJ, Williams D W, Lewis M A O, Wise M P, Frost P J, Thomas JG. The effect of oral microbes in biofilm formation by respiratory pathogens. *Journal of Dental Research*.90 (A): 148082, 2011

Appendix I: Clinical trial ethics

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14 April 2010

Dr Matthew Wise
Adult Critical Care
University Hospital of Wales
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Cardiff CF14 4XW

Dear Dr Wise

Study title:	Comparison of foam swabs versus toothbrushes in removing dental plaque from orally intubated mechanically ventilated patients
REC reference:	09/MRE09/44
Amendment number:	Protocol version 4
Amendment date:	06 March 2010

The above amendment was reviewed at the meeting of the Committee held on 08 April 2010.

The members of the Committee taking part in the review gave a favourable ethical opinion of the amendment on the basis described in the notice of amendment form and supporting documentation.

Approved documents

The documents reviewed and approved at the meeting were:

Document	Version	Date
Participant Consent Form: Patient's Representative	4	06 March 2010
Participant Consent Form: Patient	4	06 March 2010
Participant Information Sheet: Patient's Representative	4	06 March 2010
Participant Information Sheet: 4		06 March 2010
Protocol	4	06 March 2010
Notice of Substantial Amendment (non-CTIMPs)	Protocol version 4	06 March 2010
Covering Letter	signed Dr wise	07 March 2010

Membership of the Committee

The members of the Committee who took part in the review are listed on the attached sheet.

R&D approval

All investigators and research collaborators in the NHS should notify the R&D office for the relevant NHS care organisation of this amendment and check whether it affects R&D approval of the research.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees (July 2001) and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

09/MRE09/44: Please quote this number on all correspondence

Yours sincerely

Dr. Corinne Scott

Committee Co-ordinator

Enclosures:	List of names and professions of members who took part in the review
Copy to:	Prof M F Scanlon, R & D Office, Cardiff and Vale NHS Trust

REC for Wales

Attendance at Committee meeting on 08 April 2010

Name	Profession	Capacity
Dr Maurice Buchalter	Alternate Vice Chairman / Hospital Consultant (Cardiologist)	Expert
Mrs Philippa Herbert	JP	Lay
Mr HAO Hughes	Pharmacist	None
Dr Meriel Jenney	Hospital consultant (Paediatric oncologist)	Expert
Dr Mohammad Obaidullah	GP	Expert
Mr Chris Olchawski	Management consultant	Lay
Ms Susan Pope	Communications / PR	Lay
Dr V. Bapuji Rao	Hospital consultant (Psychiatrist)	Expert
Dr Gordon Taylor	Chairman / Statistician	Expert
Mr Stewart Williams	Management Consultant	Lay

Also in attendance:

Name	Position (or reason for attending)
Dr Corinne Scott	Co-ordinator

Written comments received from:

Name	Position
Dr Pete Wall	Vice Chairman / Clinical Physiologist

Appendix II: Phenotypic tests

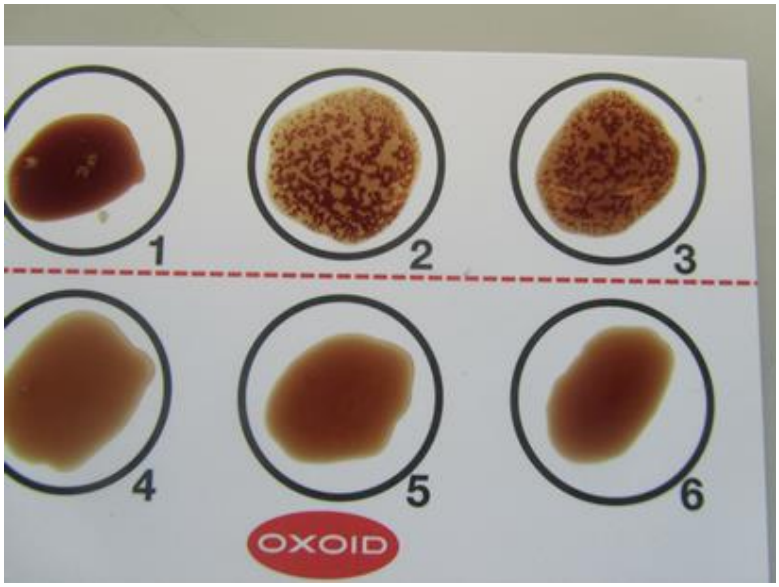
Table 1 Phenotypic test results for isolates. Table shows patient, site of origin and result for catalase, coagulase and oxidase tests as appropriate; + (positive), - (negative).

Patient	Site	Isolate	Catalase	Coagulase	Oxidase
P01	Plaque	Staphylococcus haemolyticus	+	-	
P03	Plaque	*Pseudomonas aeruginosa*			+
	NBL	*Pseudomonas aeruginosa*			+
	ETT	*Pseudomonas aeruginosa*			+
P05	Plaque	Staphylococcus epidermis	+	-	
		Echerichia coli	+	-	
	NBL	Echerichia coli	+	-	
P06	Plaque	*Staphylococcus aureus*	+	+	
	NBL	*Staphylococcus aureus*	+	+	
	ETT	*Staphylococcus aureus*	+	+	
P07	NBL	Streptococcus intermedius	-		
	ETT	Streptococcus intermedius	-		
		Streptococcus gordonii	-		
	Plaque	*Staphylococcus aureus*	+	+	
P08		Echerichia coli	+	-	
	NBL	*Staphylococcus aureus*	+	+	
P09	Plaque	Staphylococcus haemolyticus	+	-	
	ETT	Staphylococcus haemolyticus	+	-	
P10	Plaque	Enterococcus fecalis	-		
	NBL	*Staphylococcus aureus*	+	+	
	ETT	*Staphylococcus aureus*	+	+	
P14	Plaque	*Staphylococcus aureus*	+	+	
P16	Plaque	Staphylococcus haemolyticus	+	-	
	NBL	*Staphylococcus aureus*	+	+	
P19	Plaque	*Staphylococcus aureus*	+	+	
	ETT	Echerichia coli	+	-	
P20	Plaque	*Staphylococcus aureus*	+	+	
P21	Plaque	*Streptococcus mutans*	-		
		Staphylococcus aureus	+	+	
	NBL	*Staphylococcus aureus*	+	+	
	ETT	*Staphylococcus aureus*	+	+	
P24	Plaque	*Pseudomonas aeruginosa*			+
	NBL	*Pseudomonas aeruginosa*			+
P25	Plaque	Streptococcus intermedius	-		

Table 1 (continuation)

Patient	Site	Isolate	Catalase	Coagulase	Oxidase
P27	Plaque	Staphylococcus epidermis	+	-	
		Enterococcus faecium	-		
	NBL	Staphylococcus epidermis	+	-	
	ETT	Staphylococcus epidermis	+	-	
		Enterococcus faecium	-		
P28	Plaque	*Staphylococcus aureus*	+	+	
		Klebsiella pneumoniae	+	-	
	NBL	Klebsiella pneumoniae	+	-	
		Staphylococcus aureus	+	+	
	ETT	*Staphylococcus aureus*	+	+	
		Staphylococcus Lugdunensis	+	-	

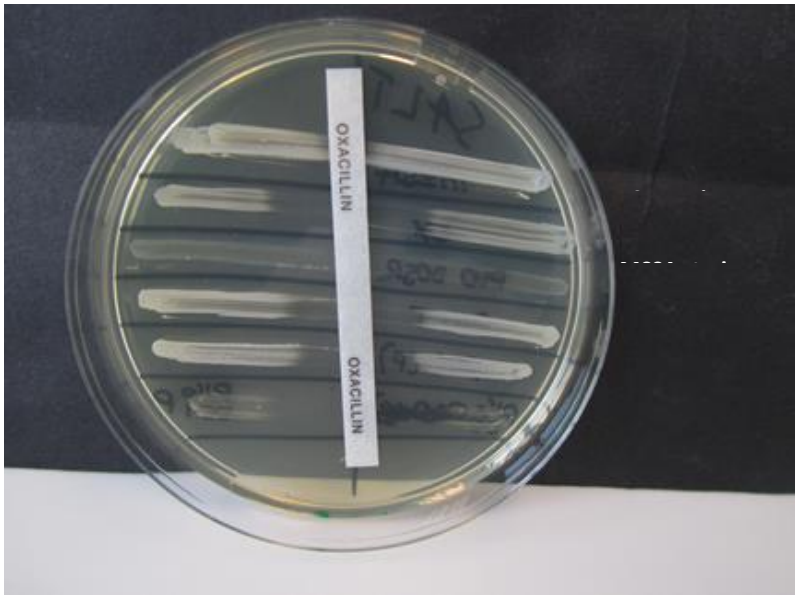
a)



b)

Figure 1 a) Coagulase test on suspected *S.aureus* isolates: #1 negative control, #2 positive control, #3 to # 6: test isolates. b) Oxidase test on suspected *Pseudomonas aeruginosa* isolates

a



b

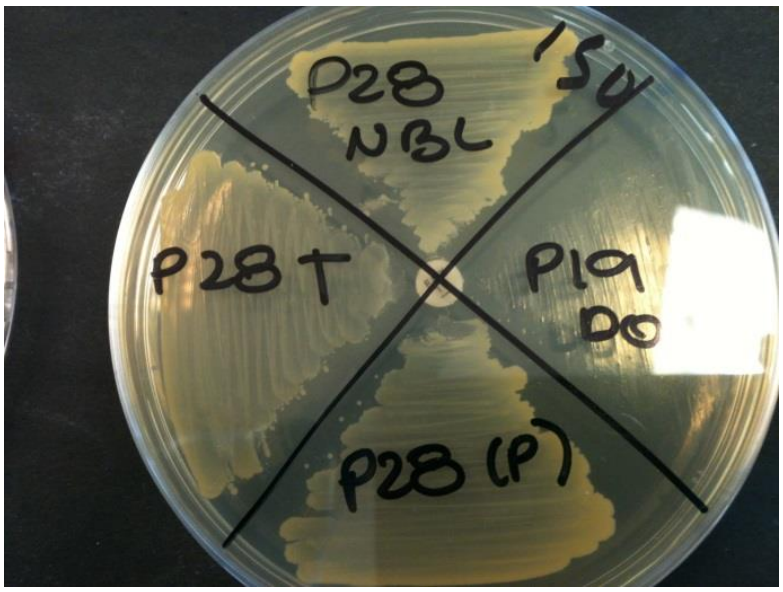


Figure 2 a) Oxacillin strip test for identifying Methicillin Resistant *Staphylococcus aureus*. MSSA (methicillin sensitive *S. aureus*). b) cefoxcitlin discs showing sensitivity for P19 isolate and resistance for P28 isolates from dental plaque (p), NBL, and endotracheal tube (T).

Table 2. Meticillin susceptibility tests results on *Staphyloccocus aureus* clinical isolates from plaque, non-directed bronchial lavages (NBL) and endotracheal tube biofikms (ETT) using an oxacillin strip and a cefoxitine disc.

Patient	Site	Cefoxitine	Oxacillin
P06	Plaque	S	S
	NBL	S	S
	ETT	S	S
P08	Plaque	S	S
	NBL	S	S
P10	Plaque	R	R
	ETT	S	S
P14	Plaque	S	S
	ETT	S	S
P19	Plaque	S	S
P20	Plaque	S	S
P21	Plaque	S	S
	NBL	S	S
	ETT	S	S
P28	Plaque	R	R
	NBL	R	R
	ETT	R	R

S indicates sensitive, R indicates resistant

Appendix III: Microbial susceptibility to mouthwash preparations

Table 1. Absorbance readings for Citroxx® assays for *S. aureus* (SA) in planktonic cultures, single biofilm and dual combinations with *S. mutans* (SM) and *C. albicans* (CA).

Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

%	SA Plank	SD	Single	SD	SA/SM	SD	SA/CA	SD
0	0.80	0.09	1.41	0.07	0.51	0.16	1.36	0.20
0.25	0.70	0.10	1.18	0.25	0.36	0.16	1.31	0.23
0.5	0.58	0.19	0.85	0.29	0.73	0.23	1.15	0.37
1	0.43	0.31	0.55	0.44	0.55	0.47	1.14	0.32
2	0.08	0.07	0.08	0.03	0.40	0.38	0.67	0.63
3	0.05	0.03	0.11	0.06	0.46	0.40	0.53	0.59
4	0.03	0.04	0.42	0.36	0.24	0.14	0.10	0.06
8			0.12	0.10	0.09	0.05	0.14	0.22

Table 2. Absorbance readings for Citroxx® assays for *P. aeruginosa* (PA) in planktonic cultures, single biofilm and dual combinations with *S. mutans* (SM) and *C. albicans* (CA). Average from 3 sets of triplicate experiments with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

	Plank	SD	PA single	SD	PA/SM	SD	PA/CA	SD
0	1.46	0.77	1.08	0.34	1.75	0.71	1.52	0.37
0.25	1.65	0.91	1.52	0.31	1.65	0.55	1.59	0.32
0.5	1.81	1.05	1.49	0.29	1.79	0.57	1.61	0.36
1	0.86	1.20	1.47	0.29	1.72	0.43	1.62	0.15
2	0.05	0.05	0.75	0.74	1.44	0.63	1.85	0.18
3	0.03	0.04	0.97	0.70	1.45	0.65	1.19	0.67
4	0.02	0.04	0.65	0.71	0.76	1.22	0.85	0.65
8			0.15	0.31	0.30	2.45	0.30	0.23

Table 3. Absorbance readings for Citroxx® assays for *C. albicans* (CA) and *S. mutans* (SM) planktonic and single species biofilms. Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

	CA Plank	SD	CA single	SD	SM Plank	SD	SM Single	SD
0	1.14	0.79	1.36	0.17	0.94	0.94	0.29	0.11
0.25	1.15	0.70	1.30	0.13	0.98	0.84	0.39	0.25
0.5	1.09	0.67	1.30	0.11	0.89	0.82	0.25	0.30
1	0.94	0.55	1.28	0.12	0.68	0.73	0.09	0.06
2	0.74	0.42	1.28	0.11	0.45	0.60	0.29	0.23
3	0.59	0.40	1.32	0.08	0.41	0.53	0.23	0.18
4	0.08	0.06	0.94	0.52	0.04	0.04	0.19	0.16
8			0.53	0.56			0.10	0.12

Table 4. Absorbance readings for Listerine assays for *S. aureus* (SA) in planktonic cultures, single biofilm and dual combinations with *S. mutans* (SM) and *C. albicans* (CA). Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

%	SA Plank	SD	Single Biofilm	SD	SA/SM	SD	SM/CA	SD
0	0.96	0.09	1.22	0.28	0.68	0.42	1.53	0.16
3.12	1.12	0.16	1.30	0.15	1.15	0.40	1.44	0.18
6.25	0.76	0.13	1.20	0.21	1.34	0.32	1.54	0.16
12.5	0.03	0.02	1.15	0.28	0.87	0.60	1.31	0.25
25	0.03	0.02	1.29	0.30	0.84	0.79	1.46	0.36
50	0.08	0.08	1.16	0.68	0.04	0.04	0.38	0.69

Table 5 Absorbance readings for Listerine assays for *P. aeruginosa* (PA) in planktonic cultures, single biofilm and dual combinations with *S. mutans* (SM) and *C. albicans* (CA). Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction. Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

%	PA Plank	SD	PA single	SD	PA/SM	SD	PA/CA	
0	1.80	0.32	1.57	0.34	1.69	0.26	1.60	0.49
3.12	1.86	0.16	1.57	0.36	1.79	0.21	1.58	0.35
6.25	1.53	0.11	1.68	0.22	1.70	0.23	1.60	0.35
12.5	1.20	0.37	1.79	0.35	1.87	0.24	1.56	0.34
25	0.99	0.19	1.69	0.40	1.92	0.34	1.69	0.52
50	0.42	0.38	1.47	0.36	1.53	0.30	1.50	0.36

Table 6. Absorbance readings for Listerine assays for *C. albicans* (CA) and *S. mutans* (SM) planktonic and single species biofilms. Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

%	*C. albicans* Planktonic	SD	Single Biofilm	SD	*S. mutans* Plank	SD	Single Biofilm	SD
0.00	0.62	0.27	1.20	0.32	0.42	0.10	0.27	0.11
3.13	0.67	0.13	1.45	0.22	0.36	0.22	0.26	0.25
6.25	0.15	0.09	1.27	0.14	0.10	0.05	0.31	0.44
12.50	0.13	0.08	1.33	0.09	0.07	0.05	0.11	0.14
25.00	0.06	0.03	1.34	0.07	0.08	0.05	0.02	0.02
50.00	0.05	0.02	0.15	0.31	0.04	0.05	0.02	0.03

Table 7. Absorbance readings for Chlorhexidine assays for *S. aureus* (SA) in planktonic cultures, single biofilm and dual combinations with *S. mutans* (SM) and *C. albicans* (CA).

Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

	SA plank	SD	SA single	SD	SA/SM	SD	SA/CA	
0	0.811	0.116	1.096	0.427	0.535	0.307	1.302	0.240
0.0002	0.368	0.380	1.181	0.100	0.718	0.323	1.281	0.225
0.0005	0.014	0.014	0.879	0.538	0.317	0.275	1.271	0.218
0.0009	0.006	0.007	1.073	0.191	0.263	0.261	1.260	0.210
0.002	0.007	0.005	1.053	0.461	0.403	0.410	1.184	0.224
0.004	0.002	0.002	0.513	0.637	0.118	0.150	1.152	0.263
0.008	0.004	0.003	0.036	0.033	0.037	0.033	0.488	0.656
0.015			0.027	0.027	0.054	0.043	0.652	0.587
0.03			0.032	0.028	0.101	0.166	0.018	0.029
0.06			0.042	0.022	0.093	0.108	0.004	0.004
0.12			0.089	0.045	0.062	0.087	0.026	0.036

Table 8. Absorbance readings for Chlorhexidine assays for *P. aeruginosa* (PA) in planktonic cultures, single biofilm and dual combinations with *S. mutans* (SM) and *C. albicans* (CA).

Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

%	Plank	SD	PA bio		PA/SM	SD	PA/CA	SD
0	2.13	0.23	1.85	0.62	1.55	0.33	1.75	0.37
0.002	1.85	0.44	2.12	0.59	1.86	0.24	2.10	0.33
0.004	0.23	0.59	1.97	0.57	1.81	0.17	1.91	2.10
0.008	0.15	0.35	1.90	0.46	1.94	0.17	2.02	0.31
0.015	0.01	0.01	1.88	0.27	1.91	0.42	2.16	0.31
0.03			1.77	0.55	1.70	0.42	1.89	0.35
0.06			1.75	0.22	1.86	0.25	2.03	0.32
0.12			1.98	0.42	1.92	0.37	1.78	0.65
0.24			1.76	0.32	1.25	0.95	1.19	0.96
0.48			1.68	0.64	1.23	0.82	1.72	0.97
0.96			0.55	0.79	1.04	0.49	1.26	1.03
1.92			0.18	0.41	2.13	0.24	0.85	0.85

Table 9. Absorbance readings for Chlorhexidine assays for *C. albicans* (CA) in planktonic cultures. Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

%	CA Plank	SD	CA Single	SD
0.000	0.767	0.096	1.236	0.401
0.001	0.551	0.368	1.025	0.037
0.002	0.004	0.006	1.354	0.124
0.004	0.006	0.004	1.329	0.346
0.008	0.006	0.005	0.619	0.611
0.015	0.013	0.012	0.718	0.624
0.030			1.240	0.603
0.060			0.821	0.695
0.120			0.743	0.731
0.240			0.706	0.836
0.480			0.451	0.617
0.960			0.280	0.357
1.920			0.188	0.173

Table 10. Absorbance readings for Chlorhexidine assays for *S. mutans* (CA) in planktonic cultures. Average from 3 sets of triplicate experiments shown with standard deviation (SD), highlighted text shows MIC/MCBI for 80% reduction.

	S. mutans Planktonic	SD	Single Biofilm	SD
0	0.43	0.22	0.31	0.21
0.0001	0.35	0.51		
0.0002	0.04	0.04	0.28	0.06
0.0005	0.04	0.04	0.46	0.23
0.0009	0.04	0.04	0.45	0.36
0.002	0.05	0.04	0.27	0.17
0.004			0.19	0.17
0.008			0.14	0.16
0.015			0.15	0.18
0.03			0.18	0.22
0.06			0.22	0.25
0.12			0.20	0.23

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