Dynamics of oral biofilms associated with mechanical ventilation

Thesis submitted in the fulfilment of the requirements of the degree of Doctor of Philosophy

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List of abbreviations

2DE – 2-dimensional electrophoresis
ACN – Acetonitrile
Agr - Accessory gene regulator
ANOVA – Analysis of variance
aPRPs – acidic proline rich proteins
AHLs – N-acyl-homoserine lactones
AI – Autoinducer
AS – Artificial saliva
BCA – Bicinchoninic acid
BLAST – Basic local alignment search tool
BM – Biofilm medium
BSA – Bovine serum albumin
BSAC – British Society for Antimicrobial Chemotherapy
CAP – Community Acquired Pneumonia
CDFF – Constant depth film fermenter
CFU – Colony forming unit
CHAPS - 3-[(3-Cholamidopropyl) dimethylammonio]-1 propanesulfonate
CHX - Chlorhexidine
CLSM – Confocal laser scanning microscopy
CPIS – Critical Pulmonary Infection Score
ddH_20 – Double distilled water (H_20)
DGGE – Denaturing gradient gel electrophoresis
DIGE – Difference gel electrophoresis
DMFT – Decayed, missing and filled teeth
DMSO – Dimethyl sulfoxide
DP – Dental plaque
DTT – Dithiothreitol
ECM – Extracellular matrix
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme linked immunosorbent assay
EPS – Extracellular polymeric substances
ETT – Endotracheal tube
EUCAST - European Committee on Antimicrobial Susceptibility Testing
E-VAP - Early onset VAP
FAA - Fastidious anaerobic agar
FAB – Fastidious anaerobic broth
FACS – Fluorescence-activated cell sorting
FDR – False discovery rate
FISH – Fluorescence *in situ* hybridisation
Gft – Glucosyltransferase
h - hour
HAP – Hospital-acquired pneumonia
HAI – Hospital-acquired infection
HRP – Horseradish peroxidase
HTA – Human tissue authority
ICU – Intensive care unit

IEF – Isoelectric focusing
IPG – Immobilised pH gradient
IQR – Interquartile range
IRAS – Integrated research application system
iTRAQ™ - Isobaric tagging for relative and absolute quantification
IV - Intravenous (antibiotics)
LC-MALDI – Liquid chromatography - MALDI
L-VAP – Late onset VAP
MALDI-TOF– Matrix Assisted Laser Desorption Ionization – Time of Flight
MDR – Multi-drug resistant
MH – Mueller Hinton agar
MHB – Mueller Hinton broth
MIC – Minimum inhibitory concentration
Min - Minutes
MOPS - Compound 3-(N-morpholino) propanesulfonic acid
MRSA – Meticillin Resistant *Staphylococcus aureus*
MS – Mass spectrometry
MSA – Mannitol salt agar
MSB – Mitis salivarius bacitracin agar
MSCRAMMS - Microbial Surface Components Recognizing Adhesive Matrix Molecules
MSDS – Material safety data sheet
MV – Mechanical ventilation
NBLs – Non-directed bronchial lavage
NCBI - National Center for Biotechnology Information
NGS – Next-generation sequencing
NH_4HCO_3 - Ammonium bicarbonate
NMDS – Non-metric multidimensional scaling
NP-40- Tergitol-type nonyl phenoxypolyethoxylethanol
NRES – National research ethics service
OOHCA – Out of hospital cardiac arrest
OTU - Operational taxonomic unit
PBS – Phosphate buffered saline
PCR – Polymerase chain reaction
PNA – Peptide nucleic acid
PPE – Protective personal equipment
PR – Proline rich
PRPs - Potential Respiratory Pathogens
PSM - Phenol-soluble modulin
PTFE – Polytetrafluoroethylene
PVC – Polyvinylchloride
QS – Quorum sensing
QTL – Quantitative tracheal lavage
R – R programming
RDP - Ribosomal Database Project
REC – Research ethics committee
SAB – Saboraud dextrose agar
SD – Standard deviation

SDS-PAGE – Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SEM – Scanning electron microscopy
SMIs – Standard Microbiological Investigations
SPSS – Statistical Package for the Social Science, IBM
STAMP – Structural time series analyser, modeller and predictor
TBE – Tris Borate EDTA
TFA – Trifluoroacetic acid
TMB - Tetramethylbenzidine
TM – Transport medium
VAP – Ventilator-associated pneumonia
ZOI – Zone of inhibition

Preface

All work unless otherwise stated was performed in The School of Dentistry, Cardiff University

The research presented in Chapter 2 has been published as follows: Sands, K et al., (2016). Respiratory pathogen colonisation of dental plaque, the lower airways and endotracheal tube biofilms during mechanical ventilation. *Journal of Critical Care,* 12 (13), pp. 30-37

The research presented in Chapter 3 has been published as follows: Sands, K. et al., (2016). Microbial profiling of dental plaque from mechanically ventilated patients. *Journal of Medical Microbiology*, 65 (2), pp. 147-59.

Abstract

Critically ill patients often require mechanical ventilation (MV) to facilitate treatment for respiratory failure or airway protection when consciousness is impaired. Whilst the endotracheal tube (ETT) is an essential interface between the patient and ventilator, it may promote VAP by impeding host defence mechanisms and by translocating microorganisms from dental plaque to the lower airways. Ventilator-associated pneumonia (VAP), which may be challenging to diagnose, is the most frequent hospital-acquired infection in critical care. It has been reported that when patients receive MV the composition of dental plaque changes to include respiratory pathogens such as Meticillin-Resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*.

The primary aim of this research was to confirm that dental plaque communities altered during MV and to identify the potential causes of these changes. A combination of culture-based microbiology, community profiling molecular techniques and proteomic analysis of saliva was performed to analyse the microbiological content of the oral cavity, and to also quantify changes in dental plaque composition and saliva.

For the first time, this study comprehensively analysed the dental plaque of mechanically ventilated patients and documented considerable species richness and diversity. Numerous potential respiratory pathogens were detected including *Staphylococcus aureus, P. aeruginosa* and *Streptococcus pneumoniae* in approximately one-third of mechanically ventilated patients. In addition, salivary flow rate was decreased and both the salivary pH and concentration of pro-inflammatory cytokines were significantly elevated during intubation.

Dental plaque is a reservoir for VAP, and preventing or reducing respiratory colonisation may play a role in the management of ventilated patients. Interventions to prevent colonisation could include the modulation of salivary parameters such as pH and volume and further work may lead to the identification of specific proteins that are significant. Reducing the incidence of VAP will not only reduce mortality in the ICU, but will also have a great impact on hospital economics by reducing inpatient stay.

Chapter 1

Literature review

1.1 The role of oral microbiome in systemic infection

The oral microbiome, the community of microorganisms and associated genetic material in the oral cavity, is extremely diverse with over 1000 microbial species currently identified (Wade, 2013a; Wade 2013b). Previous research has shown systemic infections can originate from microorganisms frequently isolated from the oral cavity (Yip & Smales, 2012). Infection mediated by oral microorganisms may occur once the number of potentially pathogenic bacteria increases to a level that can cause damage either to the tooth (*e.g.* dental caries), local tissues (*e.g.* periodontal disease) or distant sites (pneumonia or endocarditis), as highlighted in table 1.1 (Gomes-Filho et al., 2010).

Table 1.1 – The association between oral microorganisms and systemic infections (Desvarieux, 2005; Ohara-Nemoto et al., 2008; Lockhart et al., 2009; Gomes-Filho et al., 2010; Preshaw et al., 2012; Welte et al., 2012).

Systemic infection	Association with oral microbiota
Community-acquired pneumonia	Periodontal pathogens: *Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella oralis*
Hospital-acquired pneumonia	Periodontal pathogens: *Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella oralis*
Diabetes	Oral anaerobic community can influence glycaemic control, diabetes increases risk of periodontitis
Cardiac atherosclerosis	Correlation with oral infection and intimal thickening
Infective endocarditis	Gingivitis and periodontitis inflammation, dental procedures and toothbrushing - route of entry for *Streptococcus mutans* and *Streptococcus mitis* bacteraemia, staphylococci (oral and nasal origin)
Pre-term birth and low weight birth	High abundance of *Porphyromonas gingivalis, Prevotella intermedia* and *Tannerella forsythia*

The oral cavity represents a primary route of entry for microorganisms into the body and harbours microorganisms that can consequently translocate to other areas of the body. It should not be a surprise, given the contiguity of the aerodigestive tract, that bacteria within the oral cavity have been associated with pneumonia in individuals in community, nursing home, hospital or critical care settings. Dental pain or the wearing of a dental prosthesis have been identified as risk factors for community-acquired pneumonia (CAP) (Almirall et al., 2008). Community-acquired pneumonia (CAP) is defined as an acute infection of the lung occurring in an individual anywhere in the community (Raghavendran et al., 2000). A study by Rodriguez et al., 2014 enrolling 1,336 individuals with CAP and 1,326 cases of control, related the onset of CAP with oral health parameters (Rodriguez et al., 2014). In the CAP group, there was a significantly higher prevalence of dental dysaesthesia and the use of dental prosthesis. Further analysis within their study also revealed that a recent visit to the dentist (within the last month) was seen as a preventative measure for CAP.

1.2 Ventilator-associated pneumonia (VAP)

1.2.1. VAP prevalence

Critically ill patients often require mechanical ventilation (MV), to treat respiratory failure or protect the airway where conscious level is impaired. An endotracheal tube (ETT) facilitates the flow of gases from the ventilator to the lower airways, providing adequate gaseous exchange. However, whilst the ETT is an essential conduit between patient and ventilator, the placement of the ETT has been suggested to be a key factor in the development of ventilator-associated pneumonia (VAP) (Gibbs & Holzman, 2012). The placement of the ETT can impair the cough reflex, allow the pooling of secretions (contaminated with bacteria), and provides a surface for biofilm formation. VAP is a hospital-acquired infection (HAI) with reported incidences of up to 67% (Joseph et al., 2010). Recent studies suggesting the global incidence of VAP is 15% of mechanically ventilated patients (Kollef, 2015)

and attributed mortality rates are reported to be between 33-50% of cases (Nicasio et al., 2010). VAP is a bacterial pneumonia developing 48 h after intubation (Lin et al., 2013) and is the most prevalent HAI in critical care units (Joseph et al., 2010; Raad et al., 2011). There is some dispute as to the precise definition of VAP, and this leads to challenges in accurate diagnosis (Klompas et al., 2012). Correspondingly, the lack of standardisation of diagnosis can make the study of the effects of interventions difficult (Tejerina et al., 2010). Previous studies investigating prevalence rates of VAP in critical care units have highlighted the difficulties in diagnosing VAP, many features of VAP have other causes which are common in critical illness (e.g. fever, abnormal radiographs) (Grap et al., 2011). Given these issues, VAP prevalence rates from as low as 5% to as high as 67% have been reported (Brennan et al., 2004; Sundar et al., 2012). Guidelines have been generated to facilitate the reporting of VAP cases (NHSN, 2015). Reporting VAP, especially in the US, has been affected by remuneration. Cases were subject to extra billing as VAP is seen as an avoidable infection, which has undoubtedly affected reporting incidences. Although studies have shown large differences in VAP rates in intensive care units (ICUs), it is well established that pneumonia in the context of mechanical ventilation contributes significantly to the rates of HAIs. A number of risk factors have been associated with VAP development (Figure 1.1).

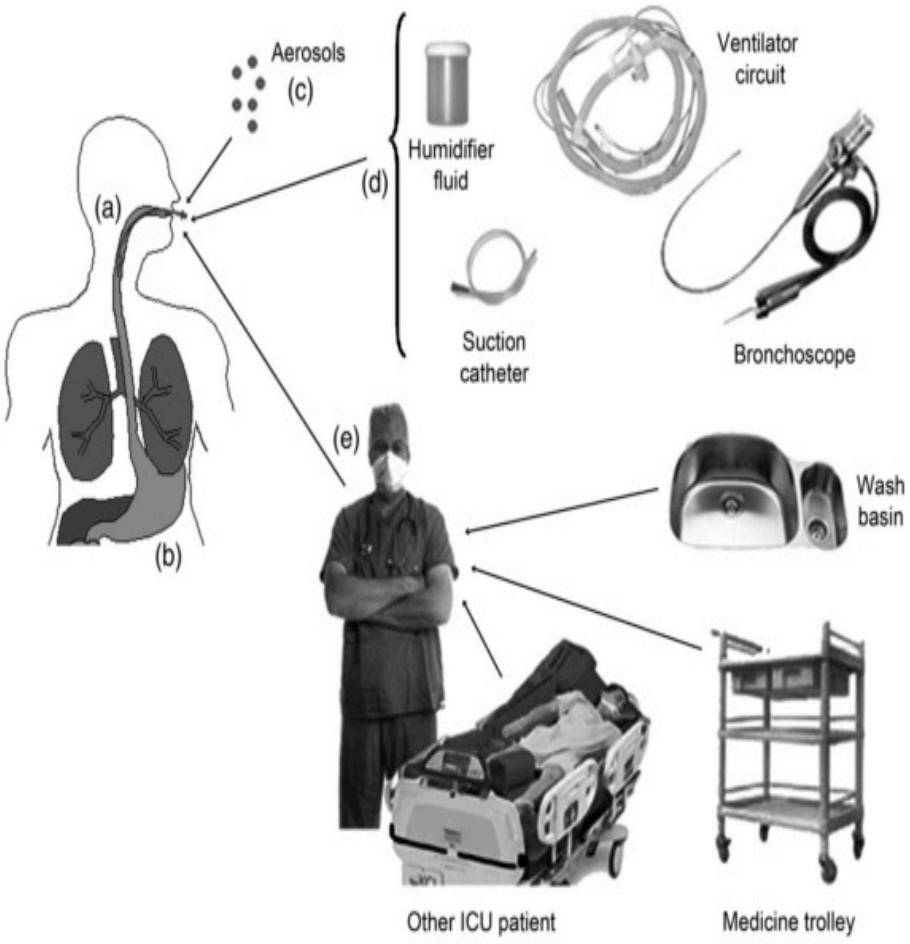

Figure 1.1 – Several factors associated with the development of VAP a; ETT b; lower airways compromised, c; aerosols, d; equipment used in critical care medicine, e; transference of organisms from biotic and abiotic sites (Joseph et al., 2010).

1.3 The Oral microbiota

1.3.1 Composition of the oral cavity

The oral cavity supports a diverse array of microorganisms with over 1000 cultivable species currently recognised (Faran Ali & Tanwir, 2012). The diversity of the oral microbiota reflects the range of microbial habitats that are present, including keratinised and non-keratinised oral mucosa, teeth, protected periodontal pockets and any existing oral prostheses (Aas et al., 2005). Environmental conditions in the oral cavity, including different oral

tissues and proteinaceous conditioning of surfaces, temperature, and pH all contribute to such microbial variety (Faran Ali & Tanwir, 2012).

The rapid development of molecular technologies has allowed relatively inexpensive high-throughput sequencing and profiling of the oral microbiome. Utilising community profiling, more than 1000 bacterial taxa have now been identified within the oral cavity, many of which coexist within polymicrobial biofilms (Kawamura & Kamiya, 2012). Microbial metabolism from diverse communities (the metabolome) is considered a key virulence factor, by way of acidic end products which may alter the local pH (Takahashi, Washio, & Mayanagi, 2012). Whilst the oral microbiome is thought to be relatively stable in terms of microbial species during adult lifetime, there can be shifts in the population dynamics, and changes to the local oral environment can lead to dental plaque-mediated diseases such as dental caries and periodontal disease (Marsh, 1994). These local perturbations can occur through alteration in diet (for example, high sugar or carbohydrate diets), accompanied by poor oral hygiene which can lead to an increase in both acid tolerant streptococci and the anaerobic community within dental plaque and other areas of the oral cavity (Kawamura & Kamiya, 2012). Medications and the disruption of local immune responses due to underlying illness may also cause changes to the dynamics of the oral microbiota (Zarco et al., 2011).

The complexity of the oral microbiome extends to the inclusion of fungi such as the yeast of the genus *Candida*. *Candida* species have been isolated from up to 60% of healthy adults, and are frequent colonisers of mucous membranes, with the most prevalent species being *Candida albicans* (Thein et al., 2006). *C. albicans'* ability to interact with bacteria adds to the diversity of the oral microbiome (Dongari-Bagtzoglou et al., 2009). *C. albicans* is an opportunistic pathogen that undergoes morphological transformation between yeast and hyphae, ultimately enhancing virulence. Although *Candida* normally colonises the human mouth harmlessly, in the presence of certain risk factors, including smoking, old age and medications, infection can occur (Gantner et al., 2005; Loyola et al., 2012).

The host tightly regulates the balance and extent of microbial colonisation. If this balance changes, for example when health declines, there is the capacity for the microenvironment to change. The oral cavity has been shown to offer a habitat to potentially pathogenic species (Zuanazzi et al., 2010). Commensal microorganisms within the oral microbiota have the ability to modulate the local environment, and in the healthy individual, such commensal oral microorganisms may therefore inhibit secondary pathogen colonisation (Wade, 2013a).

1.4 Saliva

1.4.1 Composition of saliva

Whole saliva, originates from major (submandibular, parotid and sublingual) and minor salivary glands (Huang, 2004). Saliva is a complex mixture of biomolecules including lipids, carbohydrates and proteins (Schipper et al., 2007) in addition to components from gingival crevicular fluid, epithelial cells, oral and mucosal tissues, blood components and microorganisms (Table 1.2) (Humphrey & Williamson, 2001). Protection of the oral cavity is enhanced by oral fluids such as saliva as it contains an arsenal of antimicrobial peptides (Subrahmanyam & Sangeetha, 2003). In healthy adults, up to 1500 ml of saliva is produced per day, however, physiological and pathological factors can severely alter production (Yamamoto et al., 2009).

Table 1.2 – Constituents of saliva (Humphrey & Williamson, 2001; Amerongen & Veerman, 2002).

Saliva constituent	Proportion (%)
Water	99.5
Electrolytes	<0.5%
Mucus	<0.5%
Glycoproteins	<0.5%
Antibacterial proteins	<0.5%
Enzymes (amylase)	<0.5%
Epithelial cells	<0.5%
White blood cells	<0.5%

Currently, over 2,000 proteins have been identified in saliva (Yan et al., 2009; Amado et al., 2013). Some salivary proteins have been found to adhere to the enamel surfaces of teeth (1.3). Early microbial colonisers within dental plaque, predominantly members of the *Streptococcus* genus including *Streptococcus mitis* and *Streptococcus salivarius*, can adhere to immobilised proteins such as statherin and glycoproteins anchored on the enamel surface which form the pellicle layer (Rudney et al., 2003; Kolenbrander et al., 2005; Chaudhuri et al., 2007). Salivary components therefore select for initial bacterial colonisation of the tooth surface and effect the composition of dental plaque (Sekine et al., 2004).

1.4.2 Functions of saliva

The functions of saliva include, but are not limited to, molecular transport, lubrication, antibacterial defense, and pH homeostasis (Tabak et al., 1982; Rudney, 2000). In healthy individuals, salivary flow rates vary between 0.2-2 ml/min, with a salivary pH range between 6.2-7.4 (Fenoll-Palomares et al., 2004; Dodds et al., 2005). The composition of saliva, in particular free calcium and phosphate serve as buffering agents which help maintain the integrity of the tooth under cariogenic conditions (Amerongen & Veerman,

2002; Lamkin & Oppenheim 1993). Saliva provides a transport medium for microorganisms in the oral cavity (Rudney, 2000).

1.4.3 Antimicrobial activity of saliva

Antibacterial peptides such as lysozyme and lacotransferrin provide protection and interactions against organisms and foreign debris entering the oral cavity (Table 1.3) (Silva et al., 2012; Gorr & Abdolhosseini, 2011).

Table 1.3 – Antimicrobial activity of salivary proteins

Antimicrobial protein	Main function
Lysozyme	Anti-bacterial
Lactotransferrin	Anti-bacterial
Lactoperoxidase	Anti-bacterial
Mucins	Anti-bacterial, Anti-viral
Cystatins	Anti-bacterial, Anti-viral
Histatins	Anti-fungal, Anti-bacterial
IgA	Anti-bacterial
Cytokines	Anti-bacterial
Immunoglobulins	Anti-bacterial, Anti-viral
Proline-rich glycoproteins	Anti-bacterial
Defensins	Anti-bacterial
Calprotectin	Anti-fungal, Anti-bacterial
Chromogranin A	Anti-fungal, Anti-bacterial

Salivary proteins have co-evolved with the diversity of the oral microbiome (Gorr & Abdolhosseini, 2011). Four of the most well-known antimicrobial peptides are lactotransferrin, lysozyme, salivary peroxidase and secretory IgA (Rudney et al., 1991). Lysozyme is an enzyme with antimicrobial properties, which lyses bacterial cells and limits bacterial overgrowth in the oral cavity (Pfaffe et al., 2011). However, alongside the major salivary proteins, are over 1,000 less abundant protein types present, often with poorly understood biological functions (Huang, 2004).

1.4.4 Salivary proteomics

Proteomics (the research of proteins within a 'proteome', which is a complete set of proteins expressed within a cell or organism at a certain time) focuses on protein structure and function, and is a clinically important and expanding discipline in the post-genomic era (Chevalier, 2010). With over 2,000 identified proteins in saliva, advances in proteomic platforms have enabled the study of such complex fluids (Amado et al., 2013; Jágr et al., 2014). The most abundant salivary proteins are amylase, acidic and basic proline rich proteins (PRPs) and mucin, which together make up ~75% of the salivary proteome (Figure 1.2) (Scarano et al., 2010).

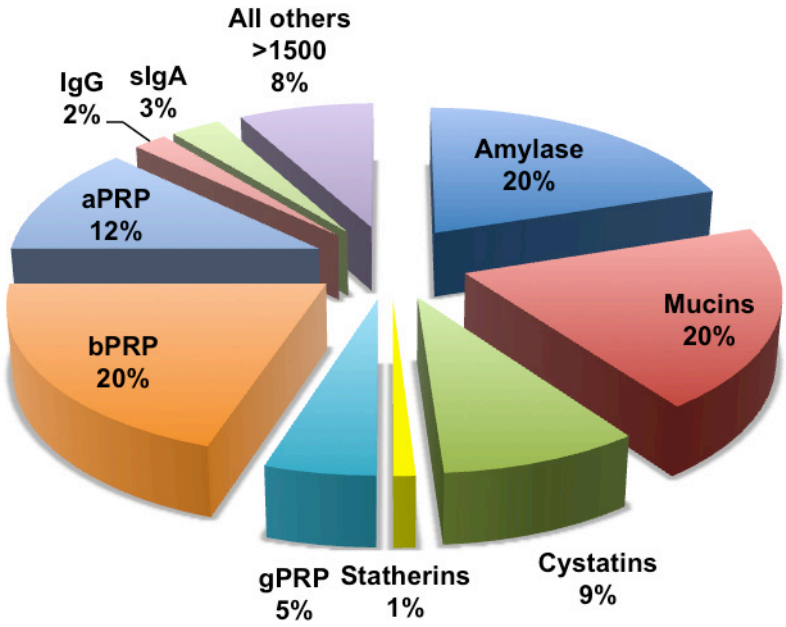

Figure 1.2 – Major protein groups in human whole saliva, adapted from (Scarano et al., 2010).

There are two main strategies to analyse the salivary proteome. Firstly, a gel based approach can separate peptides within saliva and which can be coupled with peptide-spot identification using MALDI MS. Whilst such 2D gel electrophoresis (2DE) allows separation of multiple proteins, the subsequent downstream applications, including mass protein identification are labour-

intensive compared to gel-free workflows (Chevalier, 2010). Gel-free workflows involving chromatography followed by MS can be employed for high-throughput global protein identification (Aebersold & Mann, 2003; Scarano et al., 2010; Yao et al., 2003). Gel-free proteomic workflows have the capacity to identify hundreds of peptides simultaneously, although bias can occur with the focus on specific and targeted proteins (Jehmlich et al., 2013). At each step in a proteomics workflow there are several options available, therefore, a mixed proteomic approach utilising gel-based and high-through put analysis is often used when analysing complex fluids (Shen et al., 2010).

Salivary proteins including PRPs and statherin which make up over one-third of the salivary proteome, provide a binding site for early oral colonisers and thus play an important role in modulating dental plaque formation (1.5) (Rudney, Krig, & Neuvar, 1993; Rudney, 2000).

1.5 Dental plaque an archetypal biofilm

Dental plaque was considered to be a microbial construct (*i.e*. a biofilm) in the 1970s (Costerton et al., 1978). Dental plaque specifically harbours an estimated 500 different bacterial species with differences in microbial composition occurring between (the interproximal spaces) and along the teeth, indicating that the microbial structure is not uniform, but dynamic in nature (Rosan & Lamont, 2000). *Streptococcus* species are recognised as primary pioneer colonisers of teeth and the initiators of dental plaque development (Denepitiya & Kleinberg, 1982).

Lazarevic et al., 2009 analysed the oral microbiome using molecular methods and reported that up to 70% of sequences belong to the *Streptococcus* and *Neisseria* genera. Dental plaque formation begins almost instantaneously after a tooth is mechanically cleaned via brushing (Rosan & Lamont, 2000). Largely composed of streptococci, dental plaque biofilms can be acid tolerant to a pH as low as pH 2.5 (Ritz, 1967; Mcneill & Hamilton,

2003;). The binding of *Streptococcus* species such as *Streptococcus mitis* and *Streptococcus gordonii* promotes colonisation by a range of different species including *Fusobacteria. Fusobacteria* species are considered 'bridging bacteria' as they co-adhere to other microbial species including anaerobic bacteria such as *Prevotella intermedia and Porphyromonas gingivalis* leading to polymicrobial biofilm development (Marsh, 2010), as highlighted in figure 1.3.

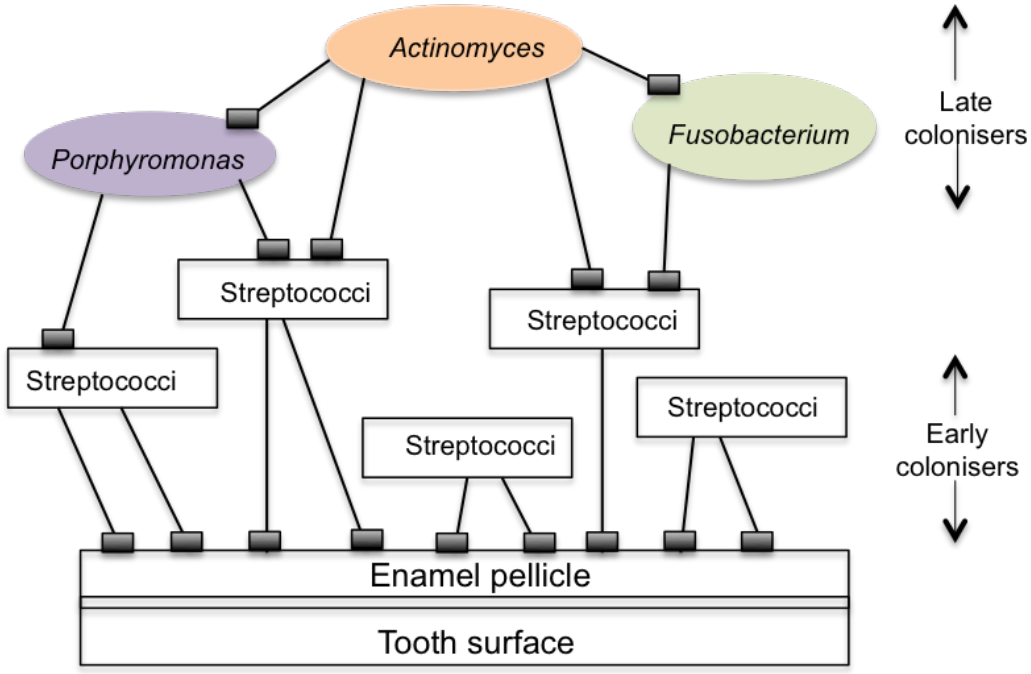

Figure 1.3 - Coaggregation between oral bacteria on the tooth surface adapted from Rickard et al 2003, with blocks representing a variety of receptors between species. Species such as *Fusobacterium* can effectively act as a 'bridge' between the early and late colonisers (Toole et al., 2000).

1.6 Biofilms

1.6.1 Biofilm composition

The term 'biofilm' describes a community of microorganisms that can be attached to living and non-living surfaces and/or to each other (Reid, 1999). Biofilms are ubiquitous in all ecosystems, forming on a variety of surfaces and within the most extreme environments including acidic and extremely hot environments (Davey & O'Toole, 2000).

Biofilm formation can be regarded as a key virulence factor, partly because of the phenotypic differences between biofilm organisms and those in planktonic (free swimming) form. Biofilm- mediated infections are difficult to treat as the cells are physically protected by the biofilm structure (Ma et al., 2009). Biofilm cells are embedded in complex matrices of extracellular polymeric substances (EPS), which enhances biofilm stability, table 1.4 (Ma et al., 2009; Kim et al., 2012; Muszanska et al., 2012).

Table 1.4 – Comparison of planktonic and biofilm cell characteristics (Ma et al., 2009).

Planktonic cells	Biofilm cells
Free floating	Attached to surface/each other
Increased sensitivity to antimicrobials	Increased virulence factor expression
Rate of growth: Cell proliferation	Cell production of EPS
	Production of ECM - structural layer
	Enhanced resistance to antimicrobials

EPS constituents can include polysaccharides, proteins, extracellular DNA (eDNA), lipids, inorganic salts and water, and the production of EPS is variable depending on the microbial species. EPS effectively provides a hydrated barrier between the cells and their external environment. Gristina et

al., 1985 documented thick adherent biofilms to both biomaterials (including catheters) and tissue.

Within the complex network of the biofilm, microchannels develop allowing fluid flow within the structure and the transfer of nutrients, chemical signals and removal of waste products through the community (Stoodley, 1999).

In addition, if biofilm microorganisms become starved of nutrients or oxygen (if required), then they can enter a 'stationary growth phase', which is protective until improved conditions arise (Fricks-Lima et al., 2011). A complex biofilm has an inherent ability to adapt to external changes, ultimately enhancing the biofilm and therefore microbial survival.

1.6.2 Biofilm development

Whilst the extent of biofilm development can vary depending upon the microbial species, a typical formation can be summarised in three universal stages (Figure 1.4). The initial phase of a biofilm development involves reversible attachment to a surface and each other (Halan et al., 2012). The adhesion of microorganisms in aqueous solutions can be explained by the Derjaguin-Landau-Verwey-Overbeek (DVLO) theory of colloidal stability to model interactions, including physical forces such as van der Waals forces (Hermansson, 1999; Garrett et al., 2008). Planktonic cells, in the initial first phase of attachment invariably become attached to a surface previously conditioned by organic and inorganic nutrients. This initial reversible attachment is the critical first step for biofilm growth (Lazar, 2011). Once cells have 'loosely' attached to the surface via available energy for adsorption specific ligand interactions (adhesins and receptors), microbial species generate covalent bonds rendering stronger and permanent adhesion. Bacterial adhesins along with pellicle receptors facilitate the process of bacterial adherence. Structurally, adhesins are either associated with fibrillar appendages or surface proteins (Rosan & Lamont, 2000). Coupled with surface colonisation, is active microbial metabolism, which uses surrounding nutrients and generates an array of by-products. This results not only in irreversible binding, but also continuous replication forming several micro-

colonies in certain niches of the body for example in between the teeth (Roger et al., 2008). Ultimately, this encourages EPS production enhancing the overall biofilm structure further anchoring the microorganisms to the attachment site (Rickard et al., 2003).

Along with communication via signaling molecules and chemical mediators, microbial cells can coaggregate together. Bacterial coaggregation is the binding of two cells via specific adhesins and receptor molecules (Rickard et al., 2003). Coaggregation is a common attribute of oral bacteria, and most notably apparent with *Fusobacterium nucleatum* and *Prevotella* species (Okuda et al., 2012). *Streptococcus* species have numerous partner-species including *Actinomyces naeslundii* and *C. albicans,* and such coaggregation willl facilitate polymicrobial biofilm development and enhancement (Bamford et al., 2009).

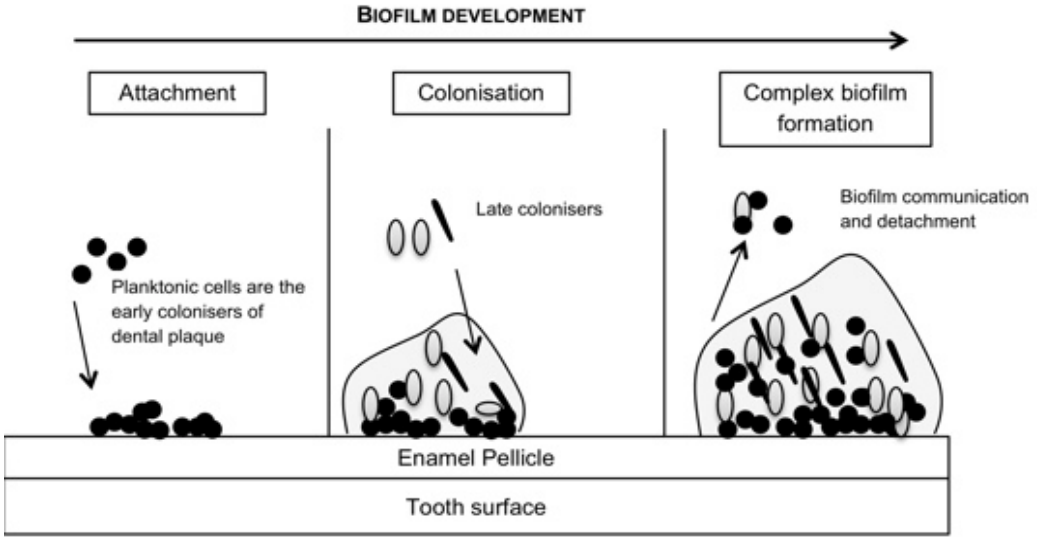

Figure 1.4 - A diagrammatic representation of biofilm formation on the tooth surface adapted from Hojo et al., 2009.

1.6.3 Biofilm detachment and dispersal

Once a biofilm has fully developed, cells can disperse to colonise neighboring and distal sites within the body. Biofilm detachment often considered a virulence factor (and sometimes referred to as the fourth stage of biofilm development), can occur as a result of either environmental forces or chemical signalling. In many cases, biofilm detachment arises due to forces from fluid or gaseous flow (Koerner, 1997). Such environmental forces can cause cellular erosion whereby small biofilm matter is sheared away from the formed biofilm, consequently moving with the direction of the flow (Morgenroth & Wilderer, 2000). Biofilm cells can also detach from the total biofilm mass via abrasion, where collisions occur between biofilm cells and particles within the surrounding materials and fluid (Gjaltema et al., 1997). In addition, biofilm sloughing can occur with increasing shear forces, resulting in big aggregates of cells detaching from the larger biofilm encased within EPS and moving as a formed biofilm (Morgenroth & Wilderer, 2000; Telgmann et al., 2004).

Biofilm dispersal or detachment can also be a virulence mechanism associated with biofilm-related infections (Kaplan, 2010), whereby detachment is regulated at a molecular and quorum sensing level and this offers a controlled strategy for biofilm survival (Lindsay & Von Holy, 2006; Boles & Horswill, 2011).

1.6.4 Quorum Sensing (QS)

With the identification of more than 1000 taxa of bacteria identified in the oral cavity, (many within close proximity to each other), it is not surprising that cell-to-cell communication and intercellular coordination occurs (Galloway et al., 2012). Quorum sensing (QS) is an umbrella term used to describe bacterial communication, mediated by chemical signaling molecules. QS is important for species survival, growth (He et al., 2012) and successful biofilm organisation (Kolenbrander et al., 2005). The subsequent microbial interactions can be mutually beneficial or antagonistic (Stacy et al., 2012).

Small species-specific diffusible molecules often mediate cellular communication. Quorum sensing (QS) molecules can directly bind to receptors on bacteria and trigger the modulation of a number of factors including virulence and biofilm regulation (Karlsson et al., 2012). Classes of QS molecules include autoinducer (AI) molecules and N-acyl homoserine lactones (AHLs). The concentration of QS molecules directly relates to the number of producing cells and when threshold concentrations are reached, signaling effects are manifested. Hence QS is a cell density based signaling process. AIs are continuously released into the EPS, and allow bacterial cells to 'monitor' their surroundings and modulate gene expression, thus contributing to biofilm survival, adaptation and competitiveness (Lazar, 2011). AHLs are more commonly produced by Gram-negative bacteria whilst Gram-positive bacteria employ peptide based systems including AIs (Waters & Bassler, 2005).

Bacteria QS signal molecules can mimic host hormones and in some situations influence and control eukaryotic expression (Pacheco & Sperandio, 2009). Studies by Telford et al., 1998 and Karlsson et al., 2012 have evaluated the role of AHLs produced by *P. aeruginosa* to modulate the behaviour in human cells. Telford et al., 1998 emphasised the role of AHL molecules in influencing Th1 and Th2 host cell responses, whereby *P. aeruginosa* AHL molecules can subsequently down regulate the production of bactericidal IL-12, thus mimicking host responses.

Studies have reported antibiotics including macrolides inhibiting QS systems. Burr et al., 2016 reported *in vitro* inhibition of *P. aeruginosa* QS by macrolide antibiotics, for example erythromycin. Burr et al., 2016 further investigated these findings in a clinical trial of *P. aeruginosa* positive sputum from non-cystic fibrosis bronchiectasis patients. Following exposure to low doses of macrolide antibiotics, they found a down regulation of *P. aeruginosa* QS gene expression, without an accompanying reduction in bacterial load.chapt

Although contributing to biofilm development, metabolic signaling via various QS systems are not the only factors controlling biofilm development. In nature, sessile polymicrobial communities need to be adaptable to external environmental conditions (Kellerberg & Molin 2002).

There are many complex and overlapping interactions within polymicrobial biofilms, where survival, growth and competitiveness thrives. Dental plaque is known for its microbial diversity and therefore, consideration of interspecies interactions is vital in determining the impact oral biofilms have in both localised and systemic infections.

1.6.5 Biofilm associated infections

Biofilm mediated infections cause up to 60% of medical and hospital associated infections (Lazar, 2011), some of which are highlighted in table 1.5. Furthermore, biofilms are recognised as leading causes for the vast majority of hospital infections requiring medical treatment (Anderson et al., 2013).

Table 1.5 – Biofilm associated-infections and causative pathogens (Otto, 2008; Toté et al., 2010; Majumdar & Padiglione, 2012; Tarquinio et al., 2014)

Bacterial species	Biofilm infections
Pseudomonas aeruginosa	Cystic fibrosis lung infection, respiratory infection, chronic wound, otitis media, catheter-associated urinary tract infections
Staphylococcus aureus	Endocarditis, respiratory infection, chronic osteomyelitis, orthopaedic implants
Escherichia coli	Urinary tract infection, catheter related urinary tract infection, biliary tract infection
Streptococcus pneumoniae	Chronic otitis media, chronic rhinosinusitis, chronic obstructive pulmonary disease
Staphylococcus epidermidis	Catheter related infections, prosthetic cardiac valve infections, joint replacement infections

Two clinically relevant examples of biofilm organisms are *Staphylococcus aureus*, a Gram-positive cocci, and *Pseudomonas aeruginosa,* a Gram-negative organism frequently producing complex biofilms within the respiratory tract.

S. aureus can form complex multi-layered biofilms both within the body and on medical devices, especially those exposed to proteinaceous fluids (Otto, 2009). Essential in *S. aureus* biofilm formation is the genetic regulator SarA, responsible for controlling key initial adhesions and promoting biofilm maturation (Abee et al., 2011). Surface components such as phenol-soluble modulin (PSM) (surfactant peptides) and Microbial Surface Components Recognising Adhesive Matrix Molecules (MSCRAMMS) are key in adhering biofilm cells to surface molecules found in the enamel pellicle conditioning film (Otto, 2009).

In biofilm-associated infections, the ability of selective de-attachment is crucial for pathogenesis, survival and competitiveness, allowing cells to 'travel' through the body (fluid, medical devices) and colonise other sites. *Staphylococcus aureus* biofilm detachment is a multi-step process, where biofilm cells are prepared for survival outside the EPS (Otto, 2009; Boles & Horswill, 2011). The accessory-gene regulator (Agr) system is a regulatory mechanism responsible for producing enzymes that degrade EPS (Mirani et al., 2013). EPS degradation allows *S. aureus* to detach from the biofilm complex (Boles & Horswill, 2011).

Pseudomonas aeruginosa an opportunistic pathogen, is often involved in respiratory infections, hospital acquired infections (HAIs) and cystic fibrosis (CF) related respiratory infections (Ma et al., 2009; Mulcahy et al., 2013). *Pseudomonas aeruginosa* forms biofilms on environmental, medical, and various surfaces within the body (Høiby et al., 2001; Fricks-Lima et al., 2011). *Pseudomonas aeruginosa* flagella enable motility, attachment and formation of bacterial monolayers (O'Toole & Kolter, 1998; Høiby et al., 2001). Type IV pili further support these initial attachments by stabilising and anchoring reactions, furthermore increasing cell to cell communications and

forming micro colonies of *P. aeruginosa*, either monomicrobial, or within a polymicrobial biofilm (O'Toole & Kolter, 1998; Barken et al., 2008).

The *P. aeruginosa* genome has a high number of regulatory genes and multiple chemotaxis systems, regulating and constantly adapting biofilm formation (Whiteley et al., 2001; Mulcahy et al., 2013). *Pseudomonas aeruginosa* can display different phenotypic forms, most notably the so-called mucoidal and non-mucoidal forms. The mucoidal phenotype exhibits increased virulence and is particularly associated with CF/respiratory infections (Sauer et al., 2002; Wiens et al., 2014). Mucoidal and virulent strains of *P. aeruginosa* demonstrate high capacity to produce alginate, which is an exopolysaccharide acting as a supporting structure within *P. aeruginosa* biofilms (Boyd & Chakrabarty, 1995).

Following bacterial adherence, and up-regulation in expression of *algC*, the gene responsible for the synthesis of alginate and EPS composition occurs (O'Toole & Kolter, 1998). Key genes involved in EPS production are those involved in alginate production, the Pel genes and polysaccharide synthesis locus (Psl). Psl is a cluster of 15 genes and promotes biofilm construction and *P. aeruginosa* cell interactions (Ma, 2007). Expression of Pel genes protects against antimicrobial agents via the production of a glucose-rich matrix extracellular matrix (Vasseur et al., 2005; Colvin et al., 2011). *P. aeruginosa* can also produce pyocyanin (a virulence factor), which attaches directly to eDNA within the matrix, providing cellular respiration through the biofilm matrix via electron transfer (Das et al., 2015). The ability of *P. aeruginosa* to lyse and detach from the biofilm is considered a virulence factor. Once *P. aeruginosa* cells detach from the biofilm as a response to the external environment, cells revert back to the planktonic form to disperse and colonise other areas of the body (Webb et al., 2003).

1.6.6 Biofilm imaging

Biofilm imaging can provide information about its structure and to a certain extent the microbial composition. With a magnification limit up to 100,000x and the ability to construct accurate and 3-dimensional images, biofilm

structures can be imaged using scanning electron microscopy (SEM) (Vernon-Parry, 2000). Electron beams are focused onto the sample surface to create an image using released scattered electrons. SEM micrographs are invaluable when imaging the structure and extent of microbial biofilms (Gil-Perotin et al., 2012). In order to generate images, specimens have to be electrically conductive at the surface and fixed through the process of sputter coating, to ensure the entire sample is coated with a thin layer of conductive material, usually gold (Hill et al., 2010).

A second form of microscopy used to analyse biofilms is confocal laser scanning microscopy (CLSM). In confocal microscopy, light is emitted by the laser source, reflecting off a dichromatic mirror scanning the sample and passing through the presence of a spatial pinhole placed at the confocal plane of the lens. This pinhole eliminates out-of-focus light and enables imaging at a range of optical planes, increasing resolution and contrast. A detector subsequently measures light that passes through the pinhole (Claxton et al., 1979). CLSM has been extensively used in medical and biological sciences since the 1970s, as there is an additional benefit of imaging throughout the section, a process called optical sectioning (Claxton et al., 1979). A wide range of fluorescent and immunohistochemcial stains can be imaged through CLSM allowing the excitation of a range of fluorochromes within the electromagnetic spectrum to increase target specificity and the generation of multiplex images. Z-stacks of optical sections can be taken through the full thickness of the biofilms and maximum intensity-type reconstructions are then prepared using confocal software packages.

Fluorescence *in situ* hybridisation (FISH) coupled with CLSM can facilitate the imaging of multiple species in real time using species-specific Peptide nucleic acid (PNA) probes in a multiplex assay. FISH is an effective and invaluable tool in microbiology that has been used frequently in research since the 1980s (Zwirglmaier, 2005; Wagner & Haider, 2012). Initially, FISH typically used DNA or RNA-based probes tagged with a fluorochrome

(Wagner & Haider, 2012). Conventional FISH methodology using DNA probes experienced several limitations including reduced sensitivity of fluorescent signals and specificity of the probe itself (Perry-O'Keefe et al., 2001). Peptide nucleic acid (PNA) probes are synthetic oligomers of nucleic acid (Figure 1.4). The uncharged sugar phosphate backbone of the PNA probe aids cell entry and reduces electrostatic repulsion between the probe and the nucleic acid target, these factors thus increase the level of hybridisation (Amann et al., 2001). The ability of the chosen probe to penetrate the cell and subsequently hybridise to the target nucleic acids ultimately provides cell fluorescence (Malic et al., 2009). PNA probes were therefore selected in this study to analyse species presence and location within biofilms.

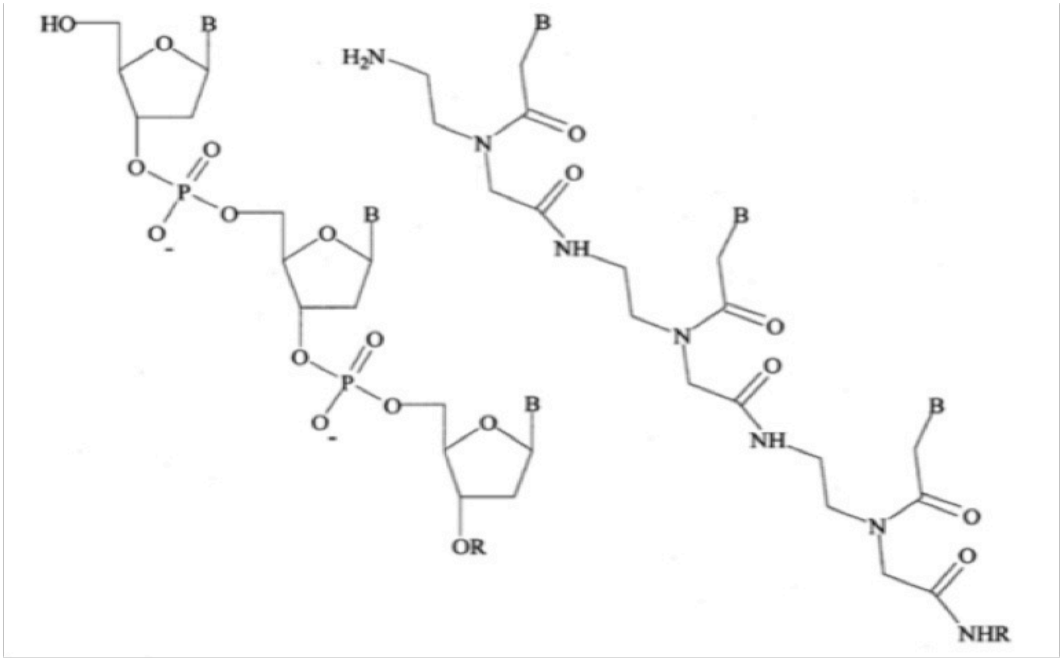

Figure 1.4 – The chemical structures of DNA and PNA. In PNA, the sugar phosphate backbone in DNA is replaced with a polyamide backbone (Perry-O'Keefe et al., 2001).

1.6.7 *In vitro* biofilm analyses

A variety of *in vitro* models can be used to develop and subsequently analyse biofilms. Microtitre plates can be used as substrata to develop biofilms, and depending on design, microbial cells may either adhere directly to the base of the microtitre plate wells or upon inserted PTFE pegs (Calgary biofilm device) which readily facilitates application of downstream analysis (Fricks-Lima et al., 2011; Hooper et al., 2011; Malic et al., 2013). Biofilms can also be formed within specifically designed models such as glass bottom chamber slides or the constant depth film fermenter (CDFF) (Wimpenny 1997; Fricks-Lima et al., 2011). Clinically relevant biofilm models can also be developed using actual medical devices such as ETTs and catheters. Furthermore, complex co-culture modeling systems can be used to allow simultaneous analyses of infection and immune response cascades (Wimpenny, 1997; Guggenheim et al., 2001; Coenye & Nelis, 2010;).

The CDFF (Figure 1.6) is a steady-state biofilm model that was initially developed in the 1970s, with further developments in 1988 by Peters and Wimpenny (Costerton et al., 1978; Kinniment et al., 1996; Wimpenny, 1997; Hill et al. 2010). The CDFF is equipped with a rotating stainless steel turntable holding polytetrafluoroethylene (PTFE) pegs in place, whereby microbial biofilms are continuously supplied with microbiological media and has proved particularly valuable in the study of dental plaque (Hope et al., 2012). The turntable is motorised during operation, and two scraper blades coated with PTFE spread media evenly across the recessed pegs, with a sample port allowing access to individual pans during operation (Wilson, 1996; Dalwai et al., 2007; Malic et al., 2009).

A major advantage of the CDFF is the generation of multiple, reproducible and representative biofilms with the ability to vary parameters such as oxygen flow, nutrient feed and temperature (Kinniment et al., 1996; Vroom et al., 1999; Hope et al., 2012).

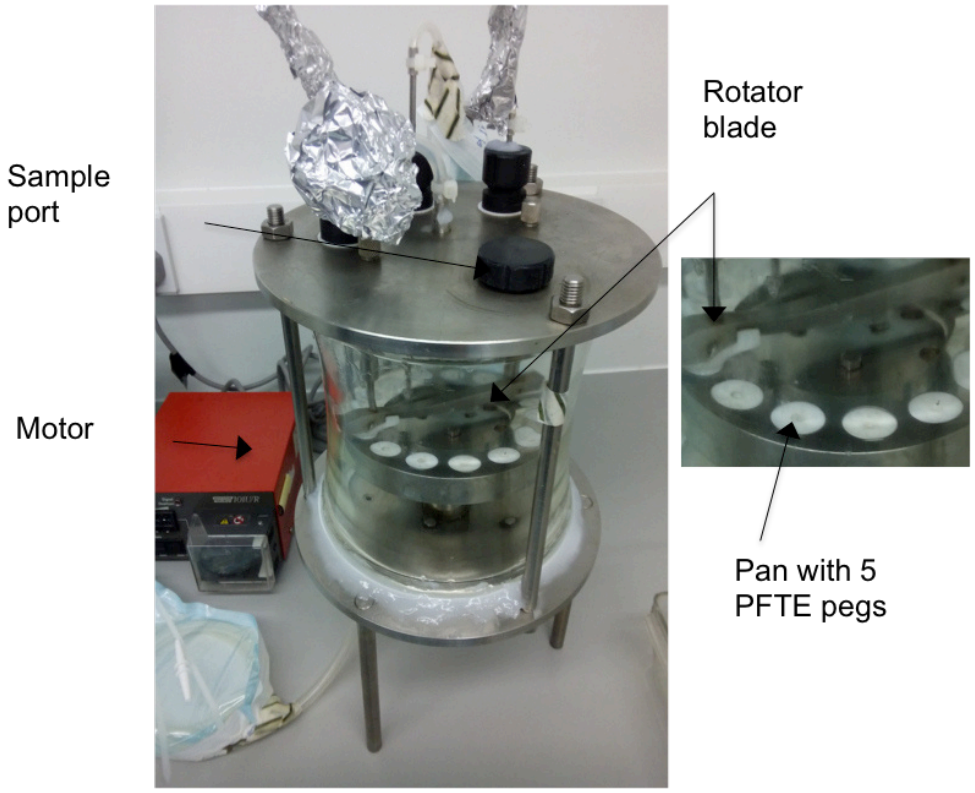

Figure 1.6 – Photograph of the Constant Depth Film Fermenter (CDFF).

1.7 The microbiological analysis of complex communities

1.7.1 Analysing the oral microbiome

The oral microbiome has previously been analysed by conventional culture and molecular techniques (Keijser et al., 2008). With advances in molecular techniques, such as microbial gene sequencing, certain uncultivable organisms have been identified by detection of their DNA (Lazarevic et al., 2009; Yamashita & Takeshita 2011). Microbial culture often underestimates the number and types of microbes within a microbial community. Conventional culture methods are unable to meet the growth requirements of all species, either through our lack of knowledge of nutrient requirements or inability to generate the correct gaseous environment *in vitro*. Despite this, microbial culture is still regarded by many as the gold standard in diagnostic medical microbiology, with the capability to culture and identify 280 microbial species from the oral microbiome (Dewhirst et al., 2010).

1.7.2 Microbial community profiling

Next-generation sequencing (NGS), an umbrella term extending beyond Sanger sequencing which was first described in the 1970s, is used to describe DNA/RNA sequencing at a high-throughput capacity (Schuster, 2008). One important application of NGS is the characterisation of mixed microbial communities. Previously, genome sequencing was both expensive and laborious. Coupled with the development of multiple platforms that can be employed to sequences of DNA, both the turnaround time and cost of producing sequence reads from unknown DNA has reduced significantly. Multiple platforms exist to perform high throughput sequencing and the choice depends to some extent on the nature of the research question. The 454 (pyrosequencing) platform first described in the 1990s paved the way for high-throughput DNA sequencing based on nucleotide detection using light emission (Amend et al., 2010). Other technologies such as the Illumina Miseq platforms, employ sequencing by synthesis and a reversible terminator chemistry process (Lazarevic et al., 2009; Caporaso et al., 2012). Further advances in these technologies have allowed the simultaneous analysis of multiple mixed DNA sequences.

1.7.3 High-through put data analysis: Bioinformatics

High-throughput DNA sequencing undoubtedly generates large quantities of data. Such data require preliminary analysis before any disease association studies for individual bacterial phylotypes can be performed. Data analysis for complex microbial sequence-based data sets can be enhanced with appropriate software programs such as R programming, allowing the generation of scripts for mass analysis and data sorting. With the number of species ranging up to several hundred for dental plaque, and perhaps considerably less within the microbiota of the lower airways, data analysis needs specific focus. For example, Bray and Jaccard are two similarity-distance diversity indices, based on identified Operational Taxonomic Units (OTU) (Lozupone & Knight, 2005; Wang et al., 2013). Both measures are

widely used in diversity analyses. The choice between these two indices is dependent upon the data to be analysed. The Jaccard approach, also known as the coefficient of community, first developed by Paul Jaccard in 1901, is one of the oldest occurrence measures. The Jaccard approach compares the occurrence of specific organisms according to DNA sequences (Lozupone et al., 2007). However, the Bray approach is based upon quantitative (abundance) data, and when used as a similarity measure of data, can be equivalent to occurrence (Wang et al., 2013). These two approaches are examples of community distance/similarity indices that can be adopted to analyse DNA profiles of the entire community.

Performing community analysis was enhanced in 2005 with the introduction of a new phylogenetic based method called UniFrac. This method is a β-diversity measure devised by Lozupone and Knight used to distinguish relationships between microbial communities (Lozupone & Knight, 2005; Lozupone et al., 2011). In addition, there are two variations of UniFrac community analysis: unweighted or weighted UniFrac, with the weighted UniFrac approach incorporating OTU measures. This is a quantitative approach commonly used in the analysis of microbiome related data (Lozupone et al., 2007).

1.8 Diagnosis and treatment of VAP

1.8.1 VAP diagnosis

Diagnosing VAP is difficult for critical care physicians as there is no effective gold standard diagnostic test (Aucar et al., 2003; Bonten, 2014; Kalanuria et al., 2014). As with many infections or diseases, a gold standard for correct, efficient and quick diagnosis is of fundamental importance. The challenges faced in diagnosing VAP may partly originate from a lack of understanding of the pathogenesis. There is often confusion between signs from underlying conditions that overlap with those of the clinical presentation of VAP. There have been multiple and conflicting reports within the last decade with regards to accurate and efficient VAP diagnosis. Chest X-rays are indicative of

hospital-acquired respiratory infection, although in the case of mechanically ventilated patients they can be taken out of context especially when considering the reasons behind the patient's initial admission to the ICU (Medford et al., 2009). Other studies have advocated the need for a clear diagnosis of VAP using lavage based testing, whereby the lung parenchyma is washed with saline and subjected to microbial analysis.

Flanagan et al, 2000 performed a prospective comparison of non-directed bronchoalveolar lavages (NBLs), bronchoalveolar lavages (BALs) and a protected specimen brush. They reported an 83% concordance of the predominant pathogen between the sampling methods, and concluded that NBLs are a simple, safe, cheap and readily available method of diagnosing VAP. The use of NBLs and BALs in the diagnosis of VAP have potential advantages over other clinical approaches, as these allow the clinician to target the microorganisms in the airways and lungs to ascertain the most effective choice of antimicrobial therapy (Jackson et al., 2008). In practice, NBLs are preferred, in comparison to BALs as a reliable and cost effective method (Flanagan et al, 2000). In certain cases, where NBLs produce negative results and the clinician suspects VAP, a BAL is often performed. Although the collection of BALs is an invasive and costly procedure, BAL sampling is thought to improve microbiological diagnostic accuracy. Zaccard et al, 2009 reported that, statistically, unilateral right lung sampling may be preferable over taking bilateral sampling in cases where there are multiple areas on CT imaging requiring investigation, and will therefore reduce patient stress with the same diagnostic accuracy. Although microbiological sampling of the lower respiratory tract (NBLs and BALs) often provides accurate and informative culture based results, the sampling is invasive for the patient and the results turn around time is at least a few days.

To improve VAP diagnosis, the Clinical Pulmonary Infection Score (CPIS) was introduced (Pugin et al., 1991; Kalanuria et al., 2014) which focuses on clinical presentations, radiographic and microbiological data to provide a

likelihood of VAP being present (Fartoukh et al., 2003). However Lauzier et al., 2008 described the lack of specificity and sensitivity in many cases using variables in the CPIS, leaving debate concerning the use of CPIS scoring. Patients presenting with a high CPIS would require a NBL/BAL, and be empirically treated with antibiotics whilst awaiting microbial culture results. An additional disadvantage of the CPIS is the lack of culture information, which is of increasing importance given the increase of VAP infection caused by multi-drug resistant (MDR) pathogens. Recently, the Center for Disease Control and Prevention (CDC) published updated criteria for 'possible and probable' cases of VAP, aiming to improve the classification and consistency between critical care units (NHSN, 2015). Patients with signs of infection and inflammation are classified as "infection-related ventilator-associated complication" (IVAC). Further evidence of purulent and pathogenic secretions from the lower airways will label the patient as possible and probable VAP (Kalanuria et al., 2014; Munro & Ruggiero, 2014).

Consideration has been given to the use of systemic biomarkers in fluids from mechanically ventilated patients to improve diagnosis of VAP. Biomarkers including procalcitonin (PCT) and C-reactive protein (CRP) have been suggested as an alternative to predict onset of VAP (Christ-Crain & Müller, 2007; Palazzo et al., 2011; Zielińska-Borkowska et al., 2012). Although there have been reported specificity issues when considering the use of biomarkers such as PCT or CRP, biomarkers identified from lower airways fluid such as neutrophil proteases could elucidate lower airways infection. A study by Wilkinson et al., 2012 found significantly increased neutrophil-derived protease levels in the alveolar spaces within patients clinically diagnosed with VAP. Recent research by Hellyer et al., 2015 has revealed the potential for biomarkers including low concentrations of the cytokines IL-1β and IL-8 to specifically exclude VAP. Improving early diagnosis of true VAP cases may in turn improve antibiotic stewardship and the timely administration of antibiotics within critical care. Reducing inappropriate antibiotic prescriptions will reduce the generation of antibiotic

resistance. Biomarker development to facilitate the diagnosis of VAP is still very much an evolving area of research.

1.8.3 VAP pathogenesis: The role of the oral microbiome

A number of investigations suggest that bacteria colonising the oral cavity may be important in the aetiology of VAP (Bahrani-Mougeot et al., 2007; Scannapieco & Binkley, 2012). Firstly, there have been recorded cases where oral microorganisms may actually directly cause VAP, especially considering the immunocompromised status of critically ill patients. The *Streptococcus anginosus* group (previously known as the *milleri* group) compromising of three species (*Streptococcus anginosus, Streptococcus constellatus* and *Streptococcus intermedius)* have been the predominant isolated species in the lower respiratory tract in patients with VAP (Shinzato & Saito, 1995).

A change in the microbial composition within the dental plaque of mechanically ventilated patients has been reported, with dental plaque becoming heavily colonised with potential respiratory pathogens able to cause VAP such as *Pseudomonas, Klebsiella, Meticilin resistant Staphylococcus aureus* (MRSA) and *Acinetobacter* (Scannapieco et al., 1992; Sachdev et al., 2013). Changes in the oral cavity of mechanically ventilated patients may be influenced by exogenous factors such as the hospital environment or contamination from equipment/people, but evidence suggesting this is the case is scarce. Previous studies indicate that VAP is primarily caused by endogenous factors from the patient's microbiome, and microenvironment (Safdar et al., 2005).

One study enrolled 228 mechanically ventilated patients to evaluate the effectiveness of respiratory pathogen decontamination in the oral cavity using a 0.2% CHX gel (Somal & Darby, 2006). Within their study they isolated highly resistant *Enterobacter* in dental plaque and the lower airways in patients with VAP. Furthermore, poor dental hygiene was apparent in 90% of

enrolled patients highlighting the apparent associations between the oral microbiome, oral health and the development of VAP. During intubation, one third of dental plaque cultures collected within their study were positive for Gram-negative rods, not commonly isolated from healthy dental plaque.

One of the earliest studies categorically demonstrating that bacteria in the oral cavity were responsible for the development of VAP used pulsed field electrophoresis of genomic DNA to study colonisation and infection in 86 mechanically ventilated patients (Garrouste-Orgeas et al., 1997). In patients that developed VAP, identical strains of bacteria were found in the oral cavity and lung.

1.8.4 VAP pathogenesis: The role of the endotracheal tube (ETT)

Although the ETT provides an essential conduit between the ventilator and patient in critical illness, the ETT is a key factor in aetiology of VAP. The placement of an ETT will impair normal host defence mechanisms such as the mucociliary clearance of microorganisms, impede cough, hold open the vocal cords and provide additional surfaces for microbial biofilm development (Perkins et al., 2010; Cairns et al., 2011). Microorganisms from the oral cavity can translocate, exteriorly to the ETT and down the trachea to pool above the inflated ETT cuff (Figure 1.7). Critically ill patients receiving MV are normally nursed in a semi-recumbent position, as this is thought to reduce gastro-acid reflux, and underlines the important of an effective ETT cuff seal of the airway. The inflated ETT cuff is designed to help retain the ETT within the trachea and will theoretically also prevent movement of subglottic fluids that accumulate above the cuff down into the lower airways. These subglottic fluids contain microorganisms including pathogens, salivary components and gastric fluids. However, in practice, microchannels frequently develop in the ETT cuff material because the fully inflated cuff is larger than the tracheal diameter (Hamilton & Grap, 2012). As subglottic fluids pool above the cuff, leakage of these fluids down the microchannels and into the lower airways will occur (Fisher, 2011; Miller et al., 2011;

Hamilton & Grap, 2012). This ultimately allows microbial access and translocation of microorganisms from the oral cavity to the lower airways (Figure 1.8). Once below the inflated ETT cuff, microorganisms can also be drawn into the ETT and then colonise the inner lumen in the form of a biofilm (Perkins et al., 2010; Vandecandelaere et al., 2012). Inside the ETT, adherent microorganisms can develop into complex multispecies biofilms which are inaccessible to administered antimicrobial agents and host defenses (Tarquinio et al., 2014). Biofilms have been identified along both the inner and outer surfaces of discarded patient endotracheal tubes and shown to form within 24 h of intubation (Perkins et al., 2010). The formation of microbial biofilms within a 24 h period highlights a rapid generation and source of VAP causing organisms. Fragments of biofilm can become detached and move directly towards the lower airways with the airflow.

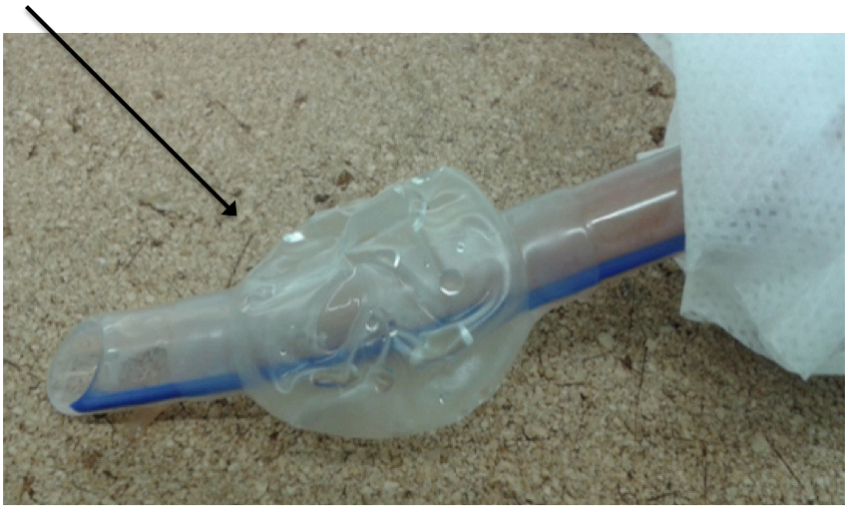

Figure 1.7 – An extubated endotracheal tube (ETT) showing a semi-inflated cuff, as indicated by the arrow.

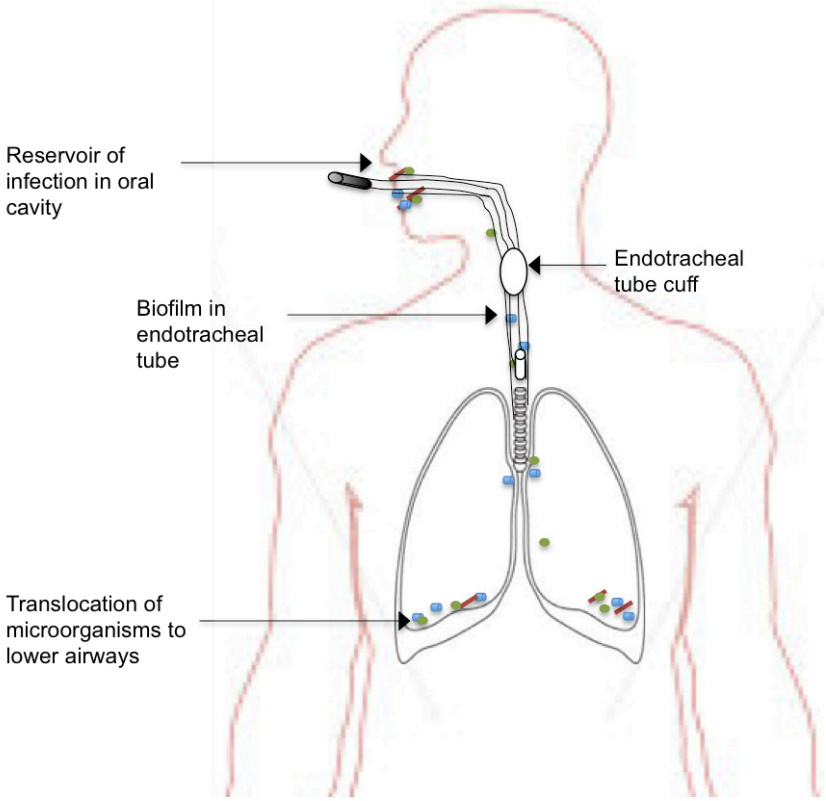

Figure 1.8 – The translocation of oral microorganisms to the lower airways adapted from E. Diaz et al., 2005.

The lungs, although once often described as a sterile environment, share microbial commonality with the oropharynx microbiota. Recent research has focused on characterising the lung microbiome in both health and disease (Dickson & Huffnagle, 2015). Once however, the lower airways become compromised via pathogenic microbial invasion, an inflammatory response is initiated (Mojon, 2002; Joseph et al., 2010). Host defense mechanisms to control microbial presence in the lung parenchyma include mucociliary clearance, a physical epithelial cell barrier, cytokine production stimulating neutrophil recruitment and phagocytic activity (Wilkinson et al., 2012). Furthermore, in intubated patients, the movement of the tongue to dislodge and clear residual microorganisms, and the swallowing mechanism are both compromised, ultimately leading to an altered environment for microorganisms to accumulate. It is likely that a combination of factors results in disruption of oral equilibrium and facilitation of microbial colonisation in the lower airways. Patient illness, and ETT intubation, along with the introduction of respiratory pathogens originating from the oral cavity can all influence the development of VAP (Marra et al., 2009).

1.8.5 Preventative measures for VAP

1.8.5.1 Host defence mechanisms for respiratory infection

The respiratory system comprises of the upper airways (nasopharynx including sinuses) and lower airways (trachea and lungs), and is responsible for gas exchange (Sato & Kiyono, 2012). The mucosal membrane lining the respiratory system acts as a physical barrier against pathogens in a similar manner to that of oral mucosal membranes (Eddens & Kolls, 2012). Epithelial cells in the lining of the trachea contain cilia on their surfaces which move foreign particles away from the lower airways, allowing expulsion via mucociliary clearance (Safdar et al., 2005). The physical actions of coughing and sneezing further expel foreign bodies and prevent them from entering the respiratory tract. In addition, epithelial cells are lined with mucins which slows pathogen translocation to the lower airways (Boyton & Openshaw,

2002; Wilkinson et al., 2012). Airway surface fluid (ASF) is produced by the lower airways, acting in a similar antimicrobial manner to that of saliva, recognising invading foreign bodies. ASF contains several antimicrobial peptides, including those produced by innate immune cells such as lactoferrin, defensins and lysozymes (specifically secreted by macrophages) (Boyton & Openshaw, 2002; Devine, 2003; Wilkinson et al., 2012).

The immune system serves to protect the host in response to a foreign substance or antigen (Alamos et al., 1986). Immune responses are tightly regulated to maintain homeostasis in the host. If too little a response is initiated, pathogen clearance is insufficient, and an overly active immune response can lead to damaging inflammatory responses (Medzhitov, 2007; Sitkovsky & Ohta, 2005). Although pathogens from the upper airways can freely escape interactions with the epithelial lining in the trachea and can invade the lower airways directly through inhalation to the alveoli, there is a cascade of cell infiltration, cytokine and mediator production, and ultimately an inflammatory response to combat them (Sato & Kiyono, 2012). An increase in cytokine production in the lower airways can be indicative of microbial invasion, or alterations in respiratory homeostasis. Critical illness may lead to immunosuppression, with the ETT altering the microenvironment leading to dental plaque overgrowth and potentially increase colonisation within the lower airways.

1.8.5.2 VAP Care bundles

Prevention of VAP is important given the impact on duration of mechanical ventilation, length of stay, attributable mortality and health care costs. The cost of VAP treatment frequently exceeds £10,000 per case (equivalent to 7 extra days in ICU), emphasising the need to focus on preventative medicine (Al-Tawfiq & Abed, 2010; Díaz et al., 2010; Wyncoll & Camporota, 2012). One of the most appropriate strategies for VAP management is to implement and adhere to certain therapeutic guidelines and VAP specific care bundles have been outlined (Rello et al., 2011). A care bundle is a small set of

evidence-based guidelines designed to improve patient outcome (Venkatram et al., 2010). Care bundles provide a prioritised and consistent list of universal based guidelines of hospital-acquired pneumonia (HAP) and VAP, such as those published by the British Society for Antimicrobial Chemotherapy (BSAC) (Masterton et al., 2008). Previous studies suggest the need for further research and training for all critical care professionals (Díaz et al., 2010), with greater education of pathogenesis, management and prevention to help maintain and reduce cases of VAP (Gallagher, 2012).

Although care bundles aimed at reducing VAP rates in critical care have been introduced, and shown benefit (Al-Tawfiq & Abed, 2010; Heck., 2012; Eom et al., 2014), the impact that care bundles have on VAP reduction rates nationwide still needs to be determined (Beattie et al., 2012). VAP based care bundles include the use of antiseptic mouthwashes educational programs, and parameters for both diagnosis and treatment choices as outlined in table 1.6

Table 1.6 – An overview of care bundles used in the prevention and standardisation of care in critical care units (Hutchins et al., 2009; Rello et al., 2011; Beattie et al., 2012; Gallagher, 2012; Heck, 2012; Eom et al., 2014; Munro & Ruggiero, 2014).

VAP care bundle preventative measures
Semi-recumbent position
Oral antisepsis - chlorhexidine
Daily sedation breaks/Minimal targeted sedation
Daily assessment - focus to wean off ventilator
Subglottic suctioning
Appropriate antibiotic use
Glucose control
Stress ulcer prophylaxis
Endotracheal cuff pressures
VAP diagnosis parameters

Other VAP preventative measures have been assessed in clinical trials, for example the design of ETTs and their efficacy in reducing VAP rates. Adjustments made to the composition of the ETT biomaterial has also been analysed. Such studies have involved changing the design of the ETT cuff, with polyurethrane cuffs being used over conventional polyvinylchloride in an attempt to avoid microchannels forming, and therefore leakage of fluidic materials (Deem & Treggiari, 2010). An observational study of 284 mechanically ventilated patients by Lorente et al., 2014 compared the effects of intermittent ETT cuff pressure control versus continuous cuff pressure control. Lorente et al showed a significantly lower ($p=0.02$) incidence of VAP with patients receiving continuous ETT cuff pressure control (11.2%) compared to intermittent ETT cuff pressure control (22%).

Secondly, adjusting the composition of the inner lumen of ETTs has been a subject of research to minimise biofilm formation. Coating the inner lumen of ETTs with antimicrobial agents such as silver or antibiotics has been assessed to prevent biofilm colonisation. Kollef et al., 2008 enrolled 2003 patients in a randomised clinical trial to receive either a silver coated ETT or an uncoated ETT (control group) during intubation. Although they reported a statistically significant reduction in the incidence of VAP in the patient cohort receiving a silver coated ETT, there were no significant differences observed in attributable mortality or duration of critical care stay. In 2011, Raad et al, specifically reported that gardine (chlorhexidine based antiseptic) successfully inhibited MRSA and multi-drug resistant (MDR) Gram-negative adherence to ETTs in *in vitro* analyses. Both the efficacy for reducing VAP incidence and the cost-effectiveness of ETT impregnated with antimicrobial agents remains unclear. Further clinical studies need to examine the extent of biofilm formation and VAP incidence when using preconditioned ETTs.

1.8.5.3 Oral and Dental scoring

Oral care assessment plans are completed within critical care units so as to provide the appropriate level of oral care needed, and these can be tailored to individual patients. For example, at the University Hospital Wales, within 4-

6 h of patient admission to critical care, medical staff complete a critical care mouth risk assessment form. This risk assessment (Appendix I) defines a series of categories upon which a score is assigned based on the clinical presentation of the patient. Categories to assess and tailor care plans include patient dependency, inflammation within the oral cavity *i.e.* mucosa and the tongue, number of natural teeth, state of ventilation and pharyngeal secretions. Once a total score is obtained it is used to place the patient into one of three groups for provision of oral care. This scoring system is to ensure the times for delivery of oral care: 12 hourly, 4-6 hourly, and 2-4 hourly. A series of oral hygiene and management strategies such as: ensuring fluid intake, provision of artificial saliva, administration of chlorhexidine gluconate as a 0.12% rinse, and antifungal medication are given to the patient.

Patients may be admitted to critical care following long-term illness deterioration or acute trauma. This ultimately leads to a degree of variation in oral hygiene status. The oral care assessment plan aims to target this variation, in an attempt to provide the highest quality of care. Maintaining oral care in critically ill patients is often, however, seen as a comfort measure by nursing staff rather than an intervention to maintain health (Dale et al., 2013).

Increases in gingival inflammation and dental plaque during MV can also reflect the changing of oral hygiene levels (Carrilho Neto et al., 2011). Measuring the gingival index, a score based on severity of gum inflammation with a score of 0 indicating gum health and a maximum of 3 indicating severe gum inflammation (Pearson & Hutton, 2002; Carrilho Neto et al., 2011), and the scoring of dental plaque by a dental professional, based on visibility of dental plaque could build upon existing information available to health care professionals and reflect potential risk of secondary infection. Measuring dental plaque scores at set intervals during critical care could further be used as an indicator of changing oral hygiene. There is a need to emphasise the importance of dental scoring such as plaque scoring and gingival index to facilitate patient specific oral interventions (Wise & Williams, 2013). Further analysis examining an association between previous oral hygiene and risk of

VAP could further highlight the importance of oral scoring in assessing a patient's risk for VAP.

A dental score that is used to assess previous oral hygiene status and a risk indictor of oral health status is the Decayed Missing and Filled Teeth (DMFT) score. A DMFT score is used to assess dental caries status and is therefore an indicator of previous oral hygiene, and can reflect current oral health status within the patient (Becker et al., 2007). DMFT scores are however not usually performed in critical care units. To obtain a DMFT score, an oral healthcare professional scores each tooth as either D, M or F, to generate a score of between 0-28 (28 being the highest score and indicative of advanced dental caries). Each incidence of a tooth resembling D, M or F results in a score of 1. DMFT scores may reflect the level of risk of dental plaque changes. It is important to complete oral profiles for patients who undergo MV as risk of VAP increases 1% with every day patient is connected to the ventilator, and can increase up to 80% chance if the patient is mechanically ventilated >10 days (Bern et al., 2013). DMFT is one example whereby an external non-invasive examination can be used to assess the patients' teeth upon admission to the ICU.

1.8.5.4 Antiseptic mouthwashes and lubricants

Antimicrobial mouthwashes and oral lubricants are also used to aid oral care in critically ill patients and have been the subject of multiple systematic reviews (Yoneyama et al., 2002; Labeau et al., 2011; Lam et al., 2012). Several oral antiseptic mouthwashes are currently available for use in oral decontamination in mechanically ventilated patients.

A 10% povidone-iodine solution was used in a study by Seguin et al., 2014, with the primarily outcome measure of VAP reduction. Povidone-iodine penetrates the cell wall of bacteria and affects the functions of bacterial proteins (Donnell, 1999), and is highly effective against Gram-negative species such as *P. aeruginosa.* Seguin et al., 2014 however concluded that

the use of povidone-iodine in high-risk patients did not reduce VAP incidence, and recommended avoiding its use due to an associated increase in acute respiratory distress syndrome (ARDS).

Essential oils menthol, thymol, methyl salicylate and eucalyptol (Listerine ™) and sodium bicarbonate combinations have also been used in oral hygiene intervention in critical care. However, there is little evidence of these combinations in reducing VAP (Berry, 2013). Eucalyptol and menthol (constituents of Listerine formulations), have previously been effective at oral biofilm penetration in *in vitro* studies (Chen et al., 2011). The overall conclusion for the use of antiseptic mouthwashes as a reduction strategy for VAP remains unclear, with studies reporting conflicting efficacy over the use of various antimicrobial mouthwashes. The most widely used and researched antiseptic mouthwash used in selective oral decontamination in mechanically ventilated patients are chlorhexidine based mouthwashes and rinses.

1.8.5.5 Chlorhexidine

A 0.12% (v/v) chlorhexidine gluconate water soluble oral rinse or gel is often applied in critical care units as an oral mouthwash (Jones, 1997; Kusahara et al., 2012). Chlorhexidine damages cell membranes of Gram-positive and Gram-negative bacteria, as well as being effective against yeasts such as *C. albicans.* The effectiveness of chlorhexidine is partly based on its ability to adsorb via its positive charge to negatively charged surfaces of the oral cavity (O'Reilly, 2003; Kusahara et al., 2012). After adsorption, chlorhexidine is released over several hours, a phenomenon called substantivity post adsorption, which serves to increase its duration of antimicrobial activity (Smith et al., 2013; Sands et al., 2014). Importantly, for chlorhexidine to be most effective there is a need to have a relatively clean surface prior to its administration (to adsorb to oral surfaces i.e. the teeth).

Early treatment with chlorhexidine, especially in the form of oral swab delivery, has been reported to reduce rates of VAP (Grap et al., 2011).

Chlorhexidine is, however, inactivated by sodium lauryl phosphate, commonly found in toothpaste, and other ingredients are known to interact with its activity (Munro et al., 2009). Care and appropriate consideration must therefore be taken when using chlorhexidine in conjunction with mechanical brushing using fluoride based toothpastes (O'Reilly, 2003).

Over the last 15 years, clinical studies, meta analyses and systematic reviews have investigated the benefits of chlorhexidine in critical care. A recent meta analysis and systematic review by Klompas et al., 2014 explored effectiveness of chlorhexidine in a critical care setting. Klompas *et al* suggested the limitations of chlorhexidine application might arise from patient variation, and specifically whether the patient was admitted due to declining health or for elective surgery. Nevertheless, the efficacy of chlorhexidine applications remains a matter of debate (Jones, 1997; Pineda et al., 2006; Zanatta et al., 2007; Bellissimo-Rodrigues et al., 2009; Scannapieco et al., 2009; Grap et al., 2011; Kumari, 2012; Scannapieco & Binkley, 2012; Bonten, 2014; Klompas et al., 2014). Such contrasting evidence over relative benefits has led to a great variation in oral care protocols worldwide. Considering the cost of VAP treatments and aftercare, preventative measures will perhaps be more cost effective for the healthcare provider, and undoubtedly reduce both patient and resource stress (Joseph et al., 2010).

1.8.5.6 Tooth brushing in mechanically ventilated patients

The accumulation of dental plaque may increase a patient's chance of developing VAP during MV (Munro et al., 2006). One of the most effective methods to prevent plaque overgrowth is the non-pharmacological approach of mechanical brushing (Johnson et al., 2012). The choice of toothbrush used can alter effectiveness of plaque removal. Research into different types of toothbrush has suggested the advantage of electric toothbrushes over manual toothbrushes in removing a greater abundance of the biofilm. A randomised study by Needleman et al., 2011 found a significant reduction in dental plaque when using a powered toothbrush. In addition, smaller toothbrushes, such as paediatric brushes have also been considered in

many ICU units as opting for a smaller brush allows access to difficult areas, especially around the mouth connector for ETTs (O'Reilly, 2003; Johnson et al., 2012; Prendergast et al., 2013). Toothbrushes with suctions and aspiration applicators are also used in critical care to simultaneously brush patients' teeth and remove any dislodged debris before application of chlorhexidine (Hutchins et al., 2009). However a major challenge of tooth brushing is the physical ability to reach tooth surfaces around the ETT apparatus (Needleman et al., 2011).

1.8.5.7 Oral care compliance

Although there is great variation in the administration of oral care in critical care units, maintaining adequate oral hygiene is essential. Performing oral care does not only limit microbial colonisation and dental plaque accumulation, but increases patient comfort and provides lubrication in the oral cavity (Jones et al., 2004).

Oral intervention for mechanically ventilated patients, although considered a vital component of the ICU oral hygiene care plan, is sometimes overlooked. In many cases the duration of oral care delivery is shorter than necessary, and many nurses are perhaps reluctant to clean the teeth mechanically, for fear of dislodging the ETT (Berry & Davidson, 2006; Lloyd et al., 2011; Munro & Grap, 2004). Maintaining adequate oral care is therefore challenging.

In order to improve oral care compliance and facilitate chlorhexidine application in critically ill patients, manufacturers (e.g. Sage Products; Illinois, USA) have produced single dispensing foam swabs incorporating chlorhexidine, referred to as a Toothette™ for use in critical care units A study by Munro et al., 2009 used the Toothette™ to apply chlorhexidine to the oral cavity within a randomised trial. Munro et al., 2009 concluded that chlorhexidine applications did reduce the incidence of pneumonia in comparison to patients receiving tooth brushing alone. The structure and composition of the swab enables both lubrication of the oral cavity with chlorhexidine or other oral care products whilst the ridged brush gently removes any anchored dental plaque.

Tooth brushing is one of the most effective management strategies for maintaining dental plaque accumulation, usually performed in conjunction with chlorhexidine application. In critical care medicine the immediate priority for nurses and physicians is patient survival and oral care is often considered a secondary intervention.

Overall, research into the benefits of mouthwash administration in mechanically ventilated patients is extensive. Oral care may reduce VAP incidence, however currently there is little evidence to suggest that there are benefits in clinical outcomes for length of ICU stay or duration of MV (Li et al., 2015).

1.8.5.8 Selective Oropharyngeal Decontamination (SOD)

Selective oropharyngeal decontamination of the oropharyngeal tract is a regime to prophylactically treat the oropharynx area with topical antibiotics and prevent pathogenic colonisation of Gram-negative bacteria and *S. aureus* (Oostdijk et al., 2013). Unlike the application of care bundles and oral antiseptics, SOD programs administer antibiotics to the entire oropharyngeal tract. For such topical applications, tobramycin and amphotericin B (for overgrowth of Yeasts) are frequently used (Bonten, 2006; de Smet et al., 2009).

1.8.5.9 Selective Digestive Decontamination (SDD)

Selective decontamination of the digestive tract (SDD) is performed to eradicate potentially pathogenic microorganisms in the oropharyngeal and digestive tract of critically ill patients (Silvestri & Saene, 2012; Oostdijk et al., 2013). SDD is the application of the combination of both topical and intravenous antibiotics to reduce incidence of exogenous and endogenous HAIs and reduce mortality rates in critical care (Silvestri & Saene, 2012).

Antibiotics used for SDD include a second-generation cephalosporin such as ceftazidime or a third-generation cephalosporin, cefotaxime, for intravenous application. If the patient was previously healthy (no underlying illness) a beta-lactam IV antibiotic is also widely used. Although there are suggested

antibiotics for SDD that are anti-*Pseudomonas* or specific antifungals, clinical choice is dependent on medical history, microbial culture results, and rates of antimicrobial resistance.

The efficacy and advantages of SDD are still debated (Bonten et al., 2000; Chaari et al., 2013), however de Smet et al., 2009 regards SDD to be as effective as SOD. With an increase in antimicrobial resistance, SOD seems preferable because it can avoid widespread systemic cephalosporin usage. Deciding whether to prophylactically administer antibiotics further complicates matters for clinicians (Laxminarayan et al., 2013).

1.8.5.10 SOD versus SDD

In a landmark study, de Smet et al., 2009 performed a crossover randomised trial of standard care, comparing SDD and SOD in 13 intensive care units in the Netherlands with 5939 patients. Rather than focusing on VAP, mortality was chosen as the outcome measure and there was an absolute reduction of 3.5% and 2.9% with both SDD and SOD respectively. There was no difference between SDD (four days intravenous antibiotics plus tobramycin, colistin, and amphotericin B to mouth and stomach) and SOD, (which used tobramycin, colistin, and amphotericin B paste four times a day). Findings of this study suggest similar survival rates with SOD and SDD programs.

Furthermore, a recent meta analysis by Zhao et al., 2015 analysed the effects of SOD and SDD in mortalitly, length of critical care stay, and carriage of Gram-negatvie bacteria in 23,822 patients. There were similar clinical outcomes in mortality and length of stay between SOD and SDD regimes, however SOD resulted in a higher carriage of Gram-negative bacteria. Due to both cost and antibiotic resistance concerns of SDD, the suggested outcome for this meta analysis was the prophyaltic SOD regime. Both these studies demonstrate and promote a reduction in mortality with a measure that aims to improve oral hygiene regimes. Ultimately, the prevention of VAP includes assessing several parameters including patient care, critical care apparatus used, oral hygiene and the training of critical care professionals.

1.8.6 Antimicrobial treatment for VAP

Pneumonia associated with mechanically ventilated patients, may be more difficult to treat, largely due to increasing incidence of antimicrobial resistance in the microorganisms involved. Once VAP has been diagnosed, or strongly suspected, antibiotic therapy is implemented. Problems can occur however, when deciding not only the choice of pharmacological agent, but also when to begin treatment. Clinicians have the choice of using empiric treatment using broad-spectrum antibiotics once symptoms of VAP appear, or wait until microbial culture results are available (Muscedere et al., 2008). Ideally, waiting for culture results would provide better evidence over which agent to use, and perhaps give an indication of the duration periods for antimicrobial chemotherapy (Magnotti et al., 2011).

Optimum antibiotic treatments for VAP often differ between critical care units and between individual patients, with multiple classes of antibiotics becoming less effective. MDR pathogens are commonly associated with causing VAP (Magnotti et al., 2011; Tseng et al., 2012; Tedja et al., 2014). Single antibiotic treatments are, in many cases of limited use, however certain antibiotics such as meropenem and tobramycin when used in conjunction with others provide a greater probability of successful treatment (Kollef, 2005; Muscedere et al., 2008; Luyt et al., 2014). The difficulty in treating VAP highlights the need of effective preventative measures for VAP in critical care.

Inappropriate and excessive exposure of microorganisms to antibiotics has occurred in recent decades. Over prescription has contributed to the generation of MDR strains for many bacterial genera. MDR organisms have disseminated globally and bacterial resistance to antibiotics is now a major health problem (Magiorakos et al., 2011; Aliberti et al., 2013). Briefly, there are 3 basic mechanisms of antimicrobial resistance (Table 1.7; figure 1.9). Firstly, cell wall, membrane and efflux pumps stop the antibiotic reaching intracellular targets. Secondly, biofilm cells can stop the antibiotic penetrating the cell, and finally changes in target site or excessive production of target, can result in the degradation of antibiotic.

Table 1.7 – Major antibiotic classes, mechanism of action and resistance mechanisms (Wolfson & Hooper, 1985; Silva, 1996; Chopra & Roberts, 2001; Courvalin, 2006; Shinkai, Henke, & Rubin, 2008; Löfmark, Edlund, & Nord, 2010; Giedraitiene, 2011; Meletis et al., 2012; Du van Veen & Luisi, 2015).

Major Antibiotic classes	static or cidal	Mechanism of action	Resistance mechanism
Penicillins	Bactericidal	Inhibits cell wall synthesis, blocks cross linking via inhibition of transpeptidase	Antibiotic hydrolysis by enzymatic β-lactamase
Cephalosporins	Bactericidal	Inhibits cell wall synthesis, blocks cross linking via inhibition of transpeptidase	Antibiotic hydrolysis by enzymatic β-lactamase
Carbapenems	Bactericidal	Cell wall inhibition	Antibiotic hydrolysis by enzymatic β-lactamase
Aminoglycosides	Bactericidal	Protein synthesis inhibition. Irreversible binding to 30S ribosomal unit	Aminoglycoside modifying enzymes
Tetracyclines	Bacteriostatic	Protein synthesis inhibition. Blocks tRNA	Efflux, altered target site (proteins bind and change active site)
Macrolides	Bacteriostatic	Protein synthesis inhibition. Reversibly binds to 50S ribosomal subunit	Altered target site, mutation reduces binding to active site. Decreased permeability
Fluoroquinolones	Bactericidal	DNA synthesis inhibitors, inhibit DNA gyrase	Altered target site, mutation reduces binding to active site. Efflux, new membrane transporters
Trimethoprim/ Sulfonamides	Bacteriostatic	Inhibits folic acid synthesis	Altered target site, mutation of gene coding
Other antibiotics:			
Metronidazole	Bactericidal	DNA synthesis inhibitors. Metabolic biproducts disrupt DNA synthesis	Decreased drug intake or efflux, change in biological target, increased oxygen scavenging abilities
Rifampin	Bactericidal	RNA synthesis inhibitor. Inhibits RNA polymerase	Altered target site, mutation changes structure of beta subunit of RNA polymerase
Chloramphenicol	Bacteriostatic	Protein synthesis inhibition. Reversibly binds to 50S ribosomal subunit	Enzymatic degradation, Efflux
Vancomycin	Bactericidal	Cell wall inhibition. Disrupts peptidogylcan cross-linkage	Altered target site. Removal of susceptible target

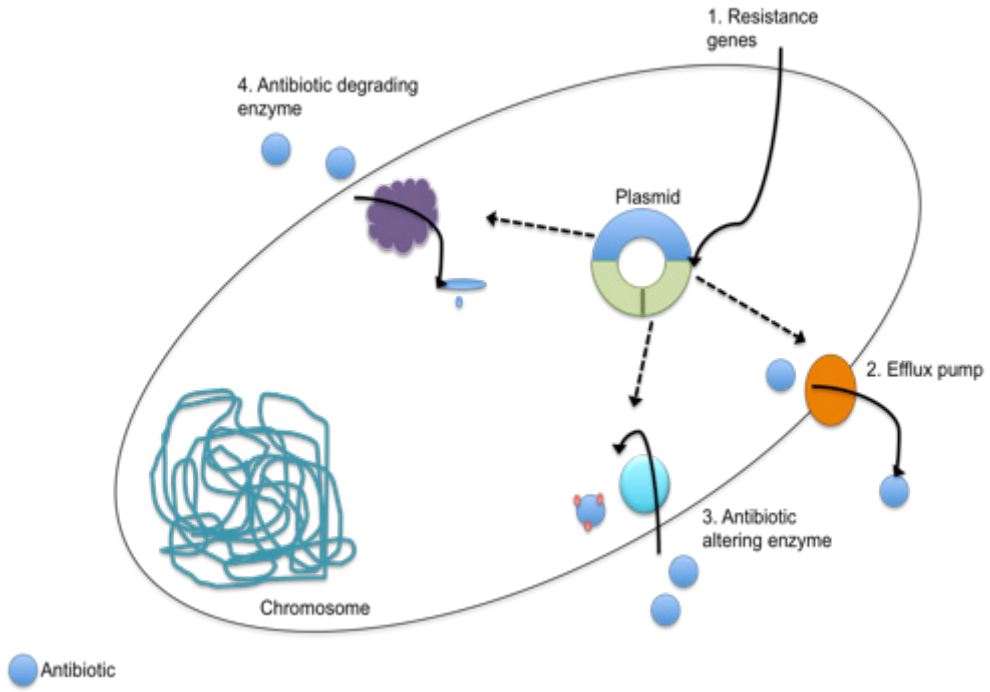

Figure 1.9 – A schematic diagram of a bacterial cell showing mechanisms of bacterial antibiotic resistance adapted from Wright, 2011.

The ability of microorganisms to acquire resistance has developed through multiple mechanisms with MDR species often having more than one mechanism (Alekshun & Levy, 2007; Wright, 2011). Antimicrobial resistance in biofilms adds a further dimension when considering antibiotic therapy. Biofilms can be up to 1,000 times more tolerant to antimicrobials compared to planktonic cells (Fricks-Lima et al., 2011), as summarised in figure 1.10. The EPS protecting the microbial species within the biofilm acts as a shield against external pressures such as antibiotics. A clinically relevant and frequently MDR resistant organism is *P. aeruginosa. Pseudomonas aeruginosa* can develop resistance through several mechanisms, resulting in resistance to carbapenems (such as meropenem) a group of broad-spectrum β-lactams. The two main mechanisms of resistance to carbapenems are use of efflux-pumps (Pai et al., 2001) and production of β-lactamases (Pitout et

al., 2005). Resistant strains of *P. aeruginosa* actively exclude antibiotics via efflux pumps, whilst altered membrane permeability can further limit delivery of antibiotic into the bacterial cell. MDR pathogens are now the cause of several antibiotic resistant bacterial outbreaks both within the hospital and the wider community (Consales et al., 2011; Tseng et al., 2012). It is essential to develop alternative treatments to reduce mortality rates of HAIs.

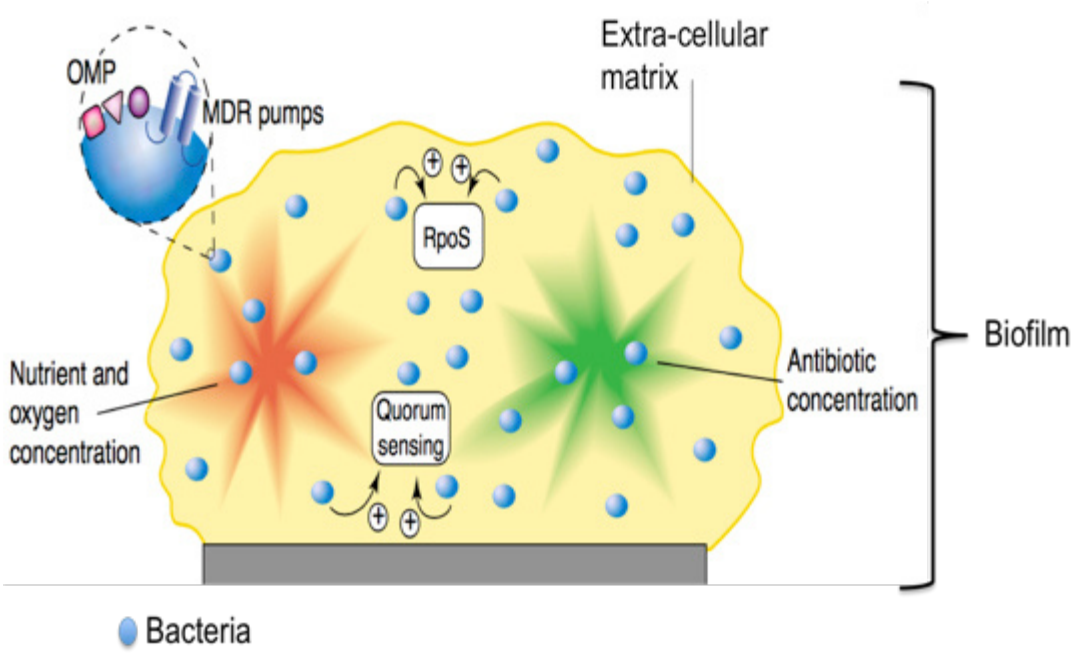

Figure 1.10 – Mechanisms of resistance in biofilms, adapted from Thien-Fah and O'Toole 2001. The bacteria within the extracellular matrix (represented in yellow) undergo changes to enhance resistance mechanisms within the biofilm community.

1.9 Summary

VAP is the most prevalent HAI in intensive care (Joseph et al., 2010). The ETT is an essential interface between patient and ventilator but its placement promotes VAP, by impeding host defence mechanisms, and supporting potentially pathogenic biofilms within its inner lumen that can subsequently disseminate into the lower airways. Timely diagnosis of VAP in mechanically ventilated patients in critical care units is often difficult. Treatment of clinically diagnosed VAP is challenging, as it is a biofilm-mediated infection that is increasingly caused by multidrug resistant organisms.

The composition of dental plaque is thought to change during MV, with respiratory pathogens dominating the polymicrobial biofilm, however the reasons for this suggested microbial shift remain poorly understood. A greater understanding of how to achieve good oral hygiene in patients and control dental plaque is essential for improving health. Microorganisms within the oral cavity have access to the lower airways, via the ETT. Examining the occurrence and extent of dental plaque community changes during MV is the overarching aim of this PhD project.

1.10 Project Aims and objectives

Reducing the prevalence of VAP requires a greater understanding of the exact pathogenesis and aetiology. Given the importance of the oral microbiome in the development of VAP and the lack of understanding of the changes of the oral microbiome during MV, within this project, there are four main aims:

CHAPTER 2: To longitudinally assess dental plaque and the microenvironment of the oral cavity throughout the course MV, and, where possible, into the post ETT-extubation recovery period of critical illness. The aim of this work is to evaluate changes to dental plaque during MV.

CHAPTER 3: Molecular characterisation of dental plaque and lower airways microbial communities will further fulfill project aims by profiling communities during MV using a new technique, without the bias of microbial culture.

CHAPTER 4: Saliva and blood (plasma extractions) will be subject to proteomic analysis to ultimately assess host immunity. The aim of this work is to ascertain whether salivary parameters are associated with dental plaque colonisation by respiratory pathogens.

CHAPTER 5: Based on clinical findings, biofilm models will be developed to mirror any dental plaque compositional changes under controlled conditions. The aim of this work is to assess the possible aetiology of compositional changes and to evaluate any associations with saliva.

Chapter 2

Characterisation of biofilms from mechanically ventilated patients by culture and imaging

2. 1 Introduction

Critical illness requiring mechanical ventilation (MV) occurs as a result of many disease processes, critical care populations are therefore heterogeneous. Up to 50% of patients admitted to critical care require MV, usually as a consequence of respiratory failure or impaired consciousness (Estern et al., 2000; Anzueto et al., 2002). Often in these patients, priorities are understandably given to addressing the immediate clinical problem and in such circumstances, provision and compliance of oral care may not be adequately delivered (Ames, 2011).

Colonisation of dental plaque by respiratory pathogens during MV has been reported previously, primarily using microbial culture techniques (Heo et al., 2008; Scannapieco et al., 2009; Needleman et al., 2011; Sachdev et al., 2013; Shi et al., 2013). Heo et al., 2008 performed pulsed-field gel electrophoresis upon DNA collected from dental plaque and the lower airways of 100 patients during ventilation, identifying several indistinguishable strains of *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia,* Acinetobacter species and Enterobacter species. Other studies including Needleman et al., 2011 and Sachdev et al., 2013, performed microbial culture analysis of dental plaque during mechanical ventilation, though with a smaller sample size (45-50 patients). Both Needleman et al., 2011 and Sachdev et al., 2013 revealed similar respiratory pathogen colonisation of dental plaque to that ascertained by Heo et al., 2008, and additional pathogens *Haemophilus influenzae*, *Proteus mirabilis* and Serratia species were also isolated from dental plaque during MV in these studies. The extent of respiratory pathogen colonisation of dental plaque and the role in the aetiology of VAP however, remains poorly understood.

In mechanically ventilated patients, the ETT provides the essential interface between the lung and the ventilator. Unfortunately, the presence of an ETT will also impair pulmonary host defences and can promote ventilator-

associated pneumonia (VAP) through supporting biofilms within its inner lumen (Raad et al., 2011; Gil-Perotin et al., 2012; Vandecandelaere et al., 2012). The physical placement of the ETT and associated equipment to secure it may also impede the ability to deliver oral care. ETT biofilms can form within a period of 24 h (Perkins et al., 2010) and may serve as a reservoir of respiratory pathogens that are largely protected from host defence mechanisms. Cairns et al., 2011 analysed the microbial communities of 24 ETT biofilms using molecular techniques including denaturing gradient gel electrophoresis (DGGE), revealing ETT colonisation by *P. aeruginosa* and *S. aureus.* In addition Cairns et al., 2011 found representative species of the oral microbiota including *Streptococcus mutans, Porphyromonas gingivalis* and *C. albicans* colonising within ETTs. Once formed, aggregates of respiratory pathogens can dislodge from the biofilm to the lower airway. It could be postulated that this facilitates the delivery of pathogenic agents already within a biofilm phenotype which, it could be argued are already more resistant to host defence mechanisms and administered antimicrobials.

In recent years, studies into the origin of VAP-causing microorganisms have primarily focused on oropharyngeal sites rather than the gastro-intestinal tract (Bonten et al., 1994; Garrouste-Orgeas et al., 1997; Bahrani-Mougeot et al., 2007; Heo et al., 2008). As a consequence, a number of current strategies aimed at preventing VAP now involve targeting oral microorganisms (Shi et al., 2013; Silvestri et al., 2014). The most obvious means of reducing the numbers of infectious microorganisms within the oral cavity is the provision of effective oral care regimes. This could be considered to work in two ways, either reducing the colonisation of plaque by potential respiratory pathogens and/or reducing the microbial load in plaque already colonised in an attempt to reduce colonisation of the lower airways.

To highlight the importance of effective oral care in mechanically ventilated patients to healthcare providers, demonstrating the complex dynamics within

oral microbial communities that culminate in respiratory pathogen colonisation is important. In addition, showing the translocation of such pathogens from the mouth to the ETT lumen and the lower airways will further strengthen the argument for effective oral care in mechanically ventilated patients. This in turn will help to facilitate the development of interventions that could reduce the colonisation of dental plaque by potential respiratory pathogens and or the translocation of respiratory pathogens from the mouth to the lower airways.

The primary aim of the work reported in this chapter was to reveal the extent of respiratory pathogen colonisation of dental plaque and lower airways of mechanically ventilated patients on a longitudinal basis, both during MV and into the post ETT-extubation (recovery from critical illness) period. Specifically, the objectives were three-fold:

(1) To ascertain the extent of respiratory pathogen colonisation within dental plaque and the lower airways during MV by culture and species-specific PCR.
(2) To ascertain whether respiratory pathogens were present in the dental plaque during the post-ETT extubation period corresponding to recovery from critical illness.
(3) To perform image analysis on ETT biofilms via SEM and CLSM coupled with FISH and species-specific PNA probes to identify and locate target respiratory pathogens within the ETT biofilm.

2.2 Materials and Methods

2.2.1 Cohort observational study: ethical approval

Ethical portfolio adoption for the observational cohort study was submitted on the 11th April 2013 to the NISCHR CRC Central Office. Ethical approval was obtained from National Research Ethics Service (NRES) within the Research Ethics Committee (REC), Wales (Ref: 13/WA/0039) for a single NHS organisation study on 20th June 2013 to evaluate dental plaque biofilms during the course of MV. To ensure sufficient statistical power (>80%) to observe a 20% change in at least one phylum in microbial profiles and associated downstream high-throughput techniques (Chapter 3), the minimum number of participants required for the study was 101.

2.2.2 Patient recruitment

Mechanically ventilated patients at a single University Hospital Critical Care Unit were eligible for inclusion in the study if they were aged >18 years, had >8 original teeth, anticipated period of MV >24 h, and an expected survival of >24 h. Informed consent for participation in the study was obtained from the next of kin by the responsible clinician. I gratefully acknowledge the research nurses within Critical Care Unit at The University Hospital of Wales, Cardiff for taking patient consent, collecting demographic data and sampling of mechanically ventilated patients recruited to the study. The following participant information was collected during the course of the study: gender, age, and diagnosis/critical care admission details. The number of decayed, missing, and filled teeth (DMFT score) was determined by a dental practitioner and used as an indirect indicator of previous general dental health status and oral hygiene (Becker et al., 2007). Standard oral care was routinely performed (every 4-12 h) following a critical care mouth assessment to determine level and frequency of oral care required.

VAP was diagnosed using the existing Clinical Pulmonary Infection Score (CPIS) score (with a score >6), with aetiology confirmed by blood culture and

quantitative microbiological culture (>10^3 colony forming units (CFU)/ml) of the lower airways samples (bronchoalveolar lavage (BAL)/non-directed bronchoalveolar lavage (NBL) (Pugin et al., 1991; Zilberberg & Shorr, 2010; Estella & Álvarez-Lerma, 2011; Kalanuria et al., 2014; Hellyer et al., 2015). Antibiotics were prescribed at the clinicians' discretion and a thrice-weekly ward round provided further stewardship with clinical microbiologists.

2.2.3 Microbial culture of dental plaque, subglottic aspirations and non-directed bronchoalveolar lavage (NBLs)

2.2.3.1 Dental plaque, subglottic aspiration and NBL collection

Subgingival and supragingival dental plaque was collected by a healthcare professional using paper points (specifically rolled absorbent and narrow strips of paper) (size 40, QED, Peterborough, UK) and dental examination kits (Minerva Dental) (Bollen & Quirynen, 1994). Paper points are also effective in collecting subgingival and supragingival dental plaque by direct mechanically scraping of target sites, minimising the risk of contamination from other colonised sites of the oral cavity (Smola, 2003). Dental plaque collection was performed on 3 occasions during the first week of admission to critical care, and then weekly until hospital discharge, for up to a maximum of 3 months post admission to the intensive care unit (ICU). To standardise collection, dental plaque was collected on the morning of the due date. A total of 9 paper points, sampling 3 teeth per area (front, middle and back) were used per collection. In cases where the patient did not have sufficient teeth for the above protocol, samples were taken from the areas closest. Dental plaque was suspended in 1 ml of filter sterilised (0.22 µm, Fisher) transport medium (TM) at a pH of 7.0 (75 ml of stock solution 1 (0.6% dipotassium phosphate – K_2PO_4), 75 ml of stock solution 2 (1.2% sodium chloride – NaCl, 1.2 ammonium sulphate, 0.6% potassium dihydrogen phosphate and 0.25% magnesium sulphate), 10 ml of 0.1 M EDTA, 5 ml of 8% sodium carbonate, 20 ml of 1% dithiothreitol (DTT) and 815 ml of distilled water (Syed & Loesche, 1972)). TM bottles were then placed in an aerobic

incubator at 37°C overnight to confirm sterility. Before use, 1 ml of TM was aliquoted into microcentrifuge tubes in the class II safety cabinet and stored at 4-8°C.

Subglottic aspirations were collected through a port in the ETT using a syringe and transferred into sterile universals. NBLs were collected using a suction catheter through the ETT, when clinically necessary as determined by the responsible Critical Care Consultant. Subglottic aspirations and NBLs were aseptically transferred into universal containers for transport to the microbiology laboratory.

2.2.3.2 Microbial culture of dental plaque and fluid collected from the lower airways

Processing and subsequent analyses of clinical specimens was undertaken in the class II safety cabinet using additional personal protective equipment (PPE) including Sheildskin nitrile PPE Category III gloves (AQL score of 0.65) when appropriate. Dental plaque was vortex mixed for 30 s and paper points removed using sterile tweezers. Aspirations and BALs/NBLs were centrifuged for 5 min at 10,000 × *g* and resuspended in 1 ml of PBS. A 500-µl volume of resuspended sample was used to inoculate Microbank™ Beads (Fisher) for subsequent storage and analysis. For microbial culture, 25µl of dental plaque, NBL and subglottic aspirate was added to selective agars for detection of *S. aureus* (Mannitol Salt Agar; MSA, LAB 007), *P. aeruginosa* (Pseudomonas agar base; Pseudo, LAB 108), *C. albicans* (Sabouraud Dextrose agar; SAB, LAB 009) and *S. mutans* (Mitis Salivarius-Bacitracin agar; MSB, Oxoid 229810). Agar media were incubated at 37°C in the presence of O_2 (aerobically) for a period of 5 days. All microbial growth was recorded for data analysis using Microsoft Excel. Suspected *Candida* species on SAB agar were subcultured on CHROMagar® Candida media (Oxoid) and incubated for 24 h at 37°C, for the selective identification of yeast species. Any culture media positive isolates of *P. aeruginosa* and *S. aureus* were subject to further biochemical identification tests. Presumptive *P.*

aeruginosa isolates were subjected to an oxidase test (Oxoid) using a non-metallic loop. For presumptive staphylococci isolates, 3 individual colonies were subjected to a catalase test (hydrogen peroxide; Sigma) to distinguish and these were subsequently tested for coagulase (with positive coagulation largely indicative of *S. aureus*; Oxoid). All confirmed species of *S. aureus* and *P. aeruginosa* were stored on Microbank™ Beads (Fisher) for subsequent species-specific PCR analysis.

2.2.3.3 Microbial analysis of ETT biofilms

ETTs were collected post extubation, wrapped in sterile moist tissue paper, and placed into an autoclaved bag for transport across to the School of Dentistry Microbiology Laboratories for processing. All analyses were performed in a Class II Safety Cabinet. The exterior of the tube was wiped with 70% ethanol and the tube was transferred onto a corkboard wrapped in sterile paper.

A sterile scalpel was used to cut 4×0.5 cm sections of the ETT from its midpoint or region where a biofilm was most evident. One section was processed for imaging (section 2.2.5 - 2.2.7), and the remaining three sections were processed for microbial culture. Biofilms were scraped from the 3 sections of the extubated ETTs using a cotton swab. The swab was re-suspended in 5 ml PBS, vortex-mixed for 30s and serial-decimally diluted. All dilutions were plated onto selective agar and incubated as described in section 2.2.3.2. The remaining ETT was discarded for incineration.

2.2.3.4 Identification of isolated respiratory pathogens using species-specific PCR

Definitive identification of *S. aureus* and *P. aeruginosa* isolates was achieved by species-specific PCR. Total DNA extraction was performed using a DNA extraction kit (Qiagen). The Gram-positive extraction protocol was followed as described by the manufacturer with minor modification. In the case of *S. aureus*, a 1 ml volume of overnight culture, in Brain-Heart Infusion (BHI) broth (Oxoid) was placed in a sterile 1.5 ml microcentrifuge tube and

centrifuged for 1 min at 16,000 × *g*. The pellet was resuspended in 300 µl of cell suspension buffer containing 1.5 µl of lytic enzyme solution (Qiagen). The tube was inverted 25 times, and incubated at 37°C for 30 min. An additional step was required for *P. aeruginosa*, an additional incubation step of 5 min at 80°C was necessary after the initial cell suspension was added to aid bacterial cell lysis. After centrifugation for 1 min at 16,000×*g*, the sample was then re-suspended in 300 µl of cell lysis solution (Qiagen). A 100-µl volume of was added and the tubes were vortex mixed for 20 s. After a 3 min centrifugation step at 16,000 × *g* the supernatant was transferred to a clean 1.5 ml microcentrifuge tube containing 300 µl of isopropanol, and gently inverted 50 times. Samples were centrifuged for a further 1 min period at 16,000 × *g*, with the supernatant being discarded and the microcentrifuge tubes drained on to a clean absorbent tissue. A 300-µl volume of 70% ethanol (v/v in water) was added to the remaining pellet and centrifuged for 1 min at 16,000 × *g*. A 100-µl volume of DNA hydration solution and 1.5 µl of RNase A solution (Qiagen) was then added, and the tubes were vortex mixed for 5 s. The tubes were incubated at 37°C for 30 min and then at 65°C for 1 h to dissolve the DNA.

PCR was performed with a total volume of 50 µl (2 µl of DNA, 22 µl of nuclease free water, 25 µl Mastermix PCR reagents (Promega) and 0.5 µl of forward primer, and 0.5 µl of reverse primer (Table 2.1)). Thermal cycling parameters for *S. aureus* PCR included an initial 5 min at 94ºC, followed by 35 cycles of 94ºC for 40s, 50ºC for 40 s and 72ºC for 1 min with a final elongation step of 72ºC for 10 min. For *P. aeruginosa* PCR, cycling parameters included an initial denaturation step of 95ºC followed by 35 cycles of 94ºC for 45 s, 58.4ºC for 45 s and 72ºC for 1 min ending with 5 min at 72ºC. Where multiple bands were encountered, repeat culture onto selective agar for re-biochemical testing and PCR amplification was undertaken to ensure single species.

Table 2.1 - PCR primers used for identification of *S. aureus* and *P. aeruginosa*

Species	Target Gene	Primers	Amplicon size	Reference
S. 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)
P. 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)

2.2.4 Dental plaque imaging

The viability and distribution of bacteria in dental plaque biofilms was assessed using fluorescent microscopy using a live/dead stain (LIVE/DEAD® BacLight™; LifeTechnologies). A microbial loop was used to transfer dental plaque vortex mixed in TM on to a microscope slide (SuperFrost, Fisher) which was air dried for 5 min. Equal volumes (50-µl) of SYTO® 9 dye (live cell stain) and propidium iodide (dead cell stain) were combined in PBS and transferred into a microcentrifuge tube and vortex-mixed for 20 s. A 25-µl volume of the dye preparation was added to each slide to cover the dental plaque smear and overlaid with a coverslip. The slides were covered with tin foil and incubated at 37°C for 15 min before imaging by confocal fluorescence microscopy (Emission green – live, red – dead) (2.2.7.3).

2.2.5 Preparation of ETTs for microscopic imaging

ETT sections (0.5 cm length) were washed in 2 ml of PBS and immersed in 2 ml of 10% (v/v) formalin for a minimum period of 24 h. The ETTs were paraffin wax embedded using routine methodology, sectioned at 20 µm and placed on to glass histology slides (SuperFrost, Fisher).

2.2.6 SEM analysis of endotracheal tube biofilms

To de-wax the sections for imaging, 3 incubation periods of 10 min each in xylene was undertaken, followed by a wash with 100% ethanol. The slides were air-dried and sputter coated with gold for 8 min in readiness for scanning electron microscopy (SEM). Biofilms were viewed in a JEOL 840A SEM at 5kV and digital images captured using SIS software (School of Medicine, Cardiff University).

2.2.7 Peptide nucleic acid fluorescent *in situ* hybridisation (PNA-FISH) confocal microscopy of endotracheal tube (ETT) biofilms

2.2.7.1 *PNA FISH* on extubated ETTs

The ETT 20 µm sections were prepared and de-waxed (2.2.6). A 100-µl volume of Sputasol (Sputum liquefying agent, Oxoid) was added to the ETT section and incubated at 37°C for 30 min. A 100-µl volume of PBS was added to wash the sections, followed by 100 µl of each of the following enzymes; proteinase K, lysostaphin or chitosan (in the volume and incubation temperature/length) as described below. Proteinase K (Sigma) was diluted from a stock solution (10 mg/ml) to 0.1 mg/ml and 100 µl added to each slide and incubated at 37°C for 30 min. Lysostaphin (Sigma) was diluted from stock solution of 1 mg/ml to 0.2 mg/ml and 100 µl added to each slide and incubated at 37°C for 1 h. Chitosan (Sigma; 1 g) was added to 50 ml of 1% acetic acid and carefully mixed to a gel. A further 50 ml of 1% acetic acid was added to create a final solution of 1% chitosan in 1% acetic acid. A 100-µl portion of this mixture was added to each slide and incubated at 20°C for 30 min. A 100-µl volume of lysozyme (10 mg/ml) was then added and incubated at 37°C for 45 min.

2.2.7.2 PNA probe hybridisation buffer and washing solution preparation

The PNA probes (Panagene) (Table 2.2) were prepared at a stock concentration of 100 µmol in sterile H_20 for storage at -20°C. Stock

concentration of probes were diluted with hybridisation solution (10% (w/v) dextran sulphate, 10 mM NaCl, 30% (w/v) formamide, 0.1% (w/v) sodium pyrophosphate, 0.2% (w/v) polyvinylpyrolidone, 0.2% (w/v) ficol, 5 mM disodium EDTA, 50 mM Tris HCl and 0.2% Triton-X at pH 7.5) to either 300 nM or 450 nM. A 1 L wash solution (5 mM Tris 15 mM NaCl and 0.1% (v/v) Triton-X-100 at pH 10) (Lefmann et al., 2006), was prepared in sterile H_20 and stored at 4°C.

Table 2.2 - Species-specific PNA probes and associated fluorescent markers

Probe	Probe sequence	Working concentration (nM)	Fluorescent marker (N-terminal)
Bacterial Universal	CTGCCTCCCGTAGGA	300	Cy3-00-
Pseudomonas aeruginosa	AACTTGCTGAACCAC	300	FITC-00-
Staphylococcus aureus	GCTTCTCGTCCGTTC	450	Cy5-00-
Streptococcus mutans	ACTCCAGACTTTCCTGAC	450	Alexa405-00-
Candida albicans	ACAGCAGAAGCCGTG	300	FITC-00-

A 100-µl volume of each PNA probe (Table 2.2) was added to a microcentrifuge tube and gently pipette mixed for hybridisation. A 100-µl of mixed PNA probes were added to 20µm sections of ETT and was covered loosely with tin foil and incubated at 55°C for 90 min for probe hybridisation. A 100-µl volume of wash solution was added to the slide and incubated at 55°C for 15 min. This process was repeated three times and the slides were removed until completely dry. A drop of Vectashield™ was added to the coverslip to mount the slide, and the slide was sealed using varnish (commercially available nail varnish) for CLSM.

2.2.7.3 Confocal Laser Scanning Microscopy (CLSM)

Confocal laser scanning microscopy (CLSM) was used to image the biofilms following PNA probe hybridisation. Biofilms were imaged using a Leica TCS SP2 AOBS spectral confocal microscope (Leica, Heidelberg, Germany) at appropriate excitation and emission settings for Fluorescein isothiocyanate (FITC) (ex max 494nm; em max 518nm); Cy 3 (ex max 550nm; em max 570nm) and Cy 5 (ex max 650nm; em max 670nm). Z-stacks of optical sections were taken through the full thickness of the biofilms and maximum intensity-type reconstructions were prepared using confocal Leica software packages. Micrographs were presented as image overlays of confocal fluorescence (colour) superimposed upon Nomarski differential interference contrast (greyscale).

2.2.8 Antimicrobial susceptibility of *S. aureus* and *P. aeruginosa* isolates

Isolated colonies of *S. aureus* and *P. aeruginosa* were cultured on Mueller Hinton (MH) agar at 37°C for 18-24 h. A 0.5 McFarland standard (10^8 CFU/ml) was created direct from these colonies to create an inoculum for antimicrobial sensitivity testing. A sterile swab was used to spread a lawn of the 0.5 McFarland standard across the MH agar plate. Cefoxitin discs were used to identify MRSA. The agar media were incubated for 18-24 h at 37°C and subsequent zones of inhibition measured (in mm) according to The

British Society for Antimicrobial Chemotherapy (BSAC) guidelines to determine isolate sensitivity to antibiotics. A total of 15 antibiotics were chosen, based on the antibiotics the patients received during MV, standards from BSAC, and standard procedures for antimicrobial testing isolates of *S. aureus* and *P. aeruginosa* (Standard microbiological investigations (SMIs)). Predefined criteria for sensitive, resistant and intermediate activities of tested antibiotics were outlined in the BSAC zone of inhibition (ZOI) measurements (BSAC, The European Committee on Antimicrobial Susceptibility Testing (EUCAST) disc diffusion method, V5). An isolate with a ZOI falling between the ranges defined as susceptible or resistant was subsequently designated as being of intermediate resistance. In the context of this study, an MDR pathogen was defined as exhibiting a resistant profile (according to BSAC guidelines) to at least three antibiotics.

2.2.9 Statistical analysis

Analysis of microbial growth was recorded and performed using Microsoft Excel. Analysis of the DMFT scoring and age group was performed using a Box and Whisker plot in IBM SPSS V20.

2.3 Results

2.3.1 Patient demographics

A total of 1016 patients' were screened over a 14-month period. Of these, 5 patients were <18 years, 20 patients had <8 teeth, 210 patients were anticipated to be mechanically ventilated for <24h, 439 patients were not mechanically ventilated, and no final consent was obtained for 232 patients. Of the 110 patients recruited to the study, 3 withdrew their consent from the study post-recovery and their details and samples were deleted and destroyed.

2.3.2 Age and gender

Informed consent was obtained from 107 patients (65 male and 42 females, mean age 54). These patients were divided into 4 age group categories for comparative analysis (Table 2.3).

Table 2.3 – Age groups categories of recruited patients. All patients were grouped into four groups for comparative analysis.

Age group (years)	No' of patients
18-29	12
30-49	30
50-69	42
70+	23
Mean age	**54**

2.3.3 Critical care admission analysis

The study was performed in a single adult critical care unit (Adult Critical Care, University Hospital of Wales). Patients were recruited to the study following ICU admission encompassing a range of illnesses and conditions (Table 2.4).

Table 2.4 – Underlying illnesses and conditions for the admission of 107 recruited patients to primary critical care[1].

Primary reason for admission	Number of patients
Respiratory failure	31
Overdose/suicide attempt	3
Stroke/brain injury/seizures	35
OOHCA	11
Poly-trauma	9
General surgery - Stomach	5
Dental/Oral cavity	1
Other	12

*OOHCA – Out of hospital cardiac arrest.

2.3.4 Antibiotic administration during critical care and MV

Seventy-seven (71%) mechanically ventilated patients received at least one antibiotic and 53 of these received more than two different antibiotics over the course of MV. Over 30 antibiotics were administered to the patients as indicated in table 2.5. Antifungals (including nystatin and fluconazole) were also administered to mechanically ventilated patients (n=12).

[1] Admission details for all patients in Appendix I

[2] +VE – positive control –VE – negative control and M represents the molecular marker.

Table 2.5 - The 10 most frequently prescribed antibiotics

Antibiotic	Number of patients
Tazocin	24
Meropenem	23
Cefuroxime	10
Co-amoxiclav	9
Augmentin	7
Clindamycin	6
Gentamicin	6
Vancomycin	6
Ciprofloxacin	4
Colomycin	2

2.3.5 Decayed, missing and filled teeth (DMFT) scoring and analysis

DMFT indices were obtained for 97 of the 107 recruited patients. DMFT scoring is shown in Appendix I. For 10 patients a DMFT was not obtained due to death of the patient. Table 2.6 summarises DMFT scores for all age groups. Results indicated that the lowest median DMFT score of 4 occurred for the 18-29 years age group, with one outlier (patient of 18 years scoring 25/28 DMFT). There was no statistical difference between gender (n=60 M, n=36 F) and DMFT score (p=0.101). Furthermore, DMFT scores increased with older age. The DMFT score was three times higher for the 70-89 year patient group compared to the youngest age group (18-29 years). Although DMFT analysis was representative for each age group, it should be noted that the sample size of patients differed for each age group recruited to the study (Figure 2.1; table 2.6).

Table 2.6 - DMFT (D – decayed, M – missing and F – filled) score for each age group category (years).

			Mean			
Age group	**No of patients**	**No of DMFTs performed**	**D**	**M**	**F**	**DMFT Score**
18-29	12	11	1.36	2.00	1.91	5.27
30-49	30	27	1.92	3.12	5.19	10.23
50-69	42	38	1.08	7.86	5.89	14.84
70-89	23	21	1.67	7.29	6.52	15.48
Total	**107**	**97**	**1.46**	**5.70**	**5.39**	**12.54**

*Individual DMFT scores in appendix I

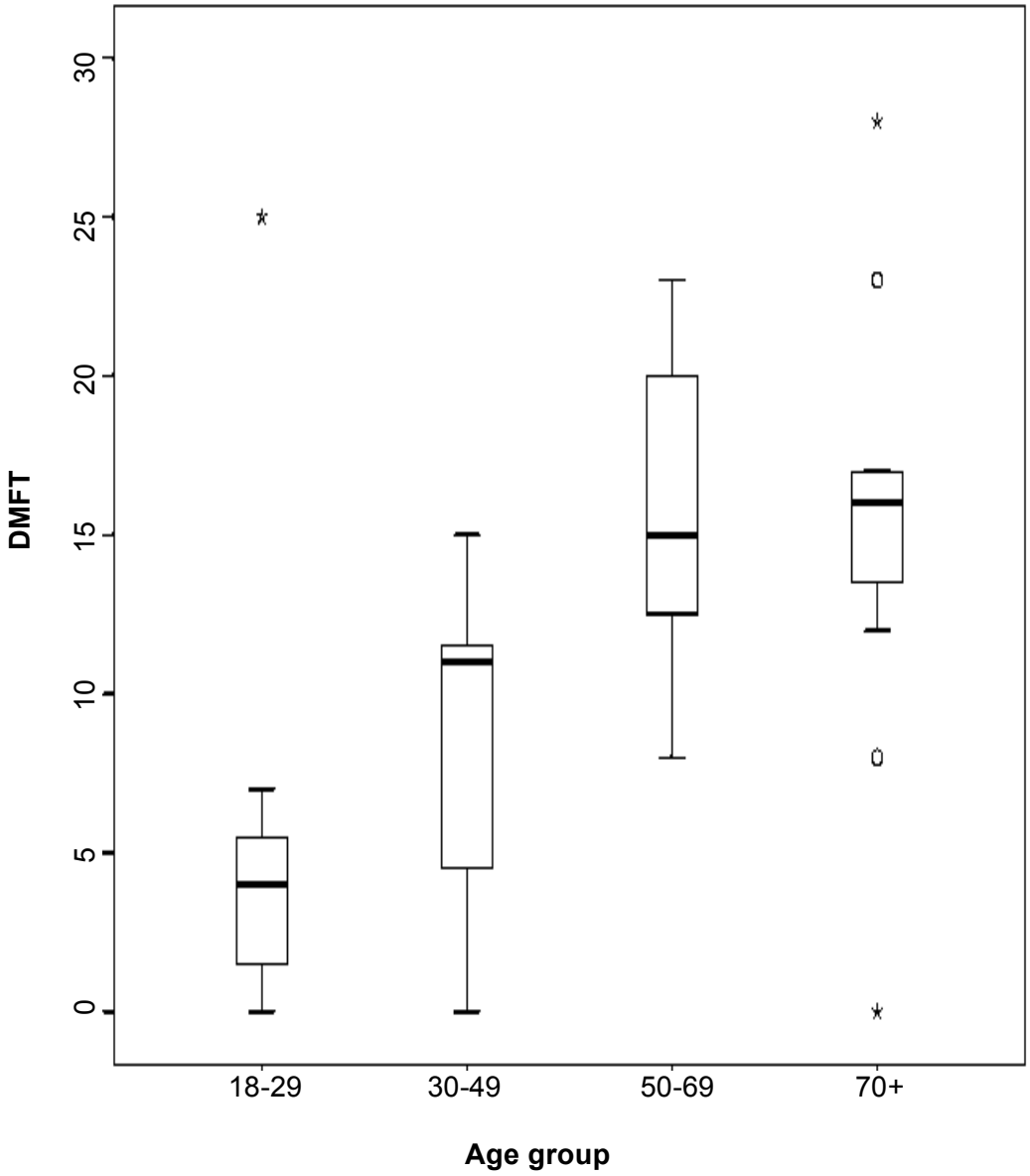

Figure 2.1 - Box and whisker plot summarising DMFT score per age group of recruited patients. The whisker bars indicate the minimum and maximum DMFT score for each age group. Data outliers are classed into two categories: 'far out' (o) and extreme values (*), 2 and 3 times outside the standard deviation around the mean respectively.

2.3.6 Molecular identification of isolates of *S. aureus* and *P. aeruginosa* from mechanically ventilated patients

In total, 177 isolates of *S. aureus* and *P. aeruginosa* were cultured from dental plaque, NBL, subglottic aspirations and ETTs. All isolates presumptively phenotypically identified as respiratory pathogens were subject to molecular confirmation by species-specific PCR. Using this approach the presumptive identity of >85% of isolates was confirmed using PCR (Table 2.7). PCR amplicons (Figure 2.2) showed that some isolates were of mixed species (multiple band presence).

Table 2.7 - Species-specific PCR for *S. aureus* and *P. aeruginosa*

Species	Total isolates	Total confirmed	% PCR Confirmed
S. aureus	114	97	85
P. aeruginosa	57	54	95

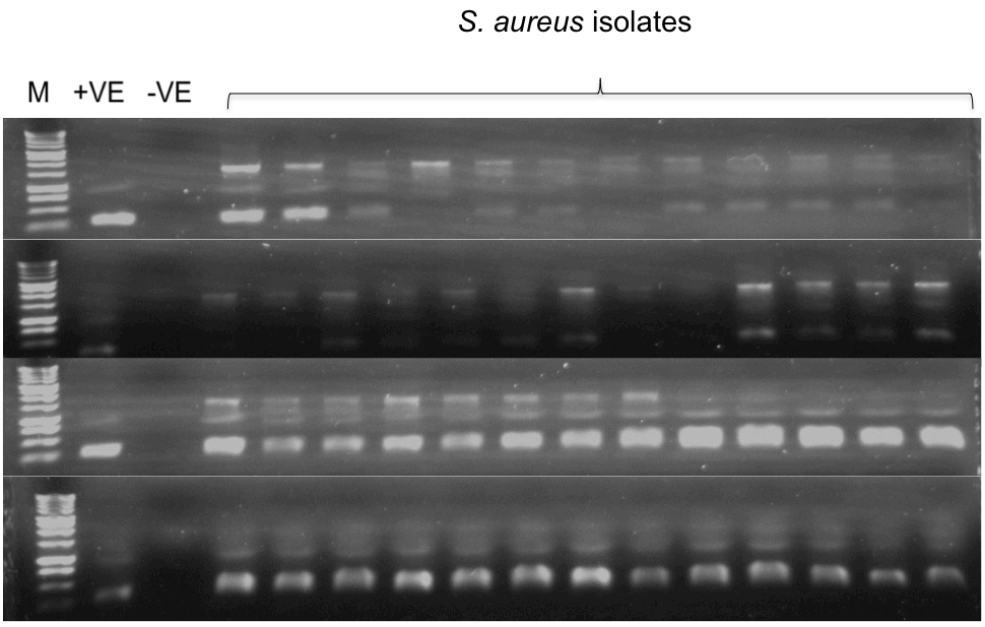

Figure 2.2 – Examples of species-specific PCR for the detection of *S. aureus* from dental plaque, non-directed bronchoalveolar lavage, subglottic aspirations, and endotracheal tubes. Individual PCR amplicons for *S. aureus* and *P. aeruginosa* are in appendix I[2].

[2] +VE – positive control –VE – negative control and M represents the molecular marker.

2.3.7 Summary of microbial culture of dental plaque:

Longitudinal microbial culture analysis results for the presence of selected microorganisms (*S. mutans* and *C. albicans, S. aureus and P. aeruginosa*) provided insight into the microbial content of dental plaque during MV. Figure 2.3 illustrates recovery of four target microorganisms from dental plaque over the course of MV (Target microbial culture for patients in Appendix I).

Co-culture of *C. albicans* and *S. mutans* from dental plaque occurred for 20 patients, whilst 14 patients harboured both *C. albicans* and *S. aureus* (Figure 2.4). For 17 patients, *P. aeruginosa* was isolated with *C. albicans* (Figure 2.4). Figure 2.5 is a typical micrograph of recovered dental plaque, highlighting microbial diversity (Image B) and live/dead proportions of cells (Figure 2.5).

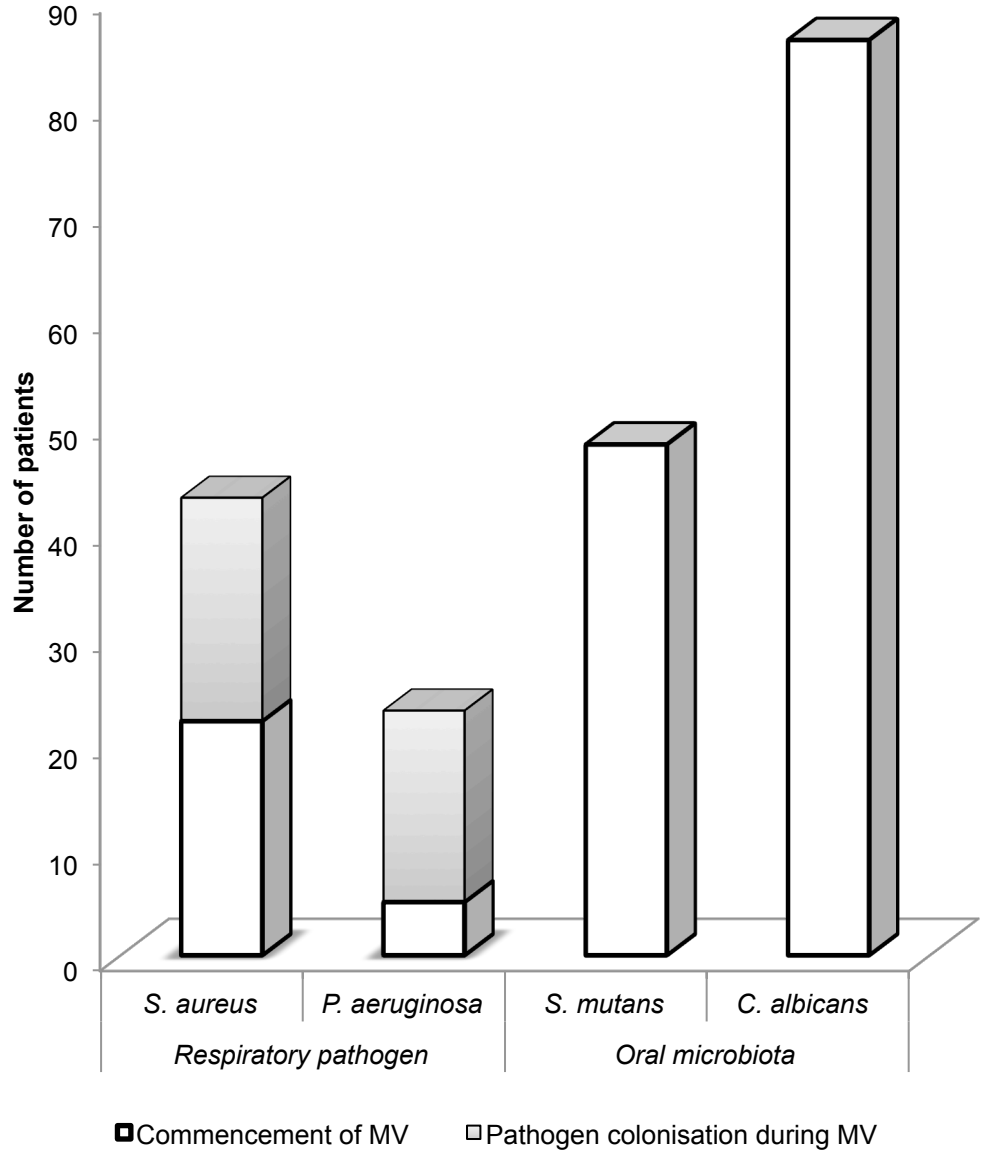

Figure 2.3 – Recovery of target microbial species at commencement and during MV. Shaded boxes represent the numbers of patients who became colonised with potential respiratory pathogens during the course of MV.

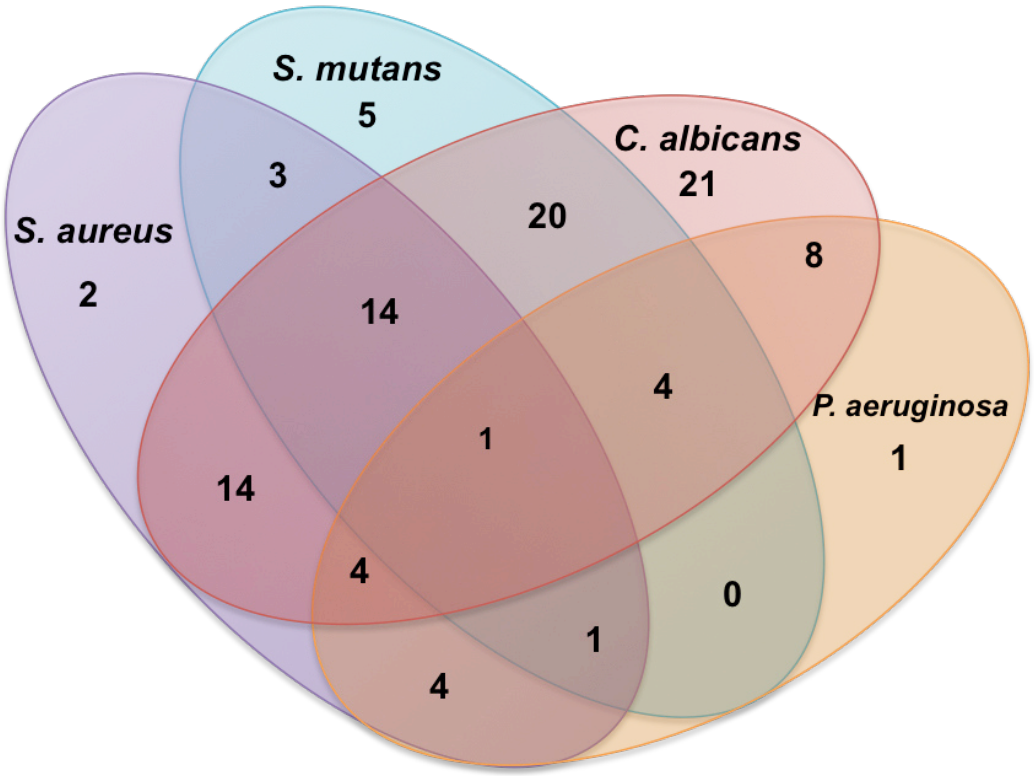

Figure 2.4 – A Venn diagram illustrating the co-isolation results of selected organisms within dental plaque during the entirety of the clinical study by microbial culture. There was a high incidence of co-colonisation between *C. albicans, S. mutans* and *S. aureus.*

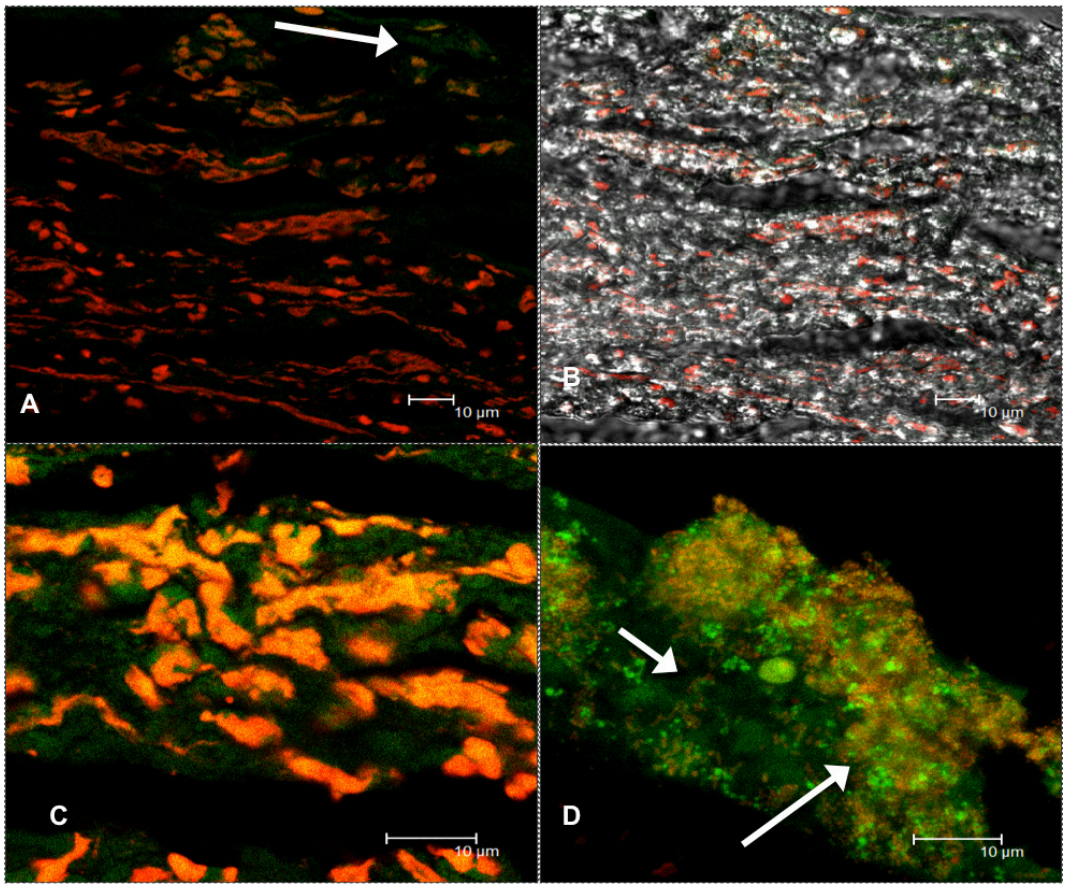

Figure 2.5 - Live/dead viability assay of dental plaque with the SYTO® 9 dye (live) and propidium iodide (red). A and B show an overlay of fluorescence (A) and light microscopy (B); C indicates that a large portion of the cells were dead (stained as red); D shows a magnified portion of dental plaque with intact live cells intact (stained green; arrowed).

2.3.8 Microbial culture of dental plaque during mechanical ventilation

A total of 848 dental plaque samples were collected from recruited patients during MV (n=592) and after ETT extubation (n=256). Dental plaque from 53 patients was culture positive for target respiratory pathogens (*S. aureus* and/or *P. aeruginosa*). Of those patients, 35 were culture negative for target respiratory pathogens at the time of ETT intubation.

Of 43 patients whose dental plaque was culture positive for *S. aureus* during MV, 22 were culture negative at time of intubation (Figure 2.3). Similarly, the dental plaque of 23 patients was culture positive for *P. aeruginosa* during MV, 18 patients (78%) culture negative at time of ETT insertion. These findings would be indicative of a microbial change in the dental plaque community to incorporate these targeted respiratory pathogens.

The dental plaque of 10 patients was culture positive for both *S. aureus* and *P. aeruginosa* during MV. *Candida albicans* and *S. mutans* were detected by culture in 86 and 48 patients, respectively during MV.

2.3.9 Prevalence of VAP and respiratory colonisation of dental plaque

Forty-one of the recruited patients were clinically diagnosed and treated for VAP during this study. The apparent high VAP rate could be related to this cohort of patients representing patients with prolonged ventilation (11 ventilated >5 d and 57 ventilated >7 d). Eighteen of these VAP patients demonstrated colonisation by either *S. aureus* and/or *P. aeruginosa* in their dental plaque during mechanical ventilation. An additional 9 VAP patients had been shown to harbour respiratory pathogens in their dental plaque from the beginning of intubation and during MV.

2.3.10 Association of dental plaque and lower airway microbiology

Figures 2.6 and 2.7 illustrate the detection of target respiratory pathogens in dental plaque, NBLs, subglottic aspirations and extubated-ETTs.

For *S. aureus* (Figure 2.6), 11 patients were culture positive for *S. aureus* only in their dental plaque during MV. Of 50 patients where *S. aureus* was cultured from any sample site, 7 patients did not have *S. aureus* in their

dental plaque. Analysis of dental plaque alongside NBL fluid showed that 43/52 patients had *S. aureus* in their dental plaque and/or the lower airways. A further 2 patients were colonised with *S. aureus* in the ETT biofilm only. However 17 patients were colonised with *S. aureus* in the ETT concurrently with the dental plaque.

Figure 2.7 shows the microbial isolation of *P. aeruginosa* at multiple sampling sites. *Pseudomonas aeruginosa* was detected in lower airways and subglottic secretions, however the majority of *P. aeruginosa* detection occurred only in the dental plaque (n=15). Ten patients were culture positive for *P. aeruginosa* within the ETT and the lower airways only (ETT n=5; NBL n=4; SUB n=1).

Clinical site	Number of patients
Dental plaque	43
Non-directed bronchoalveolar lavage	36
Subglottic aspiration	15
Endotracheal tube	21

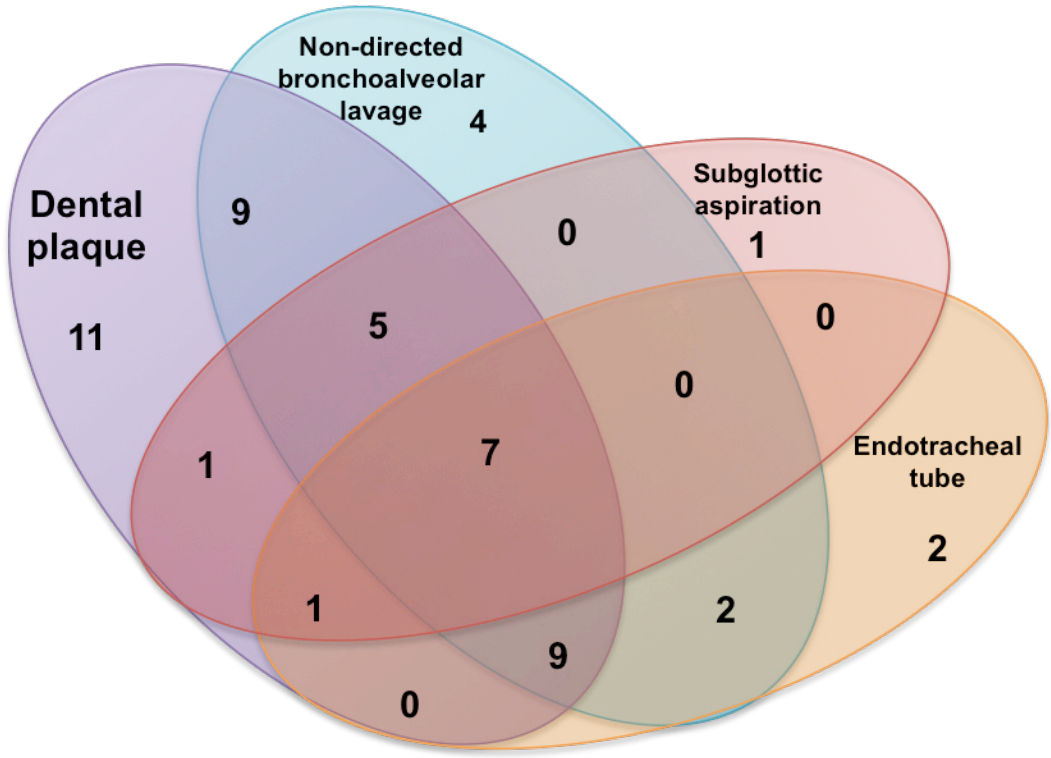

Figure 2.6 – Venn diagram representing the number of patients culture positive for *S. aureus* during MV from the 107 patients at different locations; within the dental plaque (DP – purple oval), non-directed bronchoalveolar lavage (NBL – blue oval), subglottic aspiration (SUB – red oval) and endotracheal tube (ETT – orange oval).

Sample type	Number of patients
Dental plaque	23
Non-directed bronchoalveolar lavage	12
Subglottic aspiration	4
Endotracheal tube	5

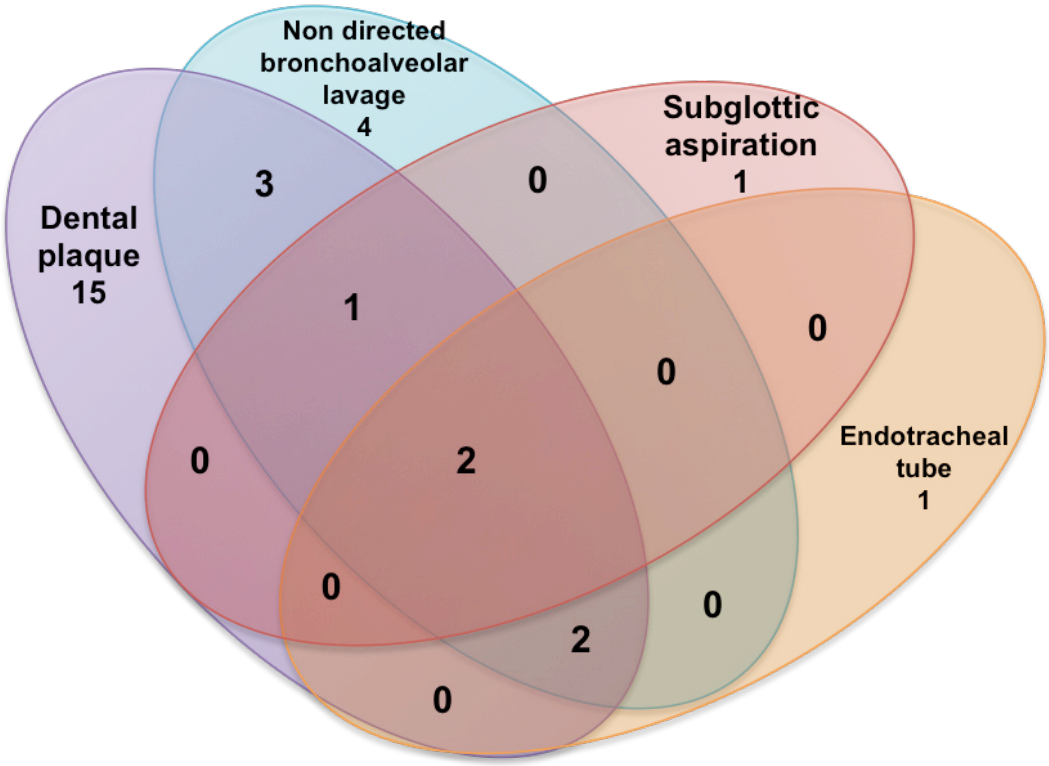

Figure 2.7 - Venn diagram representing the number of patients culture positive for *P. aeruginosa* during MV, from the 107 patients at different sites; within the dental plaque (DP – purple oval), non-directed bronchoalveolar lavage (NBL – blue oval), subglottic aspiration (SUB – red oval) and endotracheal tube (ETT – orange oval).

2.3.11 Quantitation of polymicrobial biofilm development on endotracheal tube biofilms

ETTs (n=110) were obtained from 97 mechanically ventilated patients. ETTs were not available for microbiology analysis for all patients, and some patients required >1 ETT insertion during MV. Table 2.8 summarises the findings of microbial culture and ETT biofilm communities. Respiratory pathogens were isolated from the ETTs of 21 patients, and microorganisms representing frequent colonisers of the dental plaque i.e. *S. mutans* and *C. albicans*) were recovered from 7 and 40 patients, respectively (Figure 2.8). For 7 patients, both oral microorganisms and respiratory pathogens were isolated from ETT biofilms. The ETTs from 44 patients were colonised by oral microorganisms. *Staphylococcus aureus* and *P. aeruginosa* were isolated from ETTs of 21 and 5 patients, respectively.

The number of microorganisms in ETT biofilms was quantified by culture (CFU/cm^2; Figure 2.9). Colonisation by *C. albicans* (Figure 2.9) was variable, with a minimum count of $3.08x10^3$ CFU/cm^2 and maximum of $2.45x10^7$ CFU/cm^2. Mean counts were similar for *S. aureus* ($1.04\ x10^6$ CFU/cm^2), *C. albicans* ($1.05\ x10^6$ CFU/cm^2) and *P. aeruginosa* ($9.33x10^5$ CFU/cm^2), with the lowest counts recorded for *S. mutans* ($1.61x10^5$ CFU/cm^2).

Table 2.8 – Microbial analysis of ETT biofilms (107 patients)

ETT culture	Number of patients
No ETTs collected	10
No microbial growth	29
Respiratory pathogens	21
Oral microorganisms	40
Oral & respiratory co-culture	7

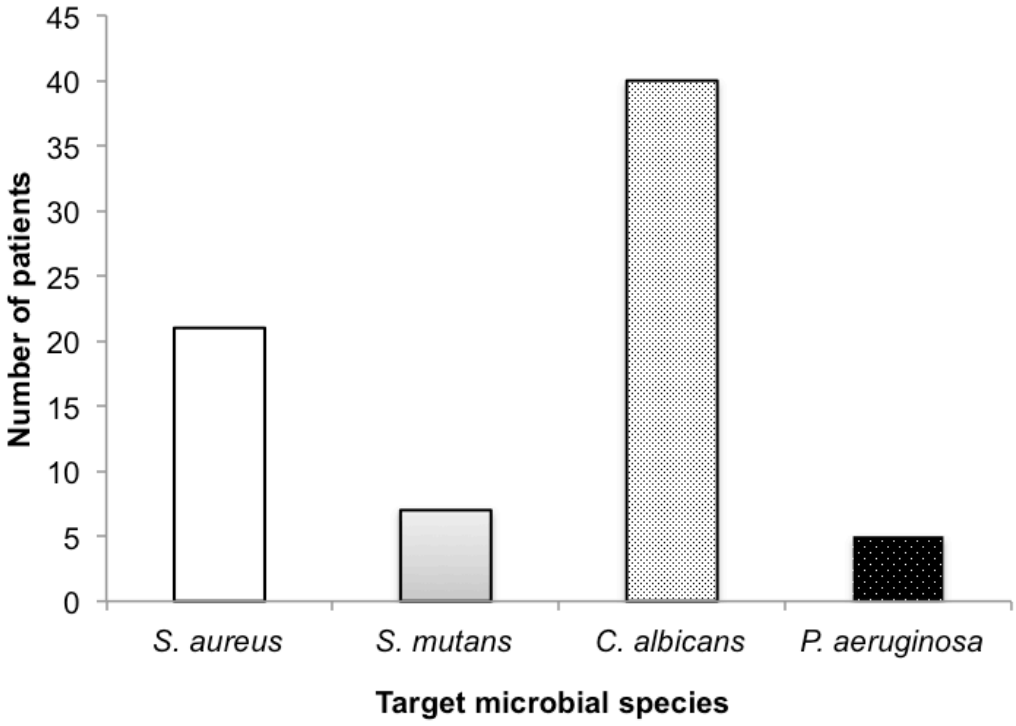

Figure 2.8 – Number of patients whose endotracheal tube (ETT) was colonised by targeted microbial species in mechanically ventilated patients. Oral species (*S. mutans* and *C. albicans*) and respiratory pathogens (*S. aureus* and *P. aeruginosa*) were found to colonise ETT biofilms.

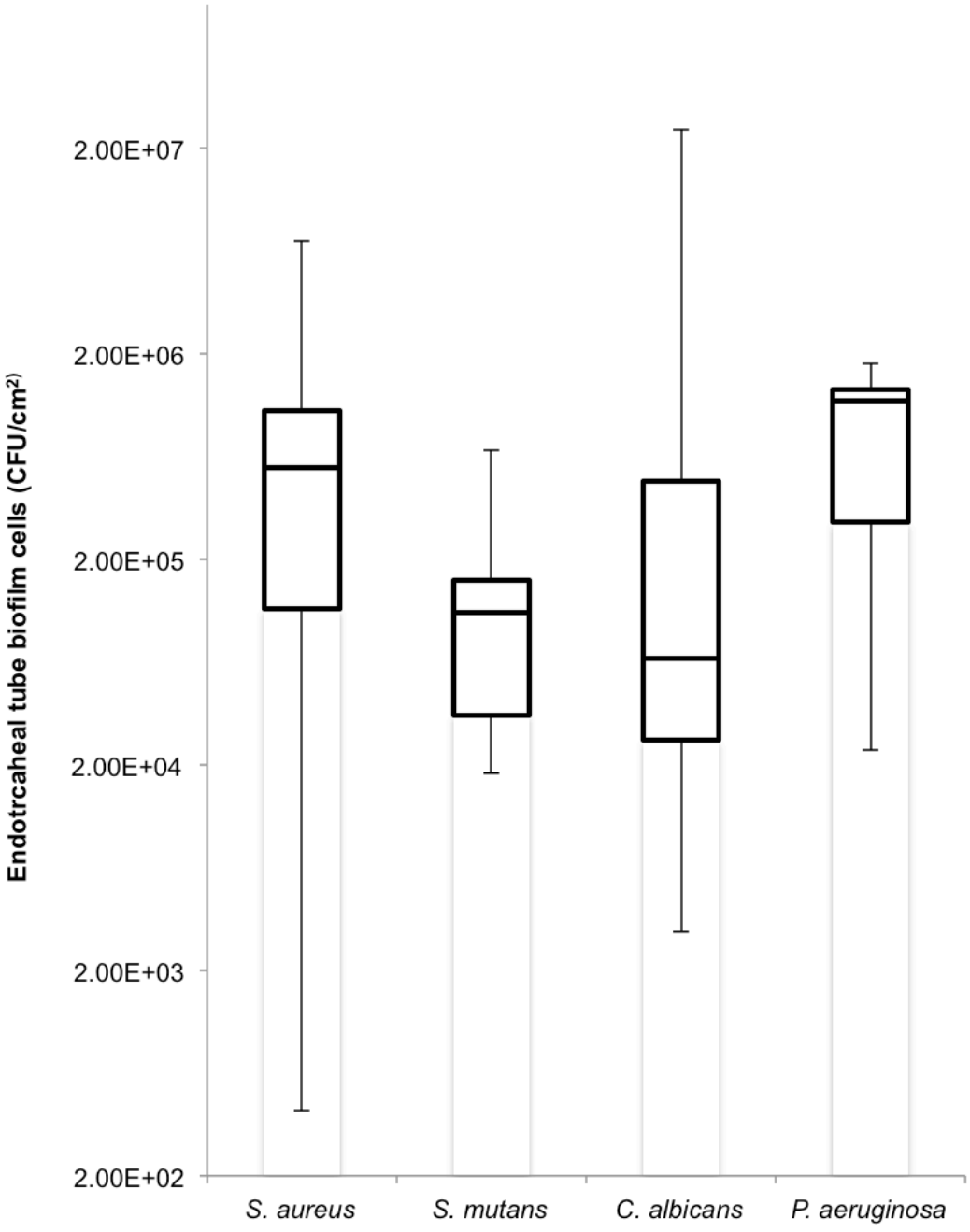

Figure 2.9 - A box and whisker plot for numbers of selected species per unit area (cm^2) of ETT colonisation. The box plot shows average ETT colonisation was greater for *P. aeruginosa* and *S. aureus,* with more variability in colonisation levels for these two respiratory pathogens**.**

2.3.12 Microscopy of ETT biofilms

2.3.12.1 Scanning electron microscopy

Extubated ETTs were subjected to microscopic analysis (Figure 2.10). ETTs were selected for biofilm analysis by microscopic imaging based on microbial culture results, whether oral-indicator organisms and/or respiratory pathogens were initially cultured (2.2.3). SEM imaging (Figures 2.10, 2.11 and 2.12) revealed mature microbial biofilms, greater than 200μm, present on ETTs from mechanically ventilated patients. In addition, EPS production and different cellular morphology was evident (Figure 2.11 and 2.12), emphasising polymicrobial biofilm formation. Biofilms were developed within ETTs after as little as 24 h through to ETTs being *in situ* for up to a period of 5-7 days (Figures 2.11 and 2.12).

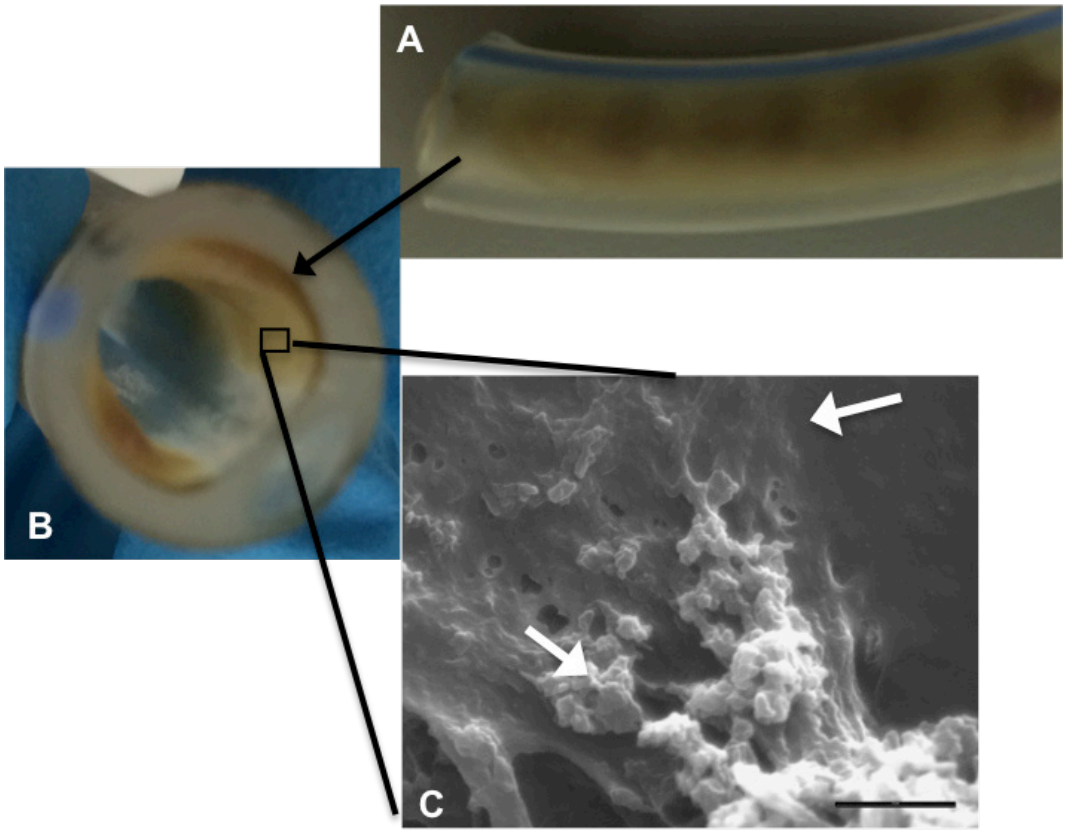

Figure 2.10 – A, B; macroscopic biofilms inside extubated endotracheal tube, C; scanning electron micrograph with distinct cellular morphology and the production of extracellular polymeric substances as indicated by the arrows.

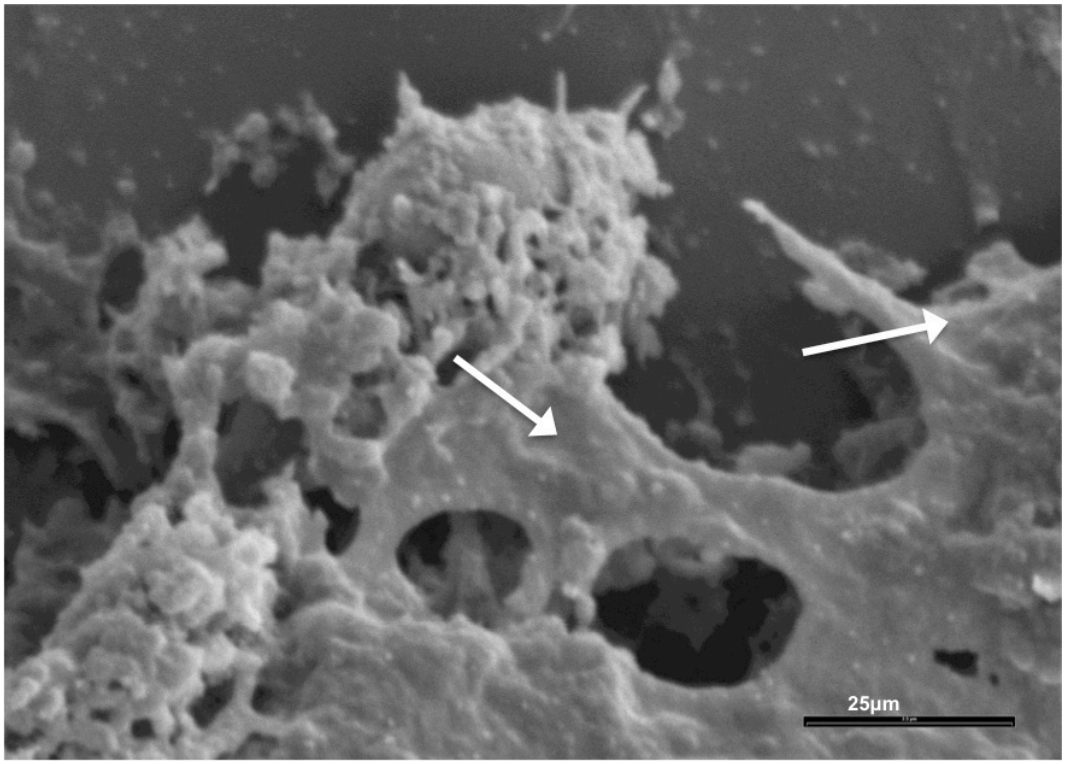

Figure 2.11 - Scanning electron micrographs of biofilms (indicated by arrows) formed on the inner lumen on endotracheal tubes from mechanically ventilated patients.

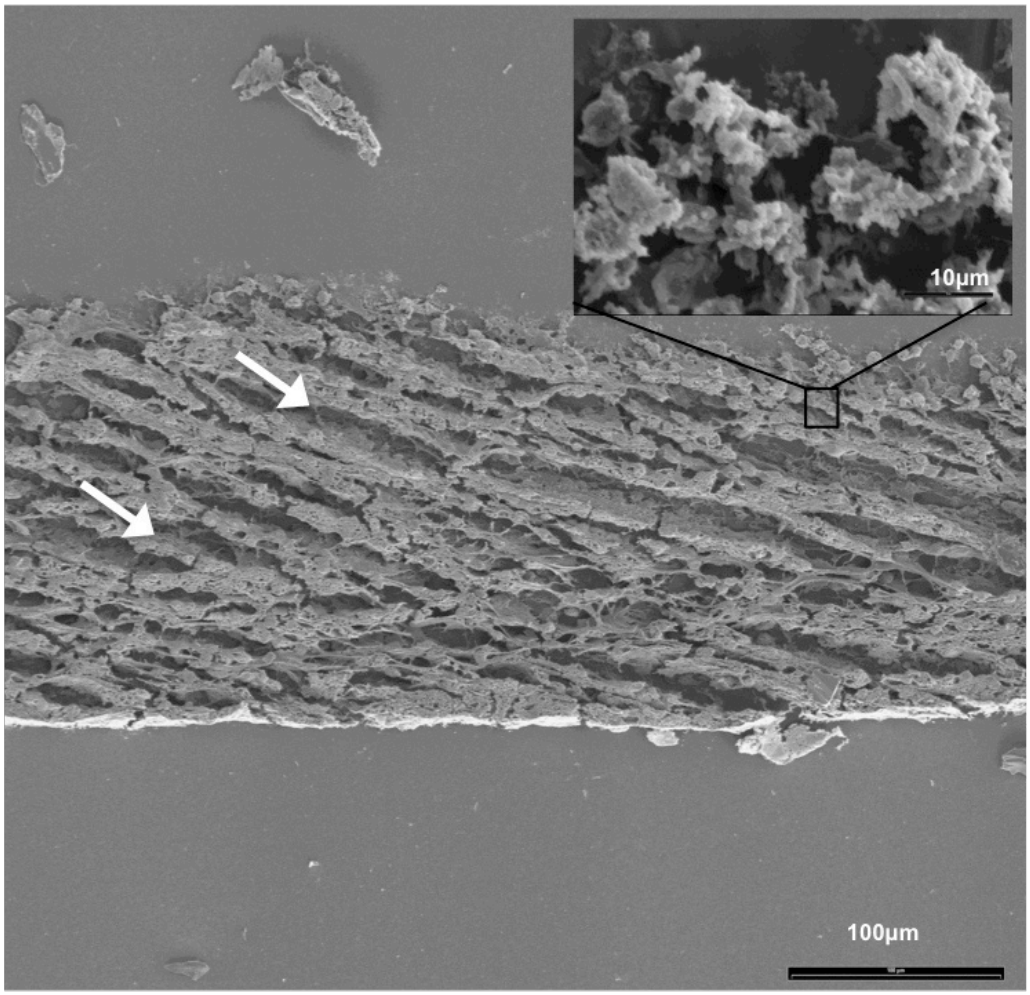

Figure 2.12 - SEM micrograph of a multi-layered and stratified biofilm (indicated by arrows) on the inner lumen of an endotracheal tube.

2.3.12.2 Peptide nucleic acid (PNA) probe fluorescent *in situ* hybridisation and confocal laser scanning microscopy (CLSM) of ETT biofilms

CLSM of ETT biofilms from 10 patients revealed aggregates of bacterial cells embedded within a complex EPS. The use of species-specific PNA probes allowed culture-independent detection of targeted microbial species in the ETT biofilm. Oral microorganisms and putative respiratory pathogens were detected in the ETT biofilm and different cellular morphologies and EPS were evident (Figure 2.13, A-D). Furthermore, various degrees of total bacterial (Universal, Cy-3) biofilm formation and aggregates of bacterial cells adjacent to the ETT lumen (Figure 2.14, image B) were apparent. PNA-FISH analysis of extubated ETTs indicated that these biofilms contained a diversity of bacterial morphologies and were therefore polymicrobial, further supporting the microbial culture results and building upon the SEM analysis initially undertaken.

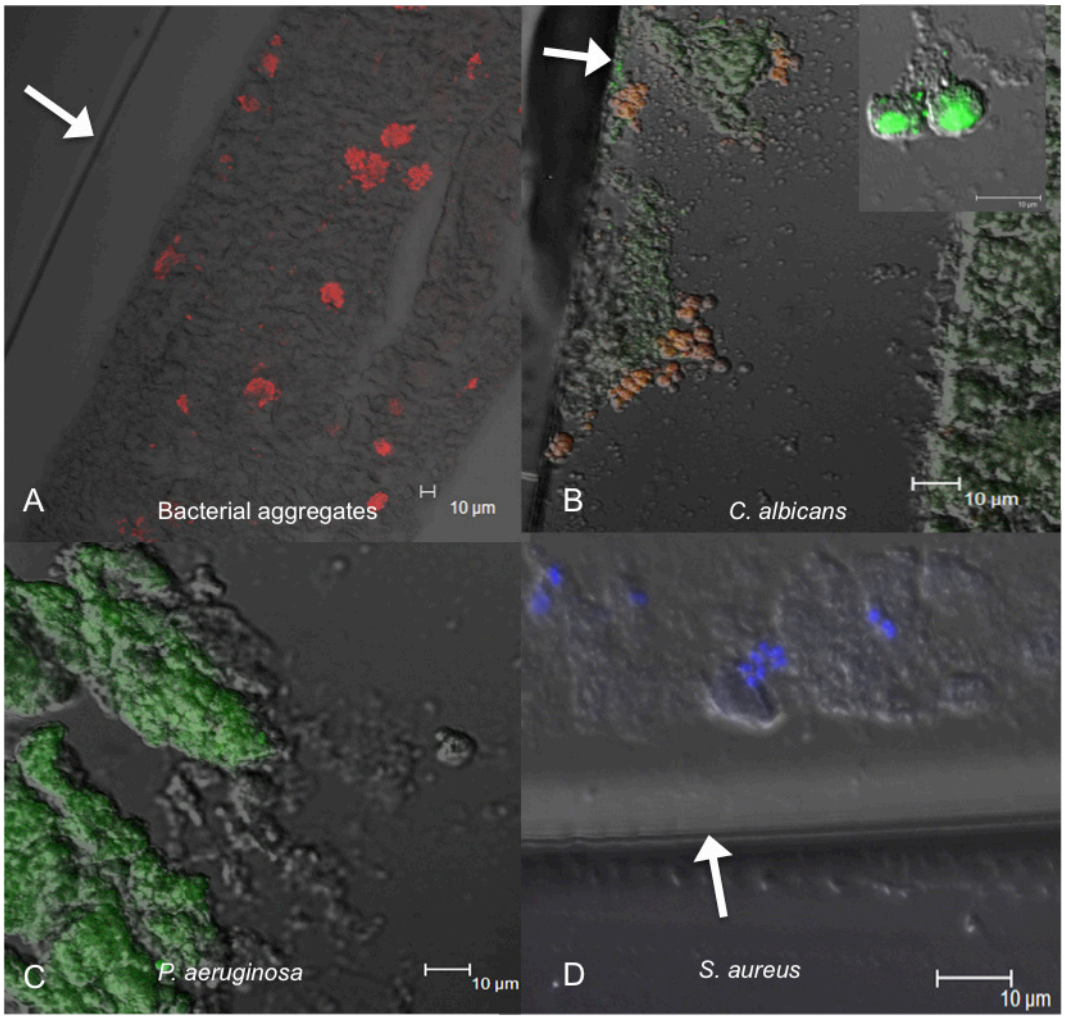

Figure 2.13 - Micrographs of endotracheal tube (ETT) biofilms obtained by confocal laser scanning microscopy (CLSM). Micrographs are overlays of the superimposed fluorescence on Nomarski differential interference (greyscale) images. A) Aggregates of bacteria hybridised with the universal bacterial Peptide Nucleic Acid (PNA) probe labelled with Cy-3 (red); B) Aggregates of *Candida albicans* hybridised with FITC conjugated PNA probe (green) and bacteria hybridised with the universal bacterial PNA probe labelled with Cy-3 (red); C) *Pseudomonas aeruginosa* hybridised with species-specific FITC labelled PNA probe (green); D) *Staphylococcus aureus* hybridised with species-specific PNA probe conjugated with Cy-5 (blue). Edge of the ETT section is arrowed.

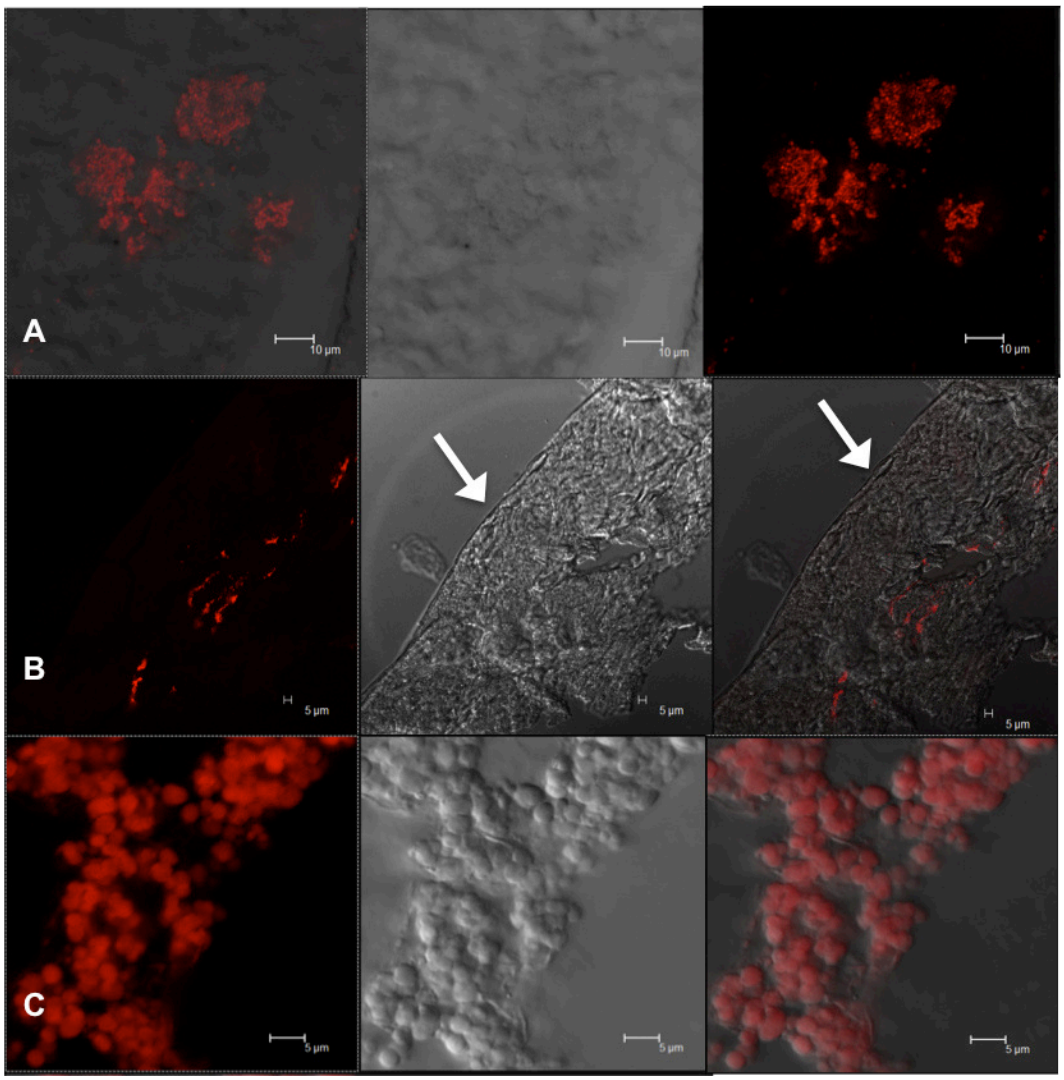

Figure 2.14 - Micrographs of endotracheal tube (ETT) biofilms obtained by confocal laser scanning microscopy (CLSM). A) Aggregates of bacteria within the microbial biofilm; B) aggregates of bacteria within a polymicrobial biofilm and; C) Clusters of cocci-shaped bacteria. Bacteria were hybridised with a universal bacterial Peptide Nucleic Acid (PNA) probe labelled with Cy-3. The edge of the ETT section is arrowed.

2.3.13 Antimicrobial susceptibility of *S. aureus* and *P. aeruginosa* detected from mechanically ventilated patients

All *S. aureus* and *P. aeruginosa* isolates confirmed by molecular techniques were subject to antimicrobial profiling. The majority (>70%) of isolates from all sites were susceptible to the antibiotics tested. Antimicrobial sensitivity patterns for recovered isolates of *P. aeruginosa* between dental plaque, the lower airways and endotracheal tube biofilms were largely similar for 7 out of 10 patients (isolates recovered at dental plaque, from NBL and ETT biofilms). Similarly, for recovered isolates of *S. aureus* between dental plaque, the lower airways and endotracheal tube biofilms, antimicrobial sensitivity patterns were related for 21 out of 30 patients (isolates recovered at dental plaque, from NBL and ETT biofilms).

Where differences occurred, *S. aureus* isolates with antibiotic resistance profiles were most frequently isolated from subglottic secretions and most frequently exhibited resistance to erythromycin, penicillin and cefepime. Of the 56 *P. aeruginosa* isolates, 35 were from dental plaque. Antimicrobial resistance patterns for all tested antibiotics ranged between 2% (ciprofloxacin) to 23% of isolates (meropenem). Whilst only 5 *P. aeruginosa* isolates were recovered from endotracheal tube sections, one of these had the most resistant profile across all antibiotics tested. *Pseudomonas aeruginosa* isolates were most sensitive to tobramycin, with 80% of ETT isolates and 97.1% of dental plaque isolates sensitive to all antimicrobials. A total of 3 patients were colonised by MDR *P. aeruginosa* and 21 with an MDR *S. aureus* (8 patients colonised with MRSA as determined by resistance to cefoxitin with a ZOI <20mm).

Table 2.9 - Antimicrobial susceptibility of 12 antibiotics for 114 *S. aureus* isolates[3].

	114 Isolates of *S. aureus*					
	Cefepime	Cefoxitin	Ceftazidime	Fusidic Acid	Gentamicin	Meropenem
Sensitive %	65	88	88	89	82	86
Intermediate %	20	0	2	4	13	6
Resistant %	15	12	11	7	4	8
	Ciprofloxacin	Clindamycin	Erythromycin	Penicillin	Tobramycin	Vancomycin
Sensitive %	86	61	62	55	79	88
Intermediate %	4	27	16	31	15	1
Resistant %	11	12	22	14	6	11

Table 2.10 - Antimicrobial susceptibility of 56 isolates of *P. aeruginosa* indicates high levels of sensitive isolates[4].

	56 Isolates of *P. aeruginosa*			
	Ceftazidime	Ciprofloxacin	Gentamicin	Tobramycin
Resistant %	8.8	1.8	1.8	1.8
Sensitive %	82.5	87.7	66.7	96.5
Intermediate %	8.8	10.5	31.6	1.8
	Meropenem	Piperacillin	Piperacillin-Tazobactam	
Resistant %	22.8	15.8	10.5	
Sensitive %	64.9	40.4	63.2	
Intermediate %	12.3	43.9	26.3	

[3] Individual isolate sensitivities in appendix I

[4] Individual isolate sensitivities in appendix I

2.3.14. Microbial culture of dental plaque after ETT extubation

Out of 848 dental plaque samples, 256 were obtained post-ETT extubation. Eighty-eight samples were collected within 1 week of ETT extubation, 66 were 2 weeks post-ETT extubation, 43 were 3 weeks post- ETT extubation and a further 59 were collected >1 month post ETT extubation. For 31 patients, it was not possible to follow up dental plaque collection and microbial analysis.

These analyses enabled assessment of persistence of respiratory pathogens in dental plaque after extubation. Of the 21 patients whose dental plaque became colonised by *S. aureus* during MV, *S. aureus* was not detectable by microbial culture in the post-ETT period for 15. For 18 patients who had *P. aeruginosa* colonisation of their dental plaque, 10 became culture negative for *P. aeruginosa* after extubation. Eight of the 35 mechanically ventilated patients exhibiting such microbial changes in total remained culture positive for respiratory pathogens post-ETT extubation.

Figure 2.15 compares the number of patients (pooled data) colonised with either *S. aureus* and/or *P. aeruginosa* at different times during MV and post-ETT extubation. As previously described, a microbial change occurred to colonisation by *S. aureus* and *P. aeruginosa* in 21 and 18 patients, respectively (as shown in the graph by the shaded section). This figure also shows the proportion of the patients in whom the change was reversed following removal of the ETT. Of the 21 patients that exhibited a microbial change to *S. aureus* dental plaque colonisation, 15 were no longer culture positive post ETT extubation.

In the case of *P. aeruginosa* dental plaque colonisation, this species appeared to be absent from dental plaque in 10 out of 18 patients post extubation. However, *P. aeruginosa* continued to be detected in 8 patients into the post ETT-extubation period. A higher proportion of patients whose dental plaque was colonised with *P. aeruginosa* retained colonisation in post-ETT extubation compared to those colonised by *S. aureus*. For the remaining 8 patients for whom an initial change in plaque microbiota during MV was documented, it was not possible to analyse dental plaque from the post-ETT

extubation period (due to patient death, removal from participation of the study, or because of a discharge to a different hospital).

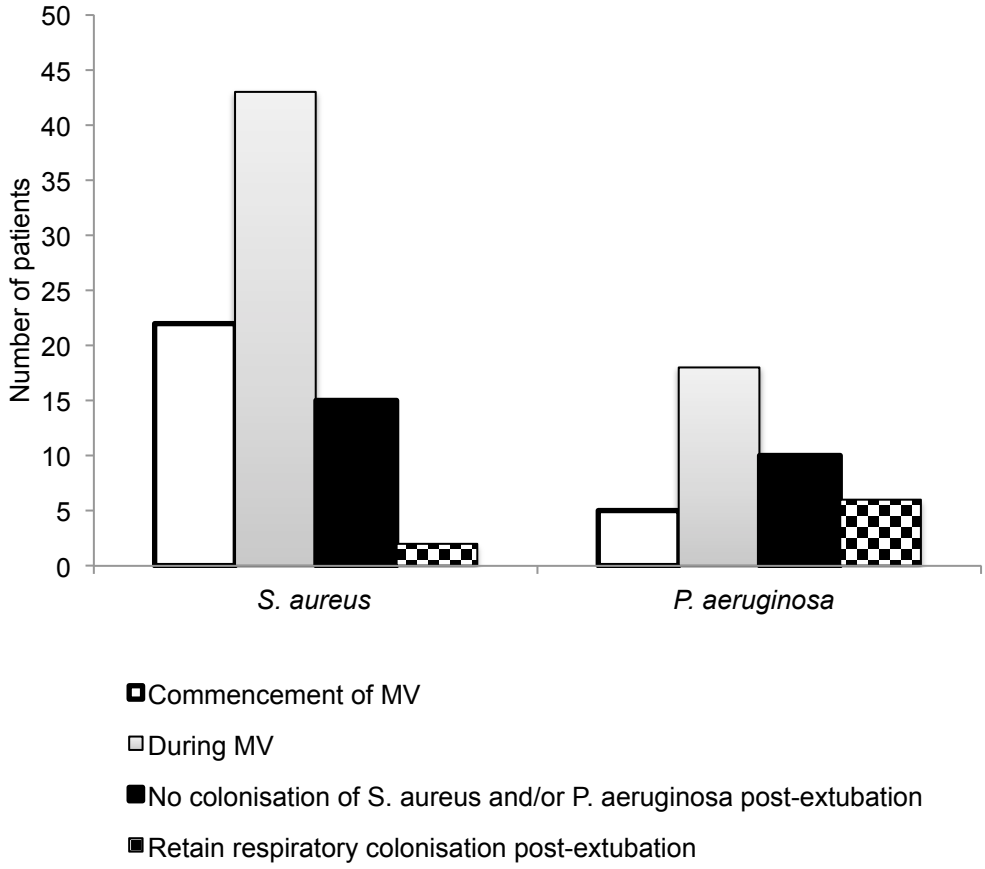

Figure 2.15 – Microbial colonisation of respiratory pathogens during endotracheal intubation and analysis up to 8 weeks post ETT- extubation.

2.4 Discussion

The microbiome of the oral cavity is both highly diverse and dynamic, primarily because of the wide range of microbial habitats that exist in the mouth and the fluctuations that can arise in these environments due to changes in diet, salivary flow and oral hygiene interventions (Dennesen et al., 2003; Wise et al., 2008; Marsh, 2010; Tada & Hanada, 2010; Wade; 2013b). Established dental plaque in healthy and stable oral environments will exhibit colonisation resistance. This is the inhibition of colonisation by new bacterial species (Wade, 2012). Synergistic and inter-relationships are formed in dental plaque communities serving to minimise the introduction of foreign microorganisms. It is however known that dental plaque community dynamics can be influenced by prolonged changes to the local environment that introduce new selective pressures that favour the presence of other species better suited to grow and persist under the new environment. Oral disease resulting from changes to the dental plaque composition is referred to as the ecological plaque hypothesis (Marsh, 1994; Marsh, 2003; Kolenbrander et al., 2010). An example of this is seen in plaque-mediated disease such as dental caries where a change in diet to one consisting of a high frequency intake of sucrose elevates the numbers of aciduric bacteria to the detriment of those species that are less tolerant of lower pH environments (Marsh, 1994). Potential pathogens, which may be present in low numbers in a healthy plaque composition, will only be able to outcompete other members of the oral microbiota following certain ecological pressures, for example a low pH or low saliva flow resulting in shifts in the microbial community (Marsh, 2006; Kolenbrander et al., 2010).

In the case of critically ill patients, there will be changes to the normal oral environment arising from several mechanisms. Firstly, mechanical ventilation (MV) via an endotracheal tube results in the mouth being partially held open, leading to dehydration of the oral environment. Secondly the presence of the endotracheal tube (ETT) physically impedes delivery of normal oral care, and

finally, local and systemic effects of any underlying illness or administered therapy may result in local and systemic changes affecting the oral cavity. The reason(s) dental plaque becomes colonised with respiratory pathogens is as yet unclear, but are likely linked to local environmental changes in the mouth. These may include plaque accumulation and gingival inflammation from inadequate delivery of oral care during MV, a reduced salivary flow as a consequence of drugs and reduced circulating saliva due to incomplete mouth closure (Wood et al., 2002; Jones et al., 2011). Furthermore, there may be patient-specific factors related to co-morbidities contributing to plaque changes, the cause of critical illness or treatment thereof.

The present study was undertaken to examine the colonisation of dental plaque by target species during and after MV. A total of 107 mechanically ventilated patients were examined over a 14-month period, generating a large quantity of data. Through targeting specific species (*S. mutans* and *C. albicans*) normally present in dental plaque, as well as species (*S. aureus and P. aeruginosa*) implicated in causing VAP, the study sought to confirm and expand upon previous research indicating compositional dental plaque changes during MV. Establishing the extent of respiratory pathogen colonisation of dental plaque could result in increased focus to maintain the oral biofilm during MV to directly limit the accumulation of dental plaque in critically ill patients and lower the numbers of respiratory pathogens within dental plaque, a risk factor for VAP. Ultimately, the purpose of targeted microbial culture analysis within mechanically ventilated patients was to assess the dynamics of the dental plaque community and by extension, the lower airways via the ETT, in the context of VAP pathogenesis. Targeted microbial culture also provided a collection of clinical isolates including respiratory pathogens, and organisms typically associated with the oral microbiota for *in vitro* biofilm dynamic studies (Chapter 5).

Previous studies have examined plaque community dynamics during critical illness, but often these studies have only been for comparatively short

periods of time during MV. A study by Sachdev et al., 2013, recruited 50 patients in a critical care unit for microbial enumeration and plaque analysis. Within their study, dental plaque was sampled at baseline, day 7 (±2 days) and day 14 (±2 days), revealing an increase in total viable dental plaque. In addition, 26% of patients exhibited colonisation by pneumonia-related pathogens including *P. aeruginosa, Klebsiella pneumoniae* and *Staphylococcus aureus*. The interaction between potential respiratory pathogens and the commensal dental plaque microbiota over a prolonged period of hospitalisation and recovery however has not previously been studied.

Patients were diagnosed and treated for VAP based on clinical presentation, X-ray changes and previously explained diagnostic criteria. In the treatment of these patients, antibiotics were frequently administered with changing clinical presentation. The VAP incidence rate for the studied patients was ~35% (n= 41) and this falls within previously reported ranges of 5-67% (Mori et al., 2006; Joseph et al., 2010; Sundar et al., 2012). Some recent studies have reported a lower average global incidence of VAP of 15% (Branch-Elliman et al., 2015; Kollef, 2015). Regardless of the true incidence, a diagnosis of VAP is significant for individual patients and the number of patients affected places considerable economic costs in healthcare systems. The most effective intervention would be the development of appropriate preventative strategies (Safdar et al., 2005; Al-Tawfiq & Abed, 2010; Heck, 2012).

During this study, mechanically ventilated patients presented with an age range between 18 and 86, and variable illness/requirement of critical care admission (Table 2.4), antibiotic administration (Table 2.5) and oral status as deemed by DMFT scores. DMFT scoring is usually performed as an indicator of dental caries (Becker et al., 2007), and dental scoring parameters such as DMFT scores, are not currently performed in ICU. A future management strategy administering tailored and specialised oral hygiene procedures by identifying ‘high risk’ patients (as indicated with large DMFT scores) could

minimise dental plaque accumulation and overgrowth of opportunistic oral organisms alongside potential respiratory pathogens. A total of 97 DMFT scores were performed within this clinical investigation. A Mean DMFT score of 12.54 for this patient cohort (Table 2.6) indicated that the oral hygiene status of these patients, was, in many causes previously poor, correlating with previous studies (Tanaka et al., 2009; Rai et al., 2015). A study by Rai et al., 2015 determined the DMFT scores of 300 hospitalised patients. A mean DMFT score of 6.18 was calculated, considerably lower than the present study. Within the study by Rai et al., 2015 however, 243 of the 300 (81%) patients were < 60 years old. Within the present study, a lower proportion of patients were < 60 years (63 out of 107 (59%)). Furthermore, a box and whisker plot evaluation (Figure 2.1) suggested that, in general, the higher the age of the patient, the higher the DMFT score. To assess localised changes in the oral cavity during MV, in particular the abundance of dental plaque and associated gingival inflammation, dental plaque scoring and gingival indexes could have been performed to further the information provided by the initial DMFT scores. Such additional information would have allowed correlation analysis between dental plaque abundance, gingival inflammation and the occurrence of respiratory pathogens in the dental plaque during MV.

A notable finding of this study was that the composition of dental plaque in a significant proportion (n=35) of mechanically ventilated patients altered with inclusion of the respiratory pathogens *S. aureus* and/or *P. aeruginosa* (Figure 2.3). Of those patients harbouring *S. aureus* and/or *P. aeruginosa* within their dental plaque during MV, 27 were clinically diagnosed with VAP and treated accordingly.

Staphylococcus aureus and *P. aeruginosa* are frequent causative pathogens of VAP often exhibiting resistance to antibiotics, *e.g.* MRSA and mucoidal *P. aeruginosa,* (Hunter, 2006; Parker et al., 2008; Bouza et al., 2012). A study by Parker et al., 2008 investigated the prevalence of *P. aeruginosa* amongst other multi-drug resistant bacterial pathogens using microbial culture techniques in 739 patients with suspected VAP. The prevalence of

Pseudomonas species was 13.4% (99/739), with 9.2% of patients (68/739) colonised with other MDR pathogens. A further study by Bouza et al., 2012 evaluated the bacterial causes of 474 cases of VAP, with 148 (31.2%) of cases caused by *P. aeruginosa* and a further 111 (23.4%) caused by MRSA (with an additional 75 cases (15.8%) caused by MSSA).

Within the present study, although only a small proportion of *S. aureus* isolates were identified as MRSA (n=8), ~25% isolates recovered from dental plaque were resistant to at least one antibiotic tested *in vitro* (Tables 2.9 and 2.10). Where differences occurred within isolate sensitivities between the dental plaque and lower airways, resistance rates were highest for *P. aeruginosa* derived from the ETT biofilm and for *S. aureus* within subglottic secretions (Table 2.10). This could imply higher rates of resistance within the airways, and if VAP were to develop in these patients then this could diminish the success of antimicrobial therapy.

Importantly, for the majority of patients where microbial changes occurred in the dental plaque, a reversal occurred once the patient was extubated and recovering from critical illness, and this was most readily evident for *S. aureus* colonisation (Figure 2.15). A higher proportion of patients colonised with *P. aeruginosa* retained the respiratory pathogen colonisation post extubation. Further investigation of bacterial strain typing and further genomic analysis of biofilm-related genes would elaborate on whether patients' who remain colonised in the post-ETT extubation period harbor the same strain. Although most dental plaque communities reverted back to a phenotype without respiratory pathogens within one week of extubation, the fact that some patients remained colonised with respiratory pathogens over a prolonged duration could represent a patient group at risk of subsequent hospital-acquired pneumonia. Dental plaque colonisation is dynamic, and during this study a reduction in respiratory colonisation occurred post-ETT extubation. With the extubation of the ETT, normal host defence mechanisms (within saliva, swallowing reflex) are re-introduced, and such defenses may facilitate further changes to the dental plaque composition.

Staphylococcus aureus colonises the nose and skin of up to 30% and 20% of healthy individuals respectively. Given the anatomical proximity of the nasal area and the oropharangeal, it is not surprising that *S. aureus* colonises the oral cavity. Although not regarded as a normal inhabitant of the oral cavity, *S. aureus* has been detected within the dental plaque of debilitated or elderly individuals (Smith et al., 2001). Furthermore, *S. aureus* has been isolated in the dental plaque community of healthy people in up to 24-36% of individuals (Heo et al., 2013). In the current study, the high incidence (43 of 107 patients) of *S. aureus* in dental plaque was nevertheless surprising, particularly as half of these patients did not have *S. aureus* in their dental plaque at the time of intubation. Similarly, dental plaque also became colonised with *P. aeruginosa* during ventilation, albeit at a lower incidence.

The current study also targeted two microbial species frequently associated with the normal oral microbiota, namely *Streptococcus mutans* and *Candida albicans*. These oral indicator species allowed tracking of oral microbial dissemination following intubation. During this study, polymicrobial colonisation of the ETT involving both oral indicator species and respiratory pathogens highlighted a link between the oral cavity and the ETT, and also the role of the ETT as source of infectious agents. Importantly, oral-indicator species were isolated from the dental plaque of 90% patients and were further detected in ETT biofilms (n=40 for *C. albicans* and n=7 for *S. mutans)*. In accordance with the present study, ETT colonisation by both respiratory pathogens and frequent members of the oral microbiota has been previously reported. Cairns et al., 2011 revealed the detection of *S. mutans* in 5 out of 20 patients' ETT biofilms, with 8 out of 20 patients' ETT biofilms being colonised with *S. aureus* and/or *P. aeruginosa.*

Subglottic secretions pool around the ETT, and these may leak below the cuff through folds and micro-channels that form in the cuff material (Hamilton & Grap, 2012). Continuity between the lower airways and the oral cavity is emphasised by the colonisation of oral microorganisms such as *S. mutans* and *C. albicans* within ETT biofilms, (and NBL fluid), shown through both

SEM (Figures 2.0 - 2.12) and CLSM imaging coupled with species-specific PNA probe hybridisation (Figures 2.13 and 2.14). Aggregates of respiratory pathogens were clearly evident in the ETT biofilm using CLSM and dissemination of these to the lower airway would deliver infectious agents already existing within a biofilm phenotype and therefore potentially more resistant to host defence mechanisms and administered antimicrobials.

To further analyse the microbial relationships between the dental plaque and lower airways (including ETT biofilms) recovered isolates of target respiratory pathogens (*S. aureus* and *P. aeruginosa*), and oral-indicator species (*C. albicans* and *S. mutans*) could be genotyped to reveal the extent of isolate matching between the dental plaque and the lower airways, and the amount of translocation occurring between these clinical sites. Such strain typing would build upon previous studies, namely an investigation by Heo et al., 2008 (as outlined in 2.1.2).

It is important to recognise that other possible respiratory pathogens were not tested for in this study (due to time, cost and the ability to assess all microbial species using tailored culture techniques), others have found that *Escherichia coli, Klebsiella* species and *Acinetobacter* species may also colonise dental plaque and ETT biofilms during MV (Gil-Perotin et al., 2012). A review by Kalanuria et al., 2014, lists the ten most frequent VAP pathogens. Within the review, *P. aeruginosa* and *S. aureus* were responsible for ~45% of VAP cases. Other VAP causative pathogens include Enterobacteriaceae (*E. coli, Klebsiella* and *Proteus* species), *Streptococcus* species, and *Acinetobacter* species. In acknowledgment of the limitations of microbial culture, community profiling of dental plaque (Chapter 3) was performed to characterise the microbiome during the course of MV.

Conclusion

In accordance with the aims of this chapter, the occurrence of respiratory pathogens within both the dental plaque and the lower airways were revealed

during this longitudinal study. Irrespective of patient demographics including age and current oral health (as indicated by the DMFT scores), microbial changes within the dental plaque composition were observed. Interestingly, respiratory pathogens were also isolated from mechanically ventilated patients in the recovery phase of critical illness following removal of the ETT. In relation to the third aim, microscopic imaging of ETT biofilms further revealed the colonisation of respiratory pathogens and oral-indicator species including *S mutans* and *C. albicans*. Furthermore, such longitudinal and target microbial culture not only revealed that extensive changes occurred within the dental plaque composition during MV, there are also polymicrobial and pathogenic biofilms within the ETTs.

Chapter 3

Molecular community profiling of oral biofilms within mechanically ventilated patients; a longitudinal study

3.1 Introduction

Mapping the oral microbiota has been the subject of numerous molecular studies over the past decade (Wade, 2013a). Since the introduction of Sanger sequencing, technological advances has allowed in-depth analysis of both whole microbial communities and of individual species genomes (Lazarevic et al., 2009; Perkins et al., 2010). The oral cavity is colonised by an array of microorganisms including bacteria, fungi and viruses and currently over 1000 bacterial species have been detected using culture-independent approaches (1.1) (Avila et al., 2009; Lazarevic et al., 2009; Dewhirst et al., 2010; Zaura, 2012; Wade, 2013a; Wang et al., 2013; Palmer, 2014). The use of contemporary DNA based technologies facilitates identification of uncultured, dormant and dying cells that are unable to replicate even on the most tailored media and as yet uncultured microorganisms whose culture requirements have yet to be determined and reproduced.

Furthermore, the diversity of microbial species within the oral cavity of healthy individuals is increasingly well characterised. The microbiome of a range of oral niches has been analysed, including the mucosal membranes, the cheeks and tongue and scrapings of dental plaque (Dewhirst et al., 2010; Liu et al., 2012). Although there are many shared species, different sites within the oral cavity have a characteristic and often unique microbial composition, relating to the different biological and physical properties of each site (Dewhirst et al., 2010; Xu et al., 2015).

There is a large surface area provided by teeth for colonisation and maturation of plaque. Areas with lowered oxygen potentials such as the interproximal spaces of teeth and in deep periodontal pockets are frequently colonised by large numbers of predominantly anaerobic species (Jefferson, 2004; Faran & Tanwir, 2012). The difference in the microbial composition at

different sites emphasises the adaptability of the microbiome to specific surroundings.

There is a natural progression in dental plaque development to maturation, with initial colonisation by 'pioneer' bacteria including *Streptococcus* and *Lactobacillus* species, and subsequently combinations of anaerobic species such as *Prevotella, Veillonella* and *Porphyromonas* are detected (Peyyala & Ebersole, 2013; Kolenbrander et al., 2005; Xie et al., 2010). Several species, including those of *Streptococcus* and *Prevotella* have been identified within the polymicrobial biofilm of dental plaque using next-generation sequencing techniques and are associated with oral diseases including dental caries and periodontitis, and are increasingly linked with systemic infections, including respiratory infections (Paju & Scannapieco, 2007; Preshaw et al., 2012).

The majority of critically ill patients require mechanical ventilation (MV) to treatment survival. An endotracheal tube provides the interface between the patient and the ventilator and following insertion of the ETT (intubation) alterations can occur in the oral microenvironment and oral microbiome. These alterations have been hypothesised to facilitate the colonisation and proliferation of both respiratory and other potentially exploitative pathogens in oral and pulmonary niches (Scannapieco et al., 1992; Perkins et al., 2010; Zuanazzi et al., 2010; Berry et al., 2011). The mechanisms underlying this 'microbial shift' are not clear, but may, in part, be due to the physical presence of the ETT, which indirectly affects plaque clearance (difficulties in oral hygiene interventions, saliva flow and mucosal drying), in addition to the interventions and medications related to the management of the underlying condition during critical illness.

Given the limitations of traditional microbiological culture, the aim of this current study was to use molecular community profiling to comprehensively analyse, on a longitudinal basis, the microbiome of dental plaque and the lower airways during MV. The use of high-throughput sequencing platforms

in this way allows the investigation of a microbiome without placing specific focus or bias towards certain genera or pathogen. It also provides a more representative profile of the community. Specifically the aims were on a longitudinal basis to:

(1). Analysis of dental plaque derived sequences was performed to assess three principle criteria:

1. Occurrence of potential respiratory pathogens during MV
2. Assessment of the most dominant species during MV
3. Microbiome analysis at four time points (covering dental plaque collection at baseline, during MV and the post-ETT extubation recovery period).

(2). The second aim of this chapter was to compare dental plaque derived sequences during MV to those of microbial sequences extracted from the lower airway fluids and ETT biofilms.

3.2 Materials and Methods

3.2.1 Samples for bacterial community profiling

DNA extraction was performed on a total of 48 samples from 13 mechanically ventilated patients for community profiling at Research and Testing Laboratory, LLC (Lubbock, Texas, USA). Dental plaque, ETTs and lower airways fluids were selected based on initial microbial culture results from Chapter 2, and available patient information including critical condition, antibiotic administration and patient demographics such as age and gender.

3.2.2 Total bacterial DNA extraction for MiSeq Illumina Sequencing

3.2.2.1 DNA extraction from dental plaque

Total bacterial DNA extraction was performed as described in 2.2.3.4. All incubation steps were extended to 1 h to maximise DNA elution. A 150-µl volume of re-suspended dental plaque was transferred into a sterile microcentrifuge tube for total microbial DNA extraction using an adapted Gram-positive protocol as described in 2.2.3.4. Once the lysis suspension was added and the pellet re-suspended, the sample was transferred to a lysis tube (Qiagen) for a 1 min bead-beating step. After centrifugation for 1 min at 16,000 × g, the supernatant was transferred to a 1.5 ml centrifuge tube and the protocol continued from the RNase A solution step, as previously described in 2.2.3.4.

3.2.2.2 DNA extraction from NBLs and subglottic aspiration

A 150-µl volume of NBL/subglottic aspiration fluid was transferred into a sterile microcentrifuge tube. An equal volume of Sputasol™ was added to the tube, gently inverted and vortex mixed for 5 s. The DNA extraction procedure was then followed (2.2.3.4, 3.2.2.1).

3.2.2.3 DNA extraction from endotracheal tube biofilms

The microbial biofilm was scraped from the lumen of the ETT using a cotton swab and suspended into 1 ml of Sputasol™ (Fisher). The tubes were vortex mixed for 20 s to disrupt the biofilm. DNA extraction procedure was followed as described above (3.2.2.2).

3.2.2.4 Bacterial 16S rRNA PCR and gel electrophoresis

To confirm the presence of DNA and to detect the amplicons before preparation for Miseq Illumina sequencing, 16S rRNA was amplified using universal primers. The primers used were: 1492r (CACGGATCCTACGGGTACCTTGTTACGACTT) and 27f (GTGCTGCAGAGAGTTTGATCCTGGCTCAG) (Eurofins MWG Operon) (Zuanazzi et al., 2010; Dalwai et al., 2007). The total volume of PCR reagents was 50 µl (25 µl PCR master mix, 2 µl DNA template, 0.5 µl reverse primer, 0.5 µl forward primer, and 22 µl of nuclease water). PCR thermal cycling parameters consisted of 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). A final single cycle extension step of 72°C for 15 min was also included. Gel electrophoresis (1.0% agarose gel) was performed at 70/Vcm^2 for 50 min, in a 0.5 × Tris Borate EDTA (TBE) buffer with 10% SafeView™ for detection of nucleic acids (NBS Biologicals NBS-SV1) alongside a 1 Kbp molecular weight ladder (Promega). A gel doc system was used to determine DNA presence pre-sequencing (Gel-DocIT_UVP; Biorad).

3.2.3 Preparation and transportation of DNA extracts for MiSeq Illumina sequencing

Amplicons were stabilised via the addition of a DNA elution reagent (Qiagen), and tubes were individually wrapped in Parafilm M™ Wrapping film (Fisher) to minimise evaporation and pressure changes.

3.2.4 MiSeq Sequencing: Illumina platform

MiSeq sequencing was performed at Research and Testing Laboratory, LLC (Lubbock, Texas, USA). Bacterial universal primers (28F; GAGTTTGATCNTGGCTCAG and 388R; TGCTGCCTCCCGTAGGAGT) were used to generate multiple sequences of 250 bp overlapping at the V4 region of the 16S rRNA gene. DNA extracts were randomly fragmented and

DNA adaptors (ligates) were added to both fragment ends. The DNA mixture was injected into the glass flow-cell channel containing multiple attached primers. The single stranded fragments were bound randomly to the surface and nucleotides and polymerases were passed through the flow cell creating double stranded bridge amplifications. The dsDNA were denatured leaving anchored single strands and several million clusters of DNA in each flow cell cycle. Four fluorescently labeled dNTPs, primers and DNA polymerase were added to determine accurate pair base using the principle of laser excitation. This cycle was repeated until 250 bp sequences were generated. The sequencing by synthesis process is summarised in figure 3.1.

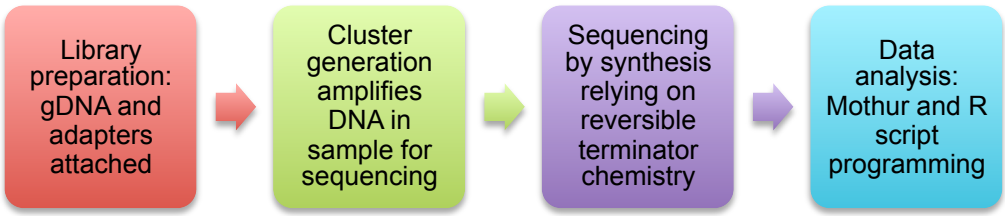

Figure 3.1 - Overview of the MiSeq Illumina DNA sequencing platform.

3.2.5 Phylogenetic identification

Data analysis from the raw sequences generated from the MiSeq Illumina platform was performed within Mothur (Schloss et al., 2009) to check the quality, pre-process, align and join the sequences to obtain a total number of sequences in each sample. Sequences were scanned for errors by a series of error command checks. Mothur was further utilised to cluster the data according to operational taxonomic units (OTUs), to a species level of 97% similarity (sequence data <97% confirmation were not identified in this study). Singletons and any OTUs that were not found more than 10 times in any

sample were collated into OTU_singletons and OTU_rare phylotypes respectively to maintain normalisation and to minimise artifacts.

3.2.6 Data analysis

Phylogenetic data from Mothur was analysed using a combination of statistical programs R-script (R Development Core Team, 2008), STAMP (Parks & Beiko, 2010), SPSS V20 and Microsoft Excel (1.1.3). The two methods used in this study to compare the similarity between each sample were Jaccard's index of similarity and the Bray-Curtis distance measure (measure of dissimilarity) (Lozupone & Knight, 2005; Wang et al., 2013). Weighted Unifrac distance matrices were analysed in R using non-metric multidimensional scaling ordination and the shared OTU file was used to determine the number of times that an OTU was observed in multiple samples and for multivariate analysis in R. OTU taxonomies (from Phylum to Genus) were determined using the RDP Multi Classifier script to generate the RDP taxonomy (Wang, et al., 2007), while species level taxonomies of the OTUs were determined using the USEARCH algorithm combined with the cultured representatives from the RDP database (Edgar, 2010). Using the Vegan package alpha and beta indices were calculated from these datasets with Mothur and R.

3.3 Results

3.3.1 Sequencing data details

Before the extracted DNA was stabilised for transport to the sequencing facility, a 2-µl aliquot was used as template for a 16S ribosomal RNA (16SrRNA) PCR reaction to confirm the presence of amplifiable DNA, and typical amplicons are shown in figure 3.2. A total of 48 samples were successfully sequenced using the MiSeq Illumina platform (one DNA sample was removed from data analysis post-sequencing due to low sequence reads producing low quality data, a total of 30 reads, compared to a mean average of >1000).

A total of 38 samples of dental plaque (DP), 4 non-directed bronchoalveolar lavage (NBL), 3 subglottic aspiration (SUB) and 3 endotracheal tubes (ETTs) were analysed by community profiling, from a total of 13 mechanically ventilated patients. A 97% or higher similarity was used as the criterion for identification of microorganisms (Mothur output in Appendix II).

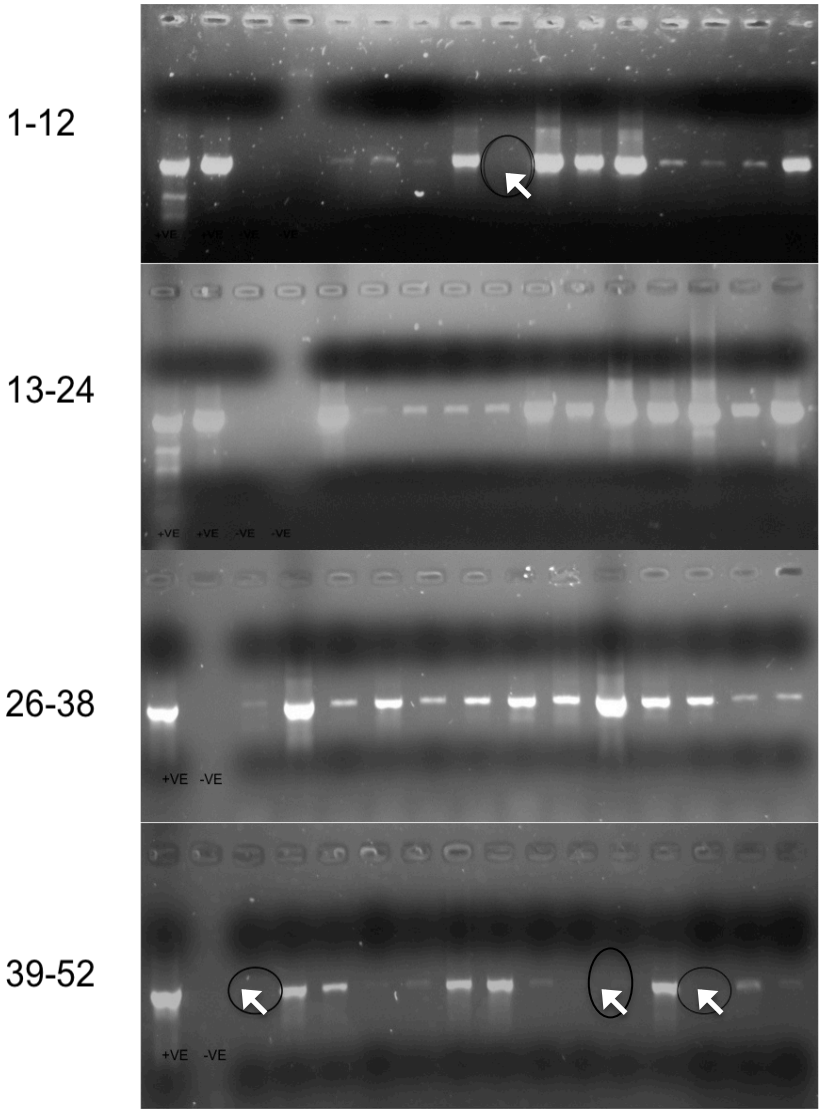

Figure 3.2 – 16S rRNA amplicons of total extracted bacterial DNA 1-52. The following amplicons were negative following 16S PCR: 5, 39 48 and 50 respectively, and were discarded from the analysis (expected band size 1,000-1,500 bp).

3.3.2 Patient demographics

The age of patients included in this analysis ranged from 18-75 years, with 5 female and 8 male subjects. The DMFT scores for 10/13 patients was >10. A total of 6/13 patients were diagnosed with VAP. An additional two patients were admitted with pre-existing respiratory disorders. Out of a total of 9 patients exhibiting respiratory pathogens within their dental plaque community during ETT intubation, 5 were treated with antibiotics >48 h after ETT intubation, and 3 patients were treated with antibiotics from the beginning of ETT intubation.

3.3.3 Community profiling of dental plaque during mechanical ventilation

3.3.3.1 Cluster analysing dental plaque bacterial sequences

All 38 samples of dental plaque were subject to initial analysis to determine distance similarity between each sample. In total 1,911,760 sequence reads (before quality control) were determined from pooled subgingival and supragingival dental plaque, obtained from 13 mechanically ventilated patients. OTUs were sub-sampled to the lowest read count of 1016, which retained 97% of all OTU counts for clustering analysis. Furthermore, clustering analysis was performed to provide an overview of the level of bacterial similarity within dental plaque. Figure 3.3 shows the clustering results using Jaccard's index of similarity and the Bray-Curtis distance methods of analysis. Figure 3.3 shows that the majority of dental plaque sequences form a tight cluster with a minority of dental plaque (7/38) sequences falling outside the tightly clustered main group.

A total of 5 bacterial phyla were detected during the analysis of dental plaque of 13 mechanically ventilated patients (Figure 3.4). Furthermore, a total of 40 different genera, and over 100 microbial species were detected (Raw sequencing data in Appendix II). Species identification was performed according to two sequence confident parameters, at 80% and 97% respectively. As previously stated, organisms identified at the 97% similarity

level were accepted as positive organism identification, and this similarity level was therefore used throughout data analysis and interpretation of the results.

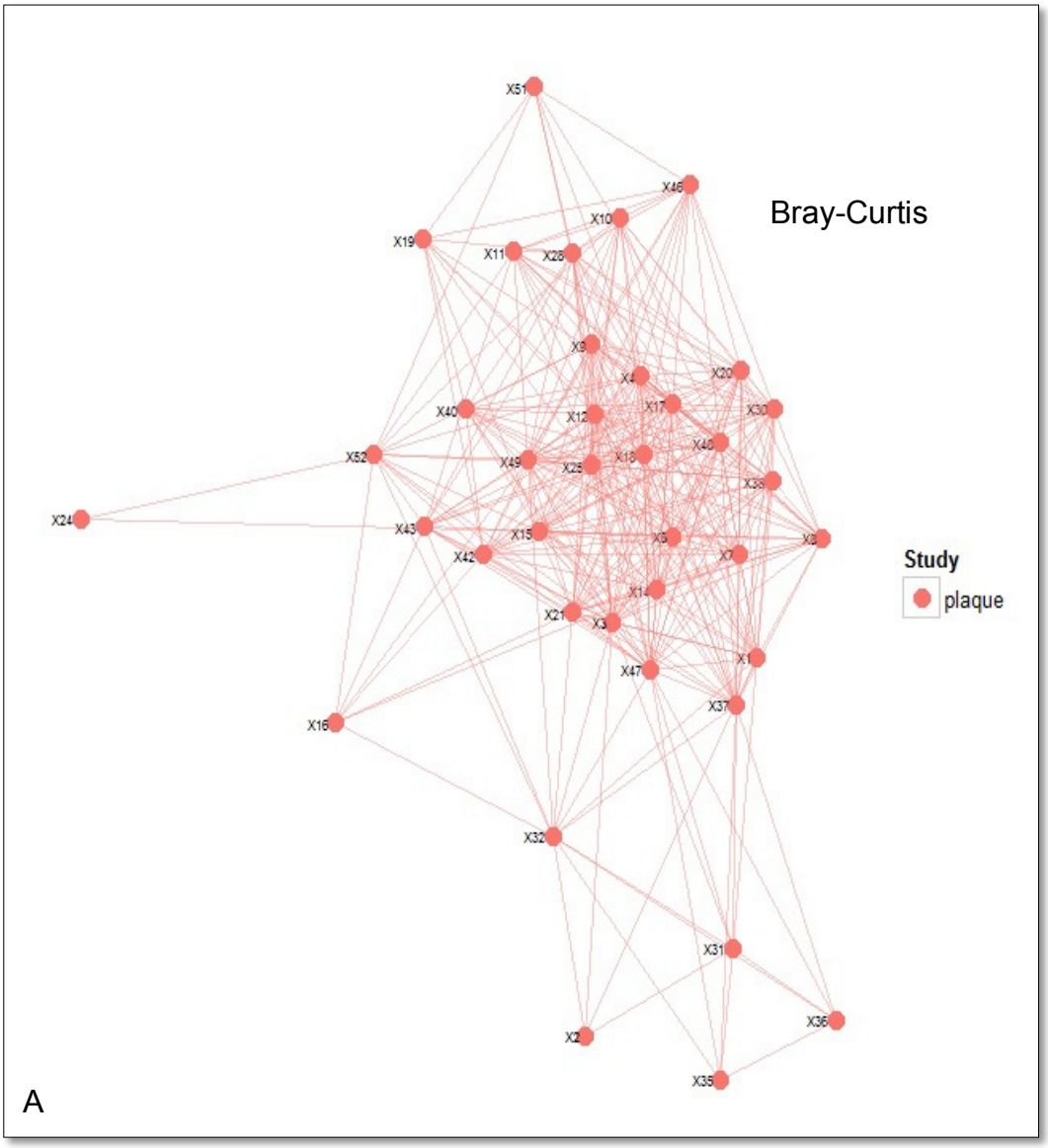

Figure 3.3A – Bray-Curtis analyses of dental plaque (n=38). A red spot represents each sample. These clustering analyses illustrate the similarity of dental plaque derived sequences based on comparisons of final species composition. By both methods, 31 communities of dental plaque were seen to cluster tightly, with seven outliers identifiable.

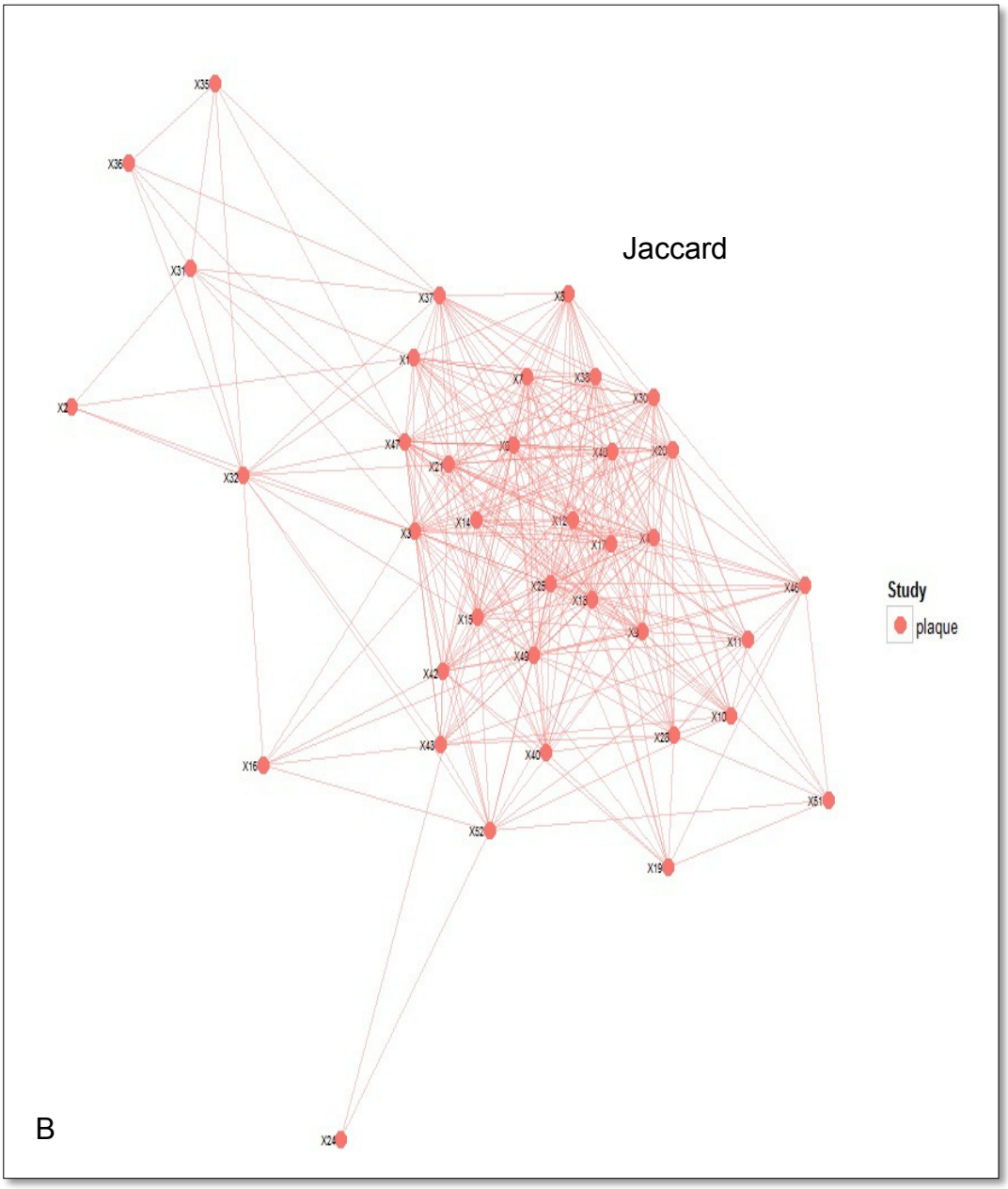

Figure 3.3B – Jaccard analyses of dental plaque (n=38). A red spot represents each sample. These clustering analyses illustrate the similarity of dental plaque derived sequences based on comparisons of final species composition. By both methods, 31 communities of dental plaque were seen to cluster tightly, with seven outliers identifiable.

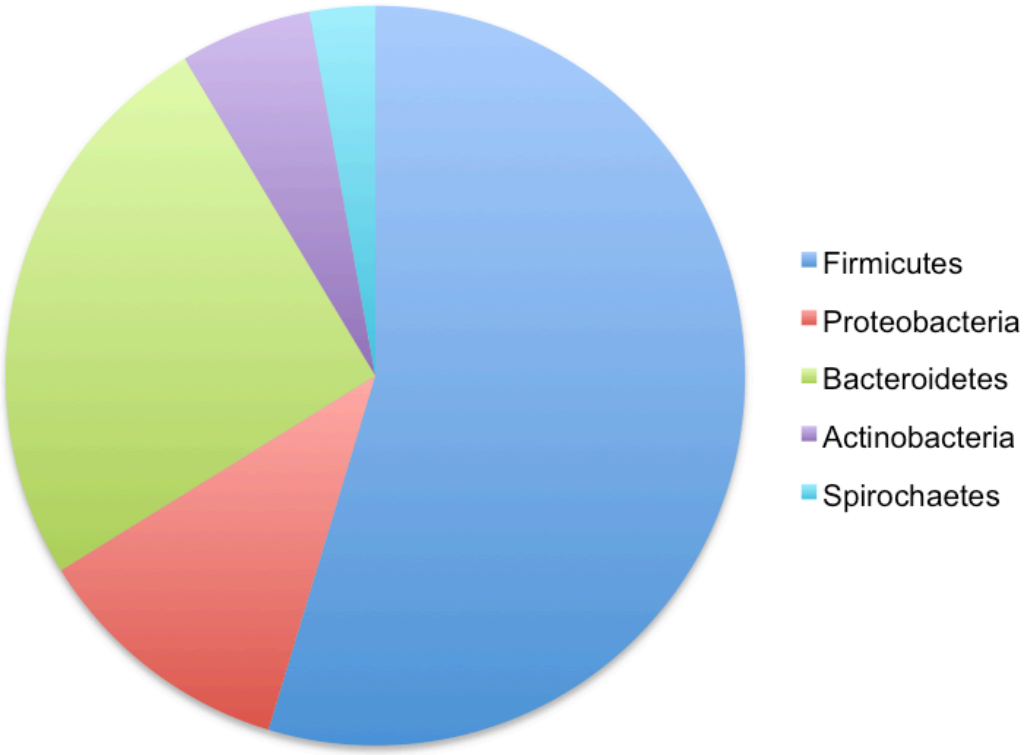

Figure 3.4 - The proportion of each of the 5 major bacterial phyla identified from dental plaque specimens. Over 50% of all organisms identified belonged to the Firmicutes phylum.

3.3.3.2 Potential respiratory pathogens detected within dental plaque

Of the 40 bacterial genera identified within this group of patients, 6 represented species not generally considered to be permanent members of the dental plaque community, including *Enterococcus* and *Staphylococcus* species.

In total, 8 potential respiratory pathogens were identified at the species level from the dental plaque. Figure 3.5 shows the prevalence of potential respiratory pathogens within dental plaque communities analysed. The four most frequently occuring species and the percentage of plaque samples from which they were isolated are as follows; *Staphylococcus aureus* (68%); *Streptococcus pseudopneumoniae* (66%); *Enterococcus faecalis* (37%); and *Escherichia coli/Shigella flexneri* (32%).

Interestingly, although *Pseudomonas aeruginosa* was identified by culture analysis of the dental plaque of mechanically ventilated patients (Chapter 2), this species was not identified using the culture-independent approaches performed within this study. Dental plaque from patients, including those patients where *P. aeruginosa* was isolated (as documented in chapter 2) were analysed via high-throughput sequencing. A total of 6/38 dental plaque communities analysed using molecular techniques were microbial culture positive for *P. aeruginosa.* Although *Pseudomonas* was detected at the genera level, albeit in low abundance, *P. aeruginosa* was not identified at species level for any of those samples. Following in house species-specific PCR, 5 out of 6 dental plaque communities were positive for *P. aeruginosa* (Figure 3.6).

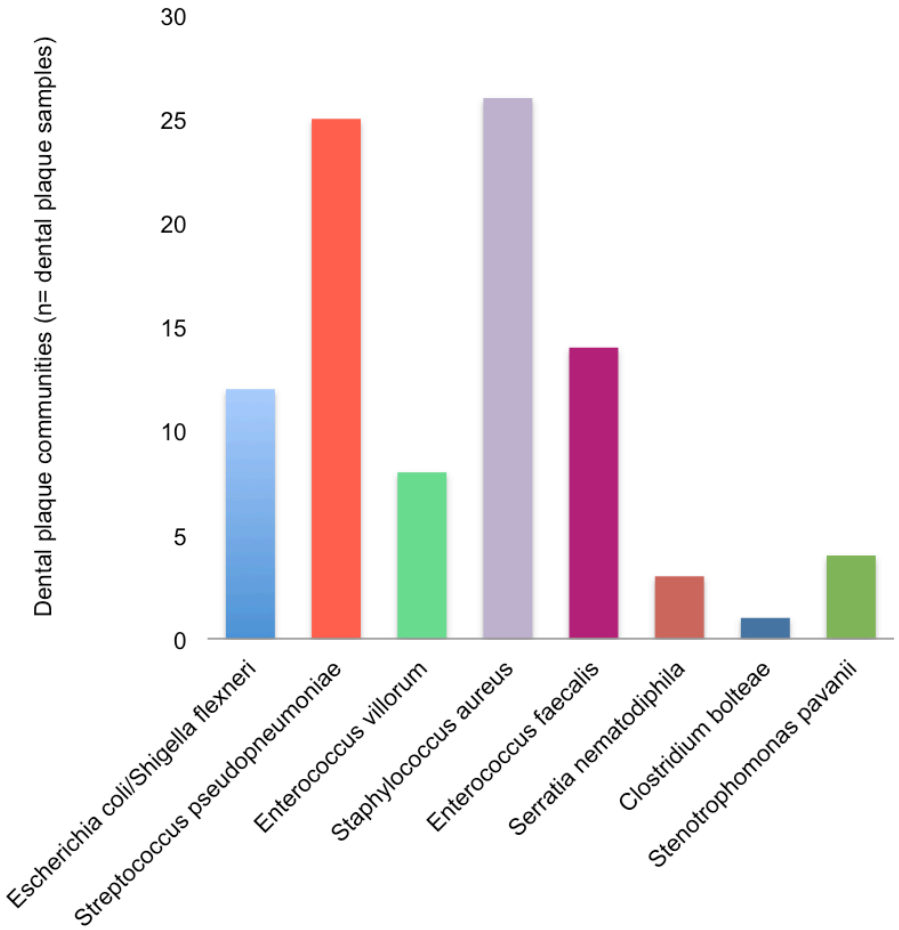

Figure 3.5 – Putative respiratory pathogen colonisation within dental plaque of mechanically ventilated patients.

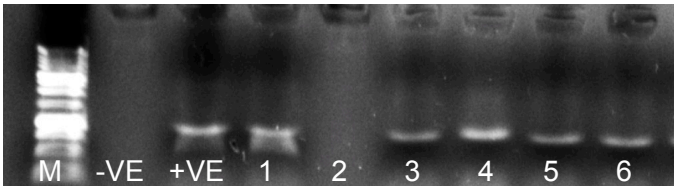

Figure 3.6 – Species-specific PCR for the detection of *Pseudomonas aeruginosa* in dental plaque. Dental plaque samples had been found to contain *P. aeruginosa* by microbial culture (but were negative by high-throughput sequencing). A total of five dental plaque samples (from the same patients represented in high-throughput sequencing) were positive for *P. aeruginosa*[5].

3.3.3.3 Profiling dental plaque communities during early stages of mechanical ventilation (d1-2)

Baseline dental plaque collected from recruited mechanically ventilated patients was taken as close to ETT intubation as possible. The time period from obtaining consent for patient recruitment to the study and the introduction of MV to the time of dental plaque collection varied between 4 h and 48 h. In order to analyse differences in microbial relationships and species for mechanically ventilated patients, a stacked bar graph depicting the 5 most abundant genera was produced. Figure 3.7 shows the 5 most abundant bacterial genera (accumulated based on the numbers of identified sequence reads) identified in dental plaque within 48 h of ETT intubation. From this analysis, we can see that abundant genera from early stages of MV included those that are recognised as common oral bacteria as well as those not frequently found in the oral cavity. The top 10 species detected in dental plaque during MV (midpoint of MV, typically 3-6 days) are listed in table 3.1, and the most abundant species belonged to the genus *Veillonella*. Within this list, 50% (5 out of 10) of the most abundant organisms during early stage MV were considered putative respiratory pathogens.

[5] M = Molecular marker, -VE = negative control, +VE = positive control

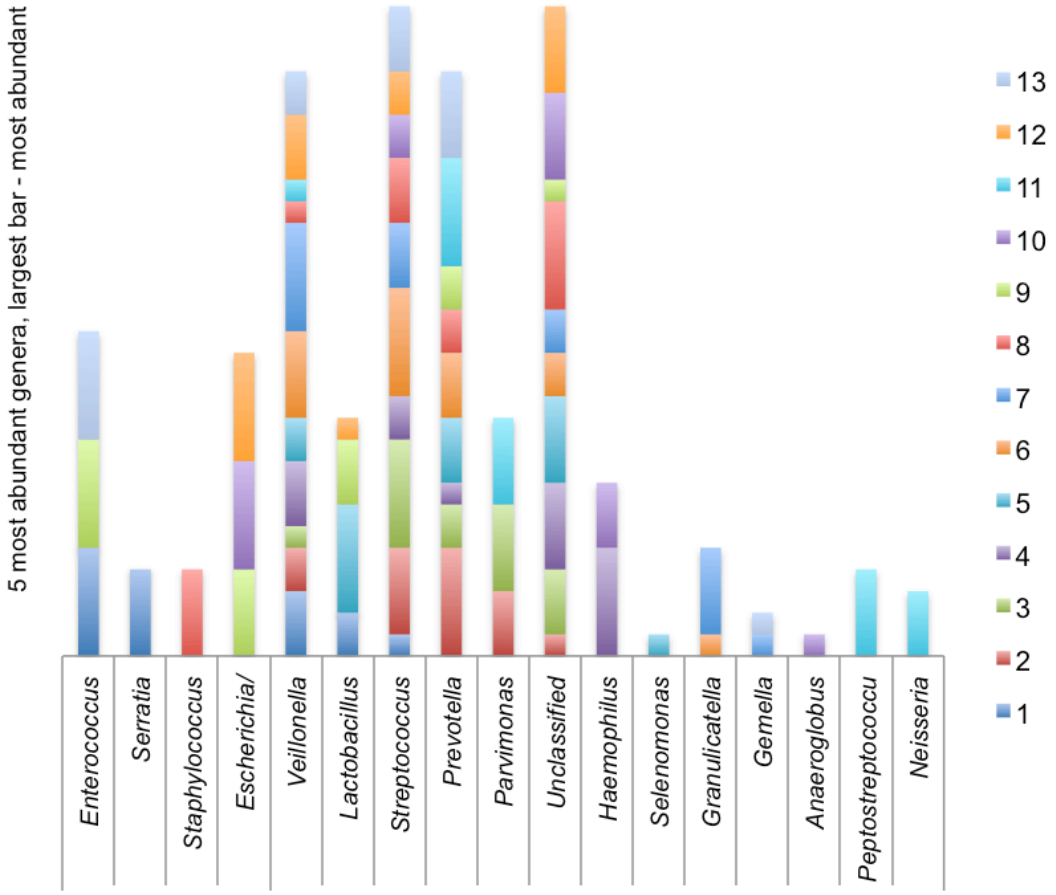

Figure 3.7 – The five most abundant organisms (genera level) present in the dental plaque community at the start of mechanical ventilation for each of the 13 patients. Each patient is represented by a different colour.

Table 3.1 – Ten most abundant bacterial species at the midpoint of MV.

Abundance	Ten most abundant species at midpoint of MV
1	*Veillonella parvula*
2	*Escherichia/Shigella flexneri**
3	*Streptococcus pseudopneumoniae**
4	*Enterococcus faecalis**
5	*Staphylococcus aureus**
6	*Granulicatella adiacens*
7	*Enterococcus villorum**
8	*Lactobacillus gasseri*
9	*Prevotella melaninogenica*
10	*Lactobacillus salivarius*

*Respiratory pathogen

3.3.3.4 Community analysis of the respiratory pathogen colonisation during mechanical ventilation

During MV, there was a degree of inter-patient microbial variability within the dental plaque community of mechanically ventilated patients. Non-Metric Multidimensional Scaling (NMDS) coupled with t-tests were performed to analyse the level of similarity between communities isolated from dental plaque during the course of MV, and into the post-ETT extubation period. Each dental plaque community was assigned coordinates based on Unifrac (phylogenetic community distance measure) analysis to generate a scatter plot to assess similarity in the microbial community of dental plaque between periods of MV and post extubation (Figure 3.8A). Dental plaque was divided into four groups for subsequent analysis focusing on the change in microbial composition or 'microbial shift' previously observed by culture analysis and reported in Chapter 2 (Group 1 – commencement of MV, group 2 – midpoint of MV typically 3-6 days, group 3 – end of MV and group 4 – post-ETT extubation recovery period). Results revealed that changes in the microbiome of dental plaque during MV occurred, and such changes were statistically significant. Despite overlapping of microbial communities (Figure 3.8 B and C), statistically significant changes in the microbial composition of dental plaque were evident during MV. Pairwise comparisons using t tests

with pooled Standard Deviation (SD) were performed to compare data for each of the time points groups. P-value adjustment was performed using the Benjamini–Hochberg (BH) method, to control the false discovery rate (FDR) when performing multiple comparisons. (FDR is a measure of true significance, controlling any false discoveries in multiple comparison data sets).

Furthermore, to analyse the microbiome of dental plaque at different time points of MV, NMDS was performed. The parameters used were Unifrac against time, to determine the level of organism diversity. The change in microbial composition of dental plaque between the start and midpoint of MV was shown to be statistically significant, p=0.0033. In addition, the community profiles between the start and end, and between the midpoint and end, were also shown to be significantly different (p=0.0403 and $p=6.3e^{-05}$ respectively). Descriptive statistics are presented in the box and whisker plot, and are visual representations of the data revealing changes in microbial composition during MV (Figure 3.9).

A stacked bar graph was generated to compare the 10 most abundant species at the four defined stages of MV and post-ETT extubation recovery period. The stacked bar graph (Figure 3.10) reveals a sweeping pattern, indicating a trend of microbial differences during the course of MV. Furthermore, the most abundant organisms detected at the start of MV differed from that at the end of MV. Bacteria detected at the start and midpoint of MV both showed a high prevalence of respiratory and non-oral pathogens.

Microbial changes and the occurrence of respiratory pathogens in the dental plaque during MV are presented in table 3.2, where the OTU abundance of respiratory pathogens is compared. Furthermore, heat maps were constructed to compare and quantify community-profiling data over time. Figure 3.11 illustrates the phyla variation in dental plaque collected from mechanically ventilated patient (PN021), and figure 3.12 extends upon this, comparing species variation between the microbiome of dental plaque and

the lower airways. Species level heat maps for all 13 mechanically ventilated patients analysed via microbial community profiling are depicted in Appendix II.

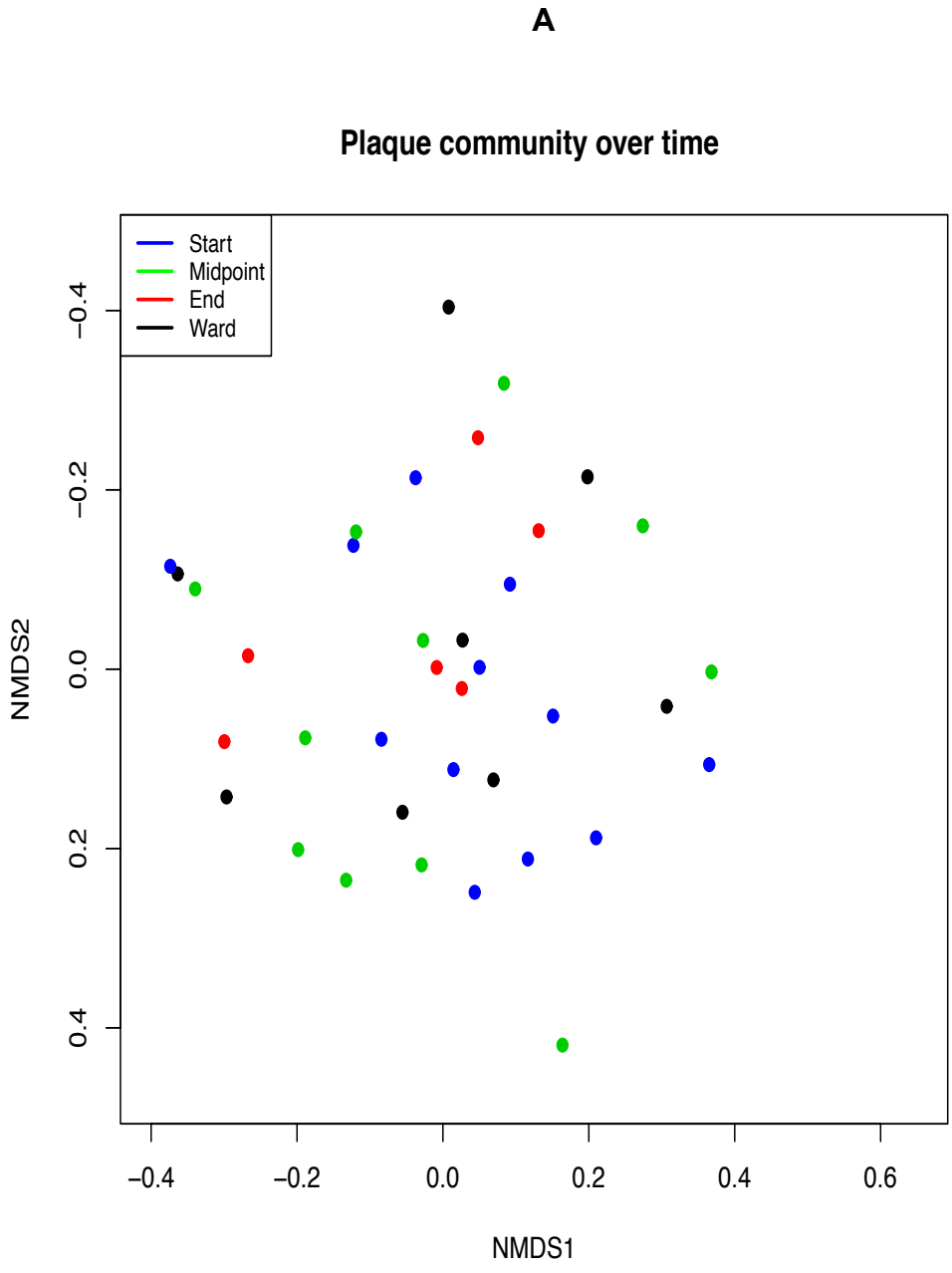

Figure 3.8A – Non-Metric Multidimensional Scaling (NMDS) analysis of dental plaque - scatter representations of dental plaque communities.

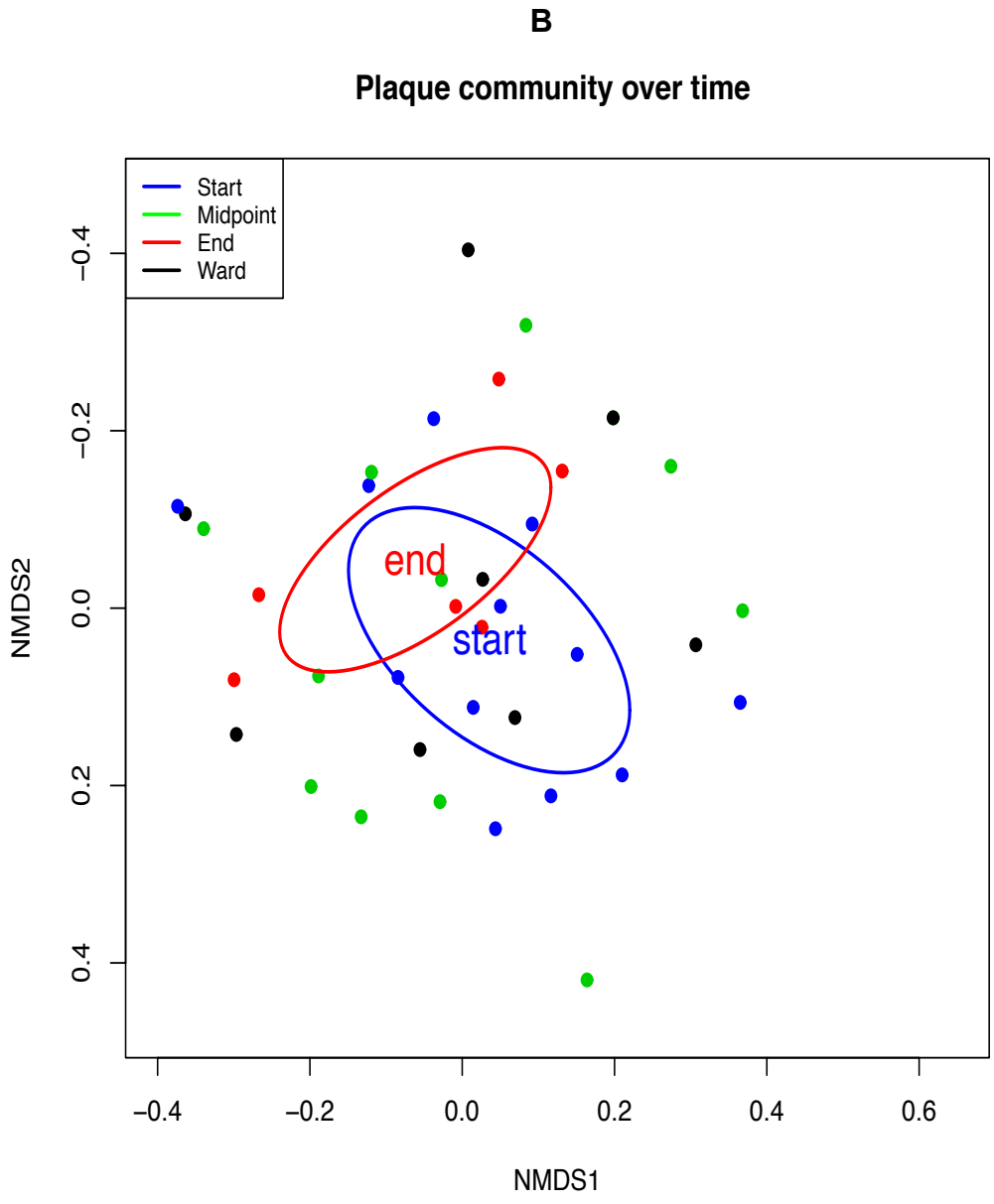

Figure 3.8B - Non-Metric Multidimensional Scaling (NMDS) analysis of dental plaque groupings and relationship between dental plaque communities from the start and end of intubation.

C

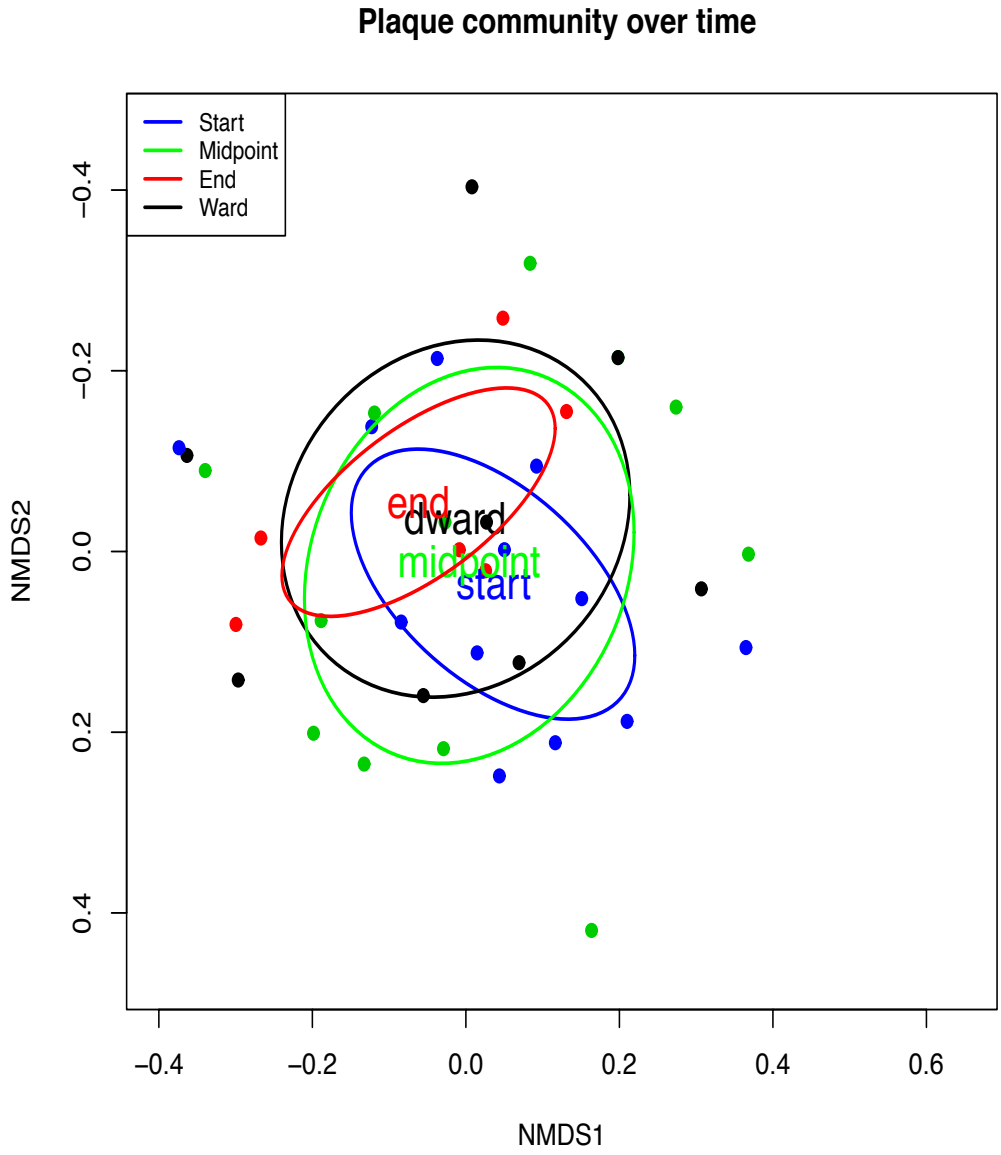

Figure 3.8C - Non-Metric Multidimensional Scaling (NMDS) overlapping analysis of dental plaque communities during MV. [6]

[6] Ward – represents dental plaque collected in the post-ETT extubation period

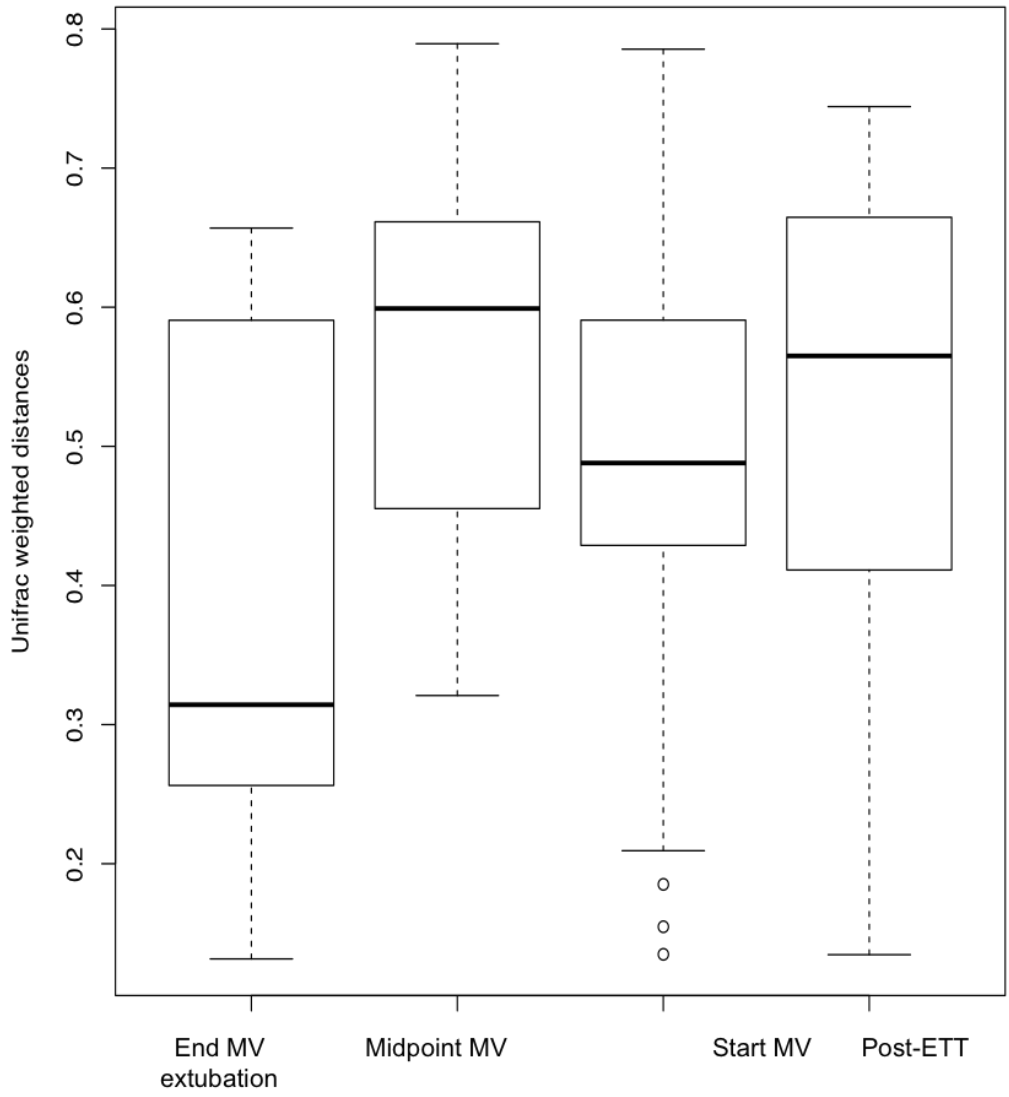

Figure 3.9 – A box and Whisker plot displaying the unifrac-weighted distances (measure to compare organism differences in communities) for the four dental plaque community time points during MV. The interquartile range (IQR), the box elements shows data dispersion for each group of dental plaque samples.

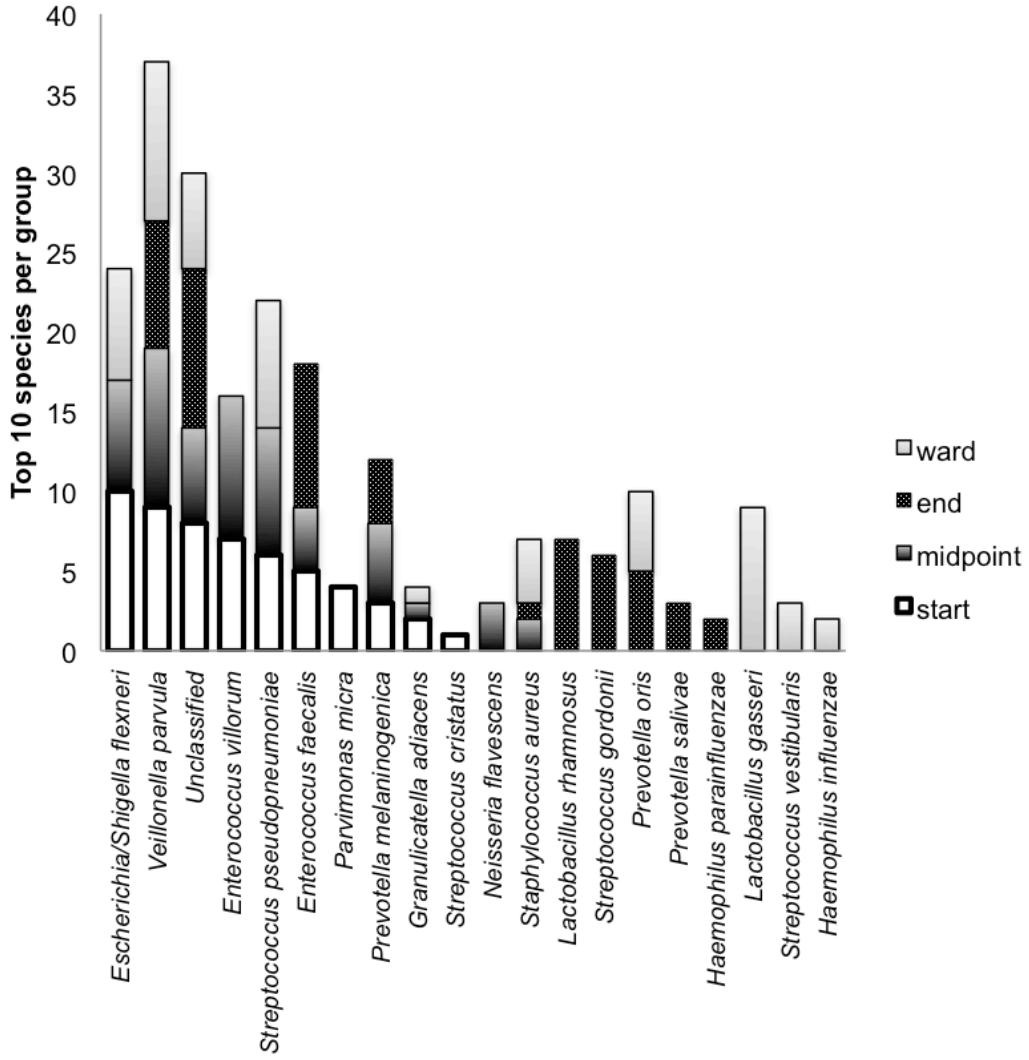

Figure 3.10 – The ten most abundant microbial species in dental plaque for each time point group. This bar graph allows the comparison between oral organisms and potential respiratory pathogens.

Table 3.2 – Abundance measurements (operational taxonomic units (OTUs)) of potential pathogens during MV.[7]

Respiratory pathogen	Total OTUs detected			
PN002	d1	d4	d5	
Enterococcus villorum	623	983	135	
Staphylococcus aureus	11	2	35	
Enterococcus faecalis	0	1	20	
Serratia nematophilia	214	0	30	
PN004	d1	d30	post-ETT	post-ETT
Streptococcus pseudopneumoniae	101	12	1	0
PN007	d1	d11	post-ETT	
Serratia nematophilia	1	0	0	
Streptococcus pseudopneumoniae	155	766	796	
Staphylococcus aureus	1	241	217	
PN018	d1			
Streptococcus pseudopneumoniae	96			
PN019	d1	post-ETT		
Streptococcus pseudopneumoniae	11	1		
Staphylococcus aureus	0	3		
Enterococcus faecalis	4	0		
PN022	d1	d6	post-ETT	
Streptococcus pseudopneumoniae	159	126	7	
Staphylococcus aureus	2	1	0	
Enterococcus faecalis	0	4	0	
PN012	d1	d6	post-ETT	
Streptococcus pseudopneumoniae	99	1	0	
Enterococcus faecalis	0	568	0	
PN021	d1	d3	post-ETT	
Streptococcus pseudopneumoniae	130	145	325	
Staphylococcus aureus	61	34	3	
PN030	d1	d5	post-ETT	
Escherichia coli	349	385	1001	
Enterococcus villorum	451	296	5	

[7]PN – patient number, d - day of mechanical ventilation, post-ETT - collection of dental plaque into the post-ETT extubation recovery period.[7]

Staphylococcus aureus	0	17	1		
Enterococcus faecalis	0	136	4		
PN032	d1	d3	d6		
Escherichia coli	949	863	13		
Enterococcus villorum	0	3	0		
Staphylococcus aureus	2	1	5		
Enterococcus faecalis	0	1	0		
PN046	d3	d13	d26	d34	d48
Escherichia coli	0	3	1	18	0
Streptococcus pseudopneumoniae	12	0	0	18	102
Enterococcus villorum	0	0	0	22	0
Staphylococcus aureus	0	27	9	23	4
Enterococcus faecalis	0	22	916	549	2
Stenotrophomonas pavanii	0	0	1	0	0
PN049	d1	d8			
Escherichia coli	422	0			
Streptococcus pseudopneumoniae	0	225			
Staphylococcus aureus	3	11			
Enterococcus faecalis	29	20			
Clostridium bolteae	20	0			
PN050	d1	d27	post-ETT		
Streptococcus pseudopneumoniae	91	0	0		
Staphylococcus aureus	0	2	104		
Enterococcus faecalis	578	0	54		
Stenotrophomonas pavanii	0	0	2		

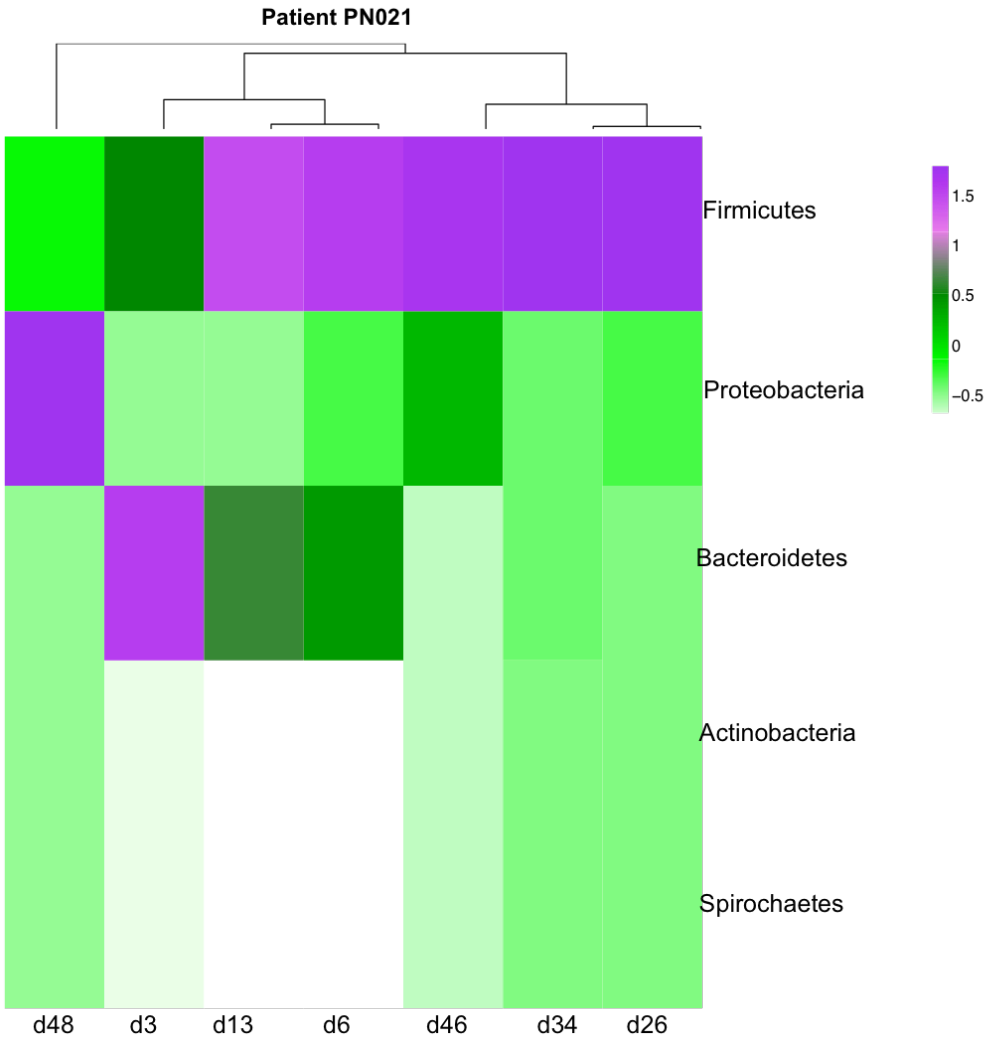

Figure 3.11 - Heat map indicating phylum variation within dental plaque and lower airways during mechanical ventilation (a total of 7 samples were pooled).

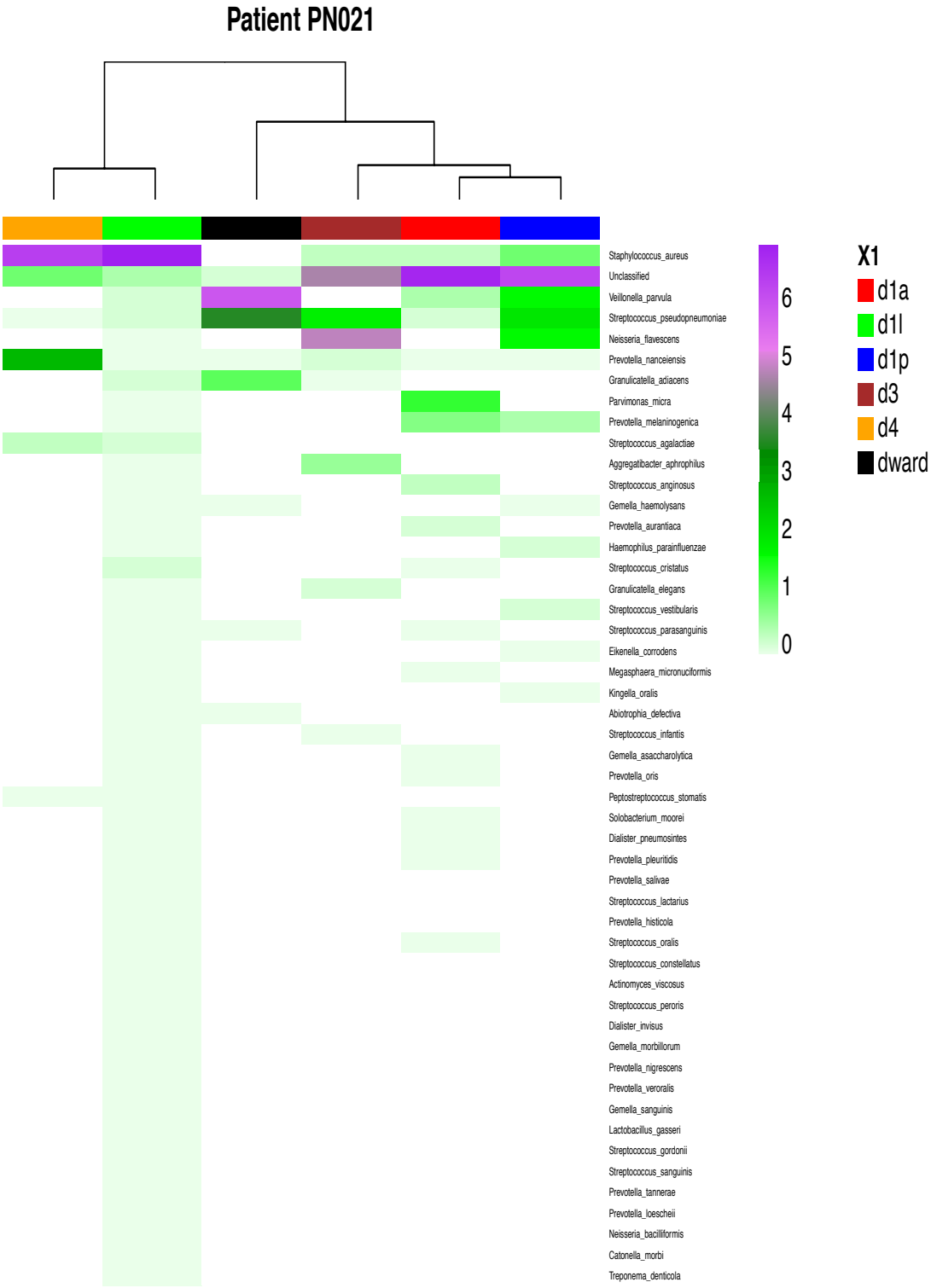

Figure 3.12 – Heat map indicating the level of phyla, species variation and species abundance during MV. In the X1 key – a=subglottic aspiration, l= NBL, and p=dental plaque. If none stated, the samples for analysis were dental plaque.

3.3.3.5 Presence of respiratory pathogens in dental plaque post-ETT extubation

The final aspect of analysis of microbial communities of dental plaque was to determine whether the bacterial communities at the start of MV (d1) were similar to that of dental plaque communities post-ETT extubation (Figure 3.13). The 10 most abundant species were compared, revealing both oral and potential respiratory pathogens were detected at the beginning of MV and post-extubation. A pairwise t-test measuring the weighted Unifrac against the data sets indicated a lack of significance (p=0.1945). Although there was no significant difference between these two-pooled microbial communities, there was a decrease in abundance of many respiratory pathogens in the post-ETT extubation recovery period. There were however similar or higher numbers of *S. pseudopneumoniae* and *S. aureus* respectively, in the post-ETT extubation period (Figure 3.13). *Veillonella* and Lactobacillus were the two most abundant genera in the post-ETT extubation period, whilst *Veillonella* and *E. coli* were the two most abundant bacterial types at the start of MV. In the post-extubation period, potential respiratory pathogens including *E. coli* and *S. aureus* were detected, however their abundance was lower compared to the start of MV. This can be seen by the smaller bar on the stacked bar graph suggesting a decrease in total abundance of pathogens (Figure 3.13). In contrast, *Enterococcus* species were not identified in the post ETT-extubation period. A total of 50% of the most abundant species in dental plaque collected during the recovery period were recognised as frequent members of the oral microbiota.

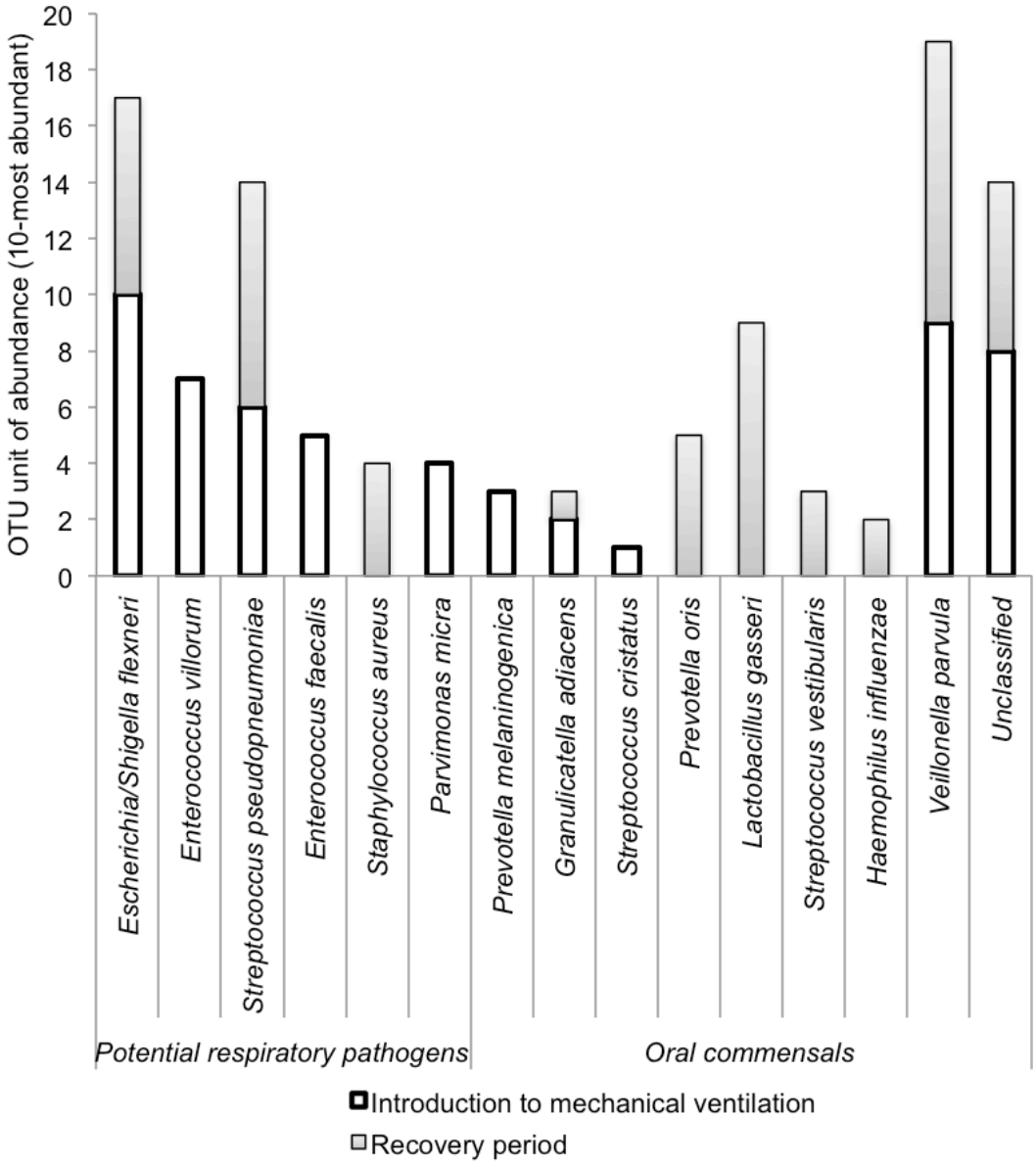

Figure 3.13 - The ten most abundant microbial species in patients' dental plaque communities at the commencement of MV (start) and into the post-ETT extubation recovery period (ward).

3.3.3.6 DMFT scoring and the abundance of dental plaque: correlation analysis

DMFT scores were recorded for 97 patients and have been reported in Chapter 2 (Section 2.3.1.6). DMFT scores for the 13 mechanically ventilated patients during microbial profiling analysis ranged from 0 to 22, with 9 out of 13 patients having a DMFT score >10. Pearson's product-moment correlation coefficient is a statistical analysis used to determine whether there is any correlation between two measured variables. Correlation analysis was performed to determine if there was a relationship between the patients' total dental plaque abundance (indicator of plaque abundance) and DMFT score (Figure 3.14). The correlation coefficient was -0.432, indicating a negative correlation between species abundance and DMFT scores within mechanically ventilated patients. The 95% confidence interval ranged from -0.637 to -0.168, and the extent of this range (~0.5) suggests a degree of patient variability. The p-value derived from the statistical analysis ($p=0.002159$) implies a very strong negative relationship between DMFT scores and bacterial abundance, suggesting with increases in DMFT scores, there are decreases in recovered bacterial abundance.

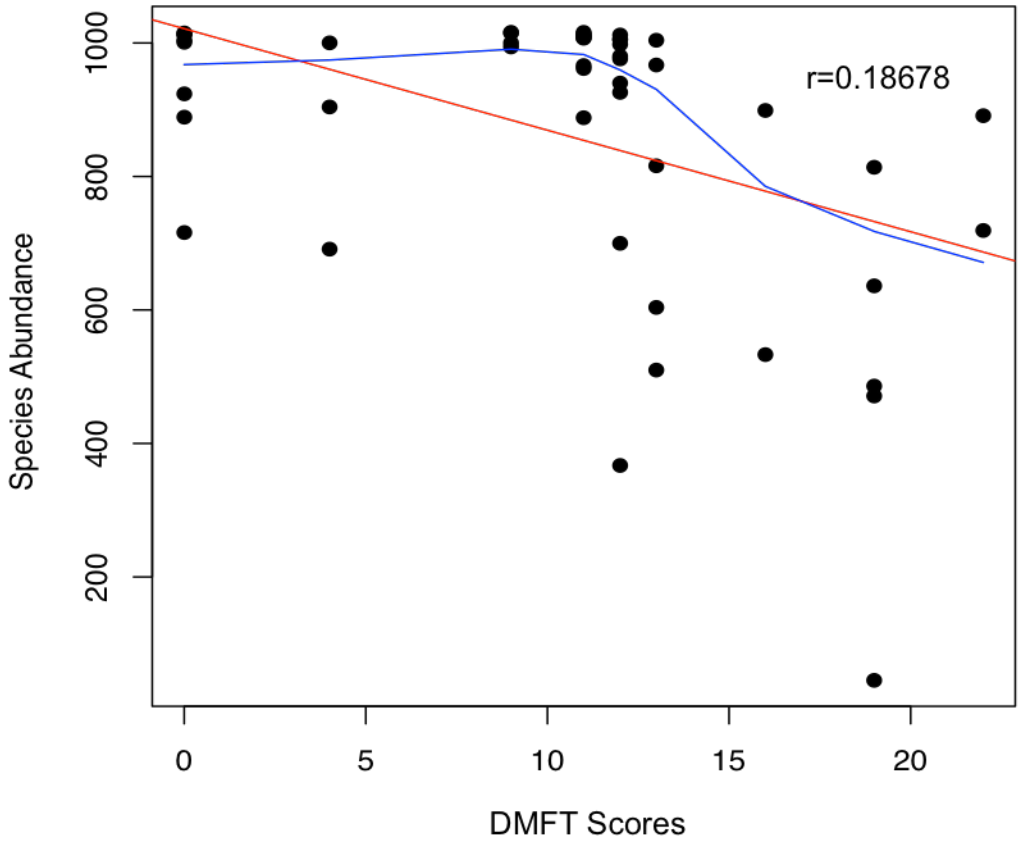

Figure 3.14 - Correlation analysis between total dental plaque species abundance and the DMFT score. This scatter plot shows the relationship between species abundance (recovered DNA sequence reads from dental plaque) with the previously recorded DMFT score.

3.3.4 Similarity analysis of dental plaque, NBL, subglottic aspirations and ETTs

Microbial community profiling was also performed to compare communities of dental plaque, NBL, subglottic aspirations and ETT biofilms. The analyses aimed to assess the degree of microbial similarity or otherwise between the oral cavity and lower airways. ANOVA Bonferroni correlation analysis (performed in STAMP) provided statistical comparison for two potential respiratory pathogens identified in mechanically ventilated patients during MV. *Streptococcus pseudopneumoniae* was identified in 11/13 patients, as represented in figure 3.15, and *E. coli* was detected in 4/13 patients (Figure 3.16). For *S. pseudopneumoniae*, the abundance from all pooled samples per patient was <25% with the exception of PN007 for whom *S. pseudopneumoniae* represented >50% of all identified sequences. The abundance of *E. coli/Shigella flexneri* when analysing total pooled samples collected per patient, ranged from <10% to >90% of total OTU reads sequenced.

To compare the microbiome at different clinical sites, a stacked bar graph representing the ten most abundant species from each sampling site was generated (Figure 3.17). The most abundant bacterium detected within pooled dental plaque was *Veillonella parvula.* For NBL and subglottic aspiration samples the most abundant organisms were *Haemophilus influenzae* and *Lactobacillus salivarius,* respectively. In the case of ETT biofilms, the most abundant organism was the potential respiratory pathogen *S. aureus*. Oral bacteria such as *Veillonella* species were detected from non-oral sites, and potential respiratory pathogens such as *S. aureus* were also identified in multiple sites.

The abundance of both the genera *Staphylococcus* and for *S. aureus* between dental plaque, NBL, SUB and ETTs was analysed via one-way ANOVA (Figure 3.18). The proportion of *Staphylococcus* species sequences was significantly higher for ETT biofilms compared with dental plaque ($p= 0.00019$). *Staphylococcus aureus* was detected at all patient sample sites, with a significantly increased proportion of *S. aureus* sequences detected

within ETT biofilms, compared with dental plaque and the lower airways (NBL and SUB).

Furthermore, ANOVA was performed for an oral-indicator species. Interestingly, ANOVA comparing the sequence proportion of *Prevotella nanceiensis* between dental plaque and ETTs revealed a statistically ($p=0.023$) greater detection of this oral species from the ETT biofilm compared to dental plaque. Figure 3.19 represents this graphically, showing a proportion of sequences for ETTs >25% for *Prevotella nanceiensis*, and <5% from all other sampled sites.

NMDS analysis was performed to compare the microbiome of dental plaque the lower airways, and ETT biofilms. Figure 3.20 (A) shows the NMDS analysis assigned scatter plot per each sample analysed via community profiling. Figure 3.20 (B) demonstrates the microbial relationship between the dental plaque microbiota and ETT biofilms. In addition, to compare similarity between microbial species in dental plaque and ETTs, a series of t-tests were performed on 5 of the most abundant species detected in both sample types. Figure 3.21 compares the sequence coverage (abundance) between 6 microbial species isolated within both dental plaque and ETT biofilms. T-tests explored the level of significance between the relative abundance of species between the dental plaque and ETT biofilms, with q-values for all t-tests revealing all were statistically significant (<0.05). A q-value is the analogue of the false discovery rate (to correct for multiple testing and comparisons), and represents the p-values. Analysis indicates significant differences between the species level at different sample sites. All species examined, including *S. aureus, Streptococcus agalactiae, Prevotella nigrescens* and *Aggregatibacter segnis* were isolated in a significantly higher abundance in ETT biofilms except *Veillonella parvula,* which was significantly more abundant within the dental plaque.

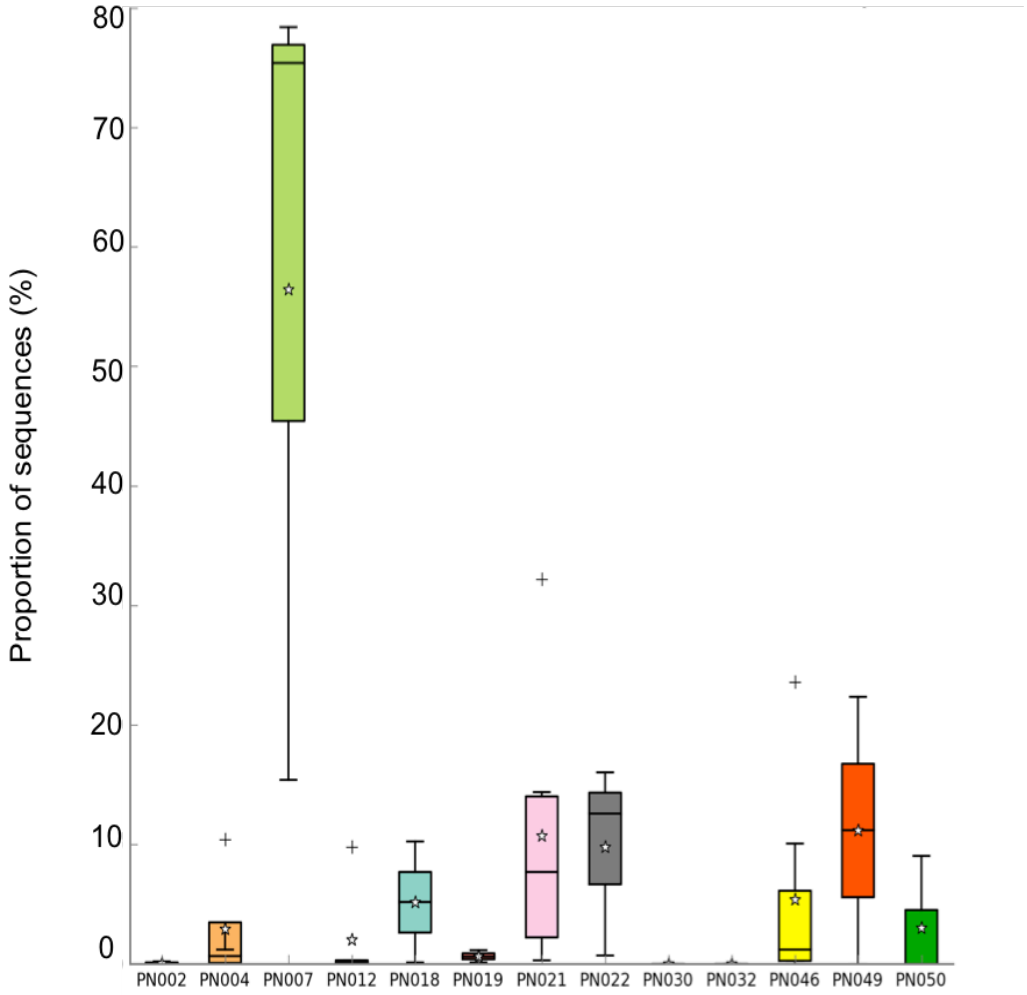

Figure 3.15 - Box and whisker plots comparing the proportion of total sequences identified as *Streptococcus pseudopneumoniae.* ANOVA Bonferroni correlation analysis compared the abundance of *S. pseudopneumoniae* against all other microbial species identified. A corrected p-value of 2.893^{-3} indicated the abundance of *S. pseudopneumoniae* in total sequences identified was statistically higher than other microbial species.

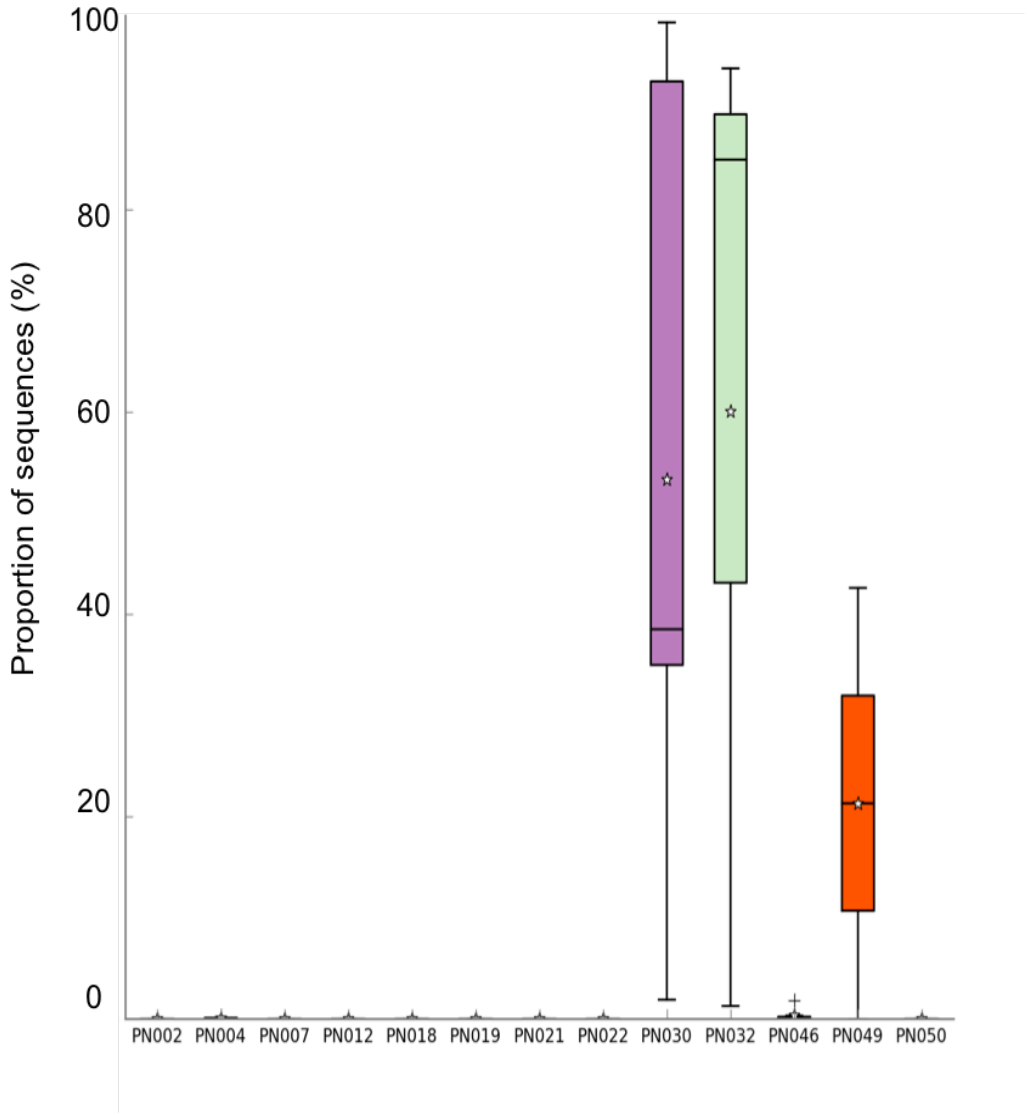

Figure 3.16 - Box and whisker plots comparing the detection of *E. coli* from total sequences collected from clinical samples from patients during mechanical ventilation. ANOVA Bonferroni correlation analysis compared the abundance of *E.coli/Shigella flexneri* against all other microbial species identified. A corrected p-value of 0.014 indicated the abundance of *E. coli/ S. flexneri* in total sequences identified was statistically higher than other microbial species.

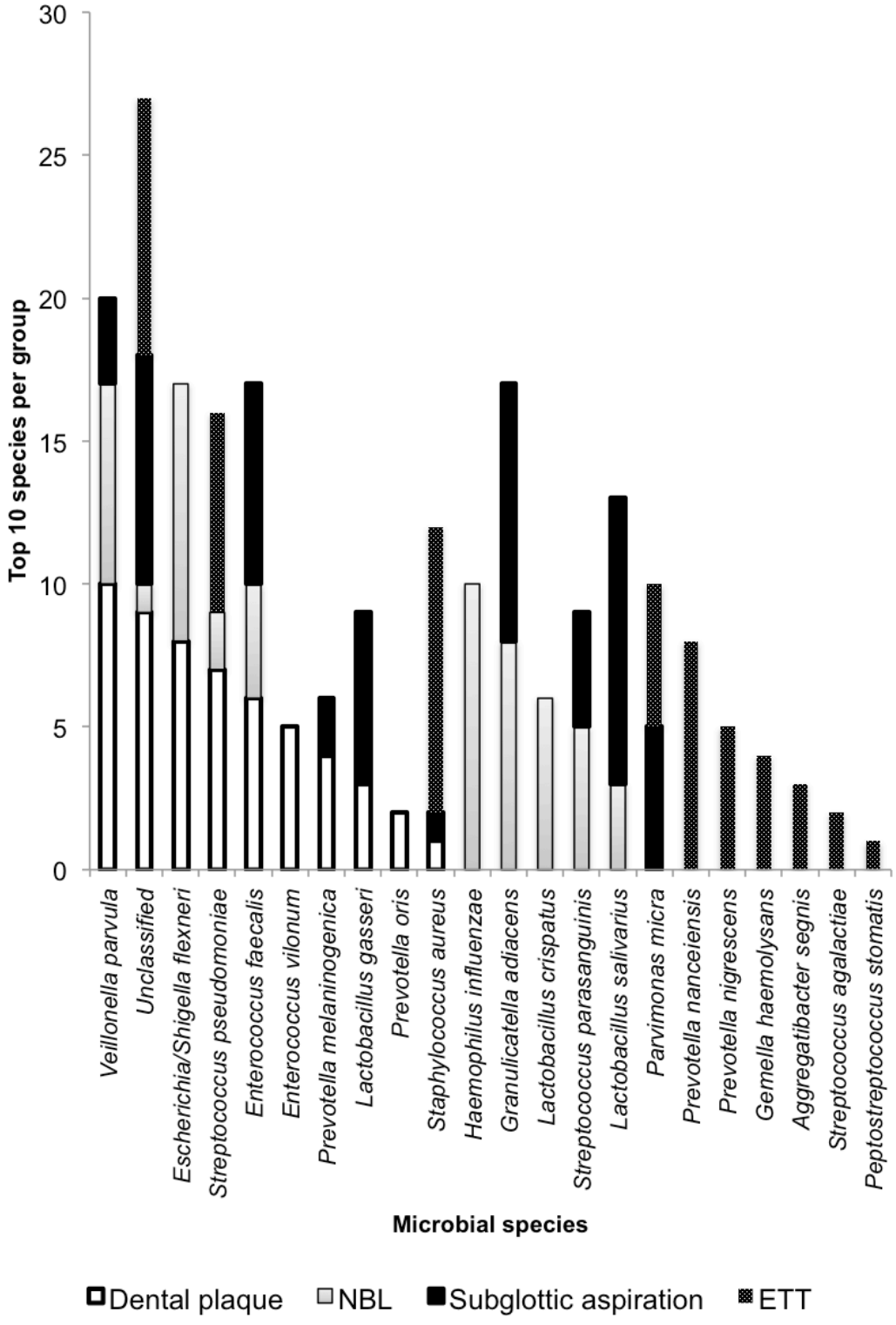

Figure 3.17 - The ten most abundant microbial species within dental plaque, NBL, subglottic aspirations and ETT biofilms.

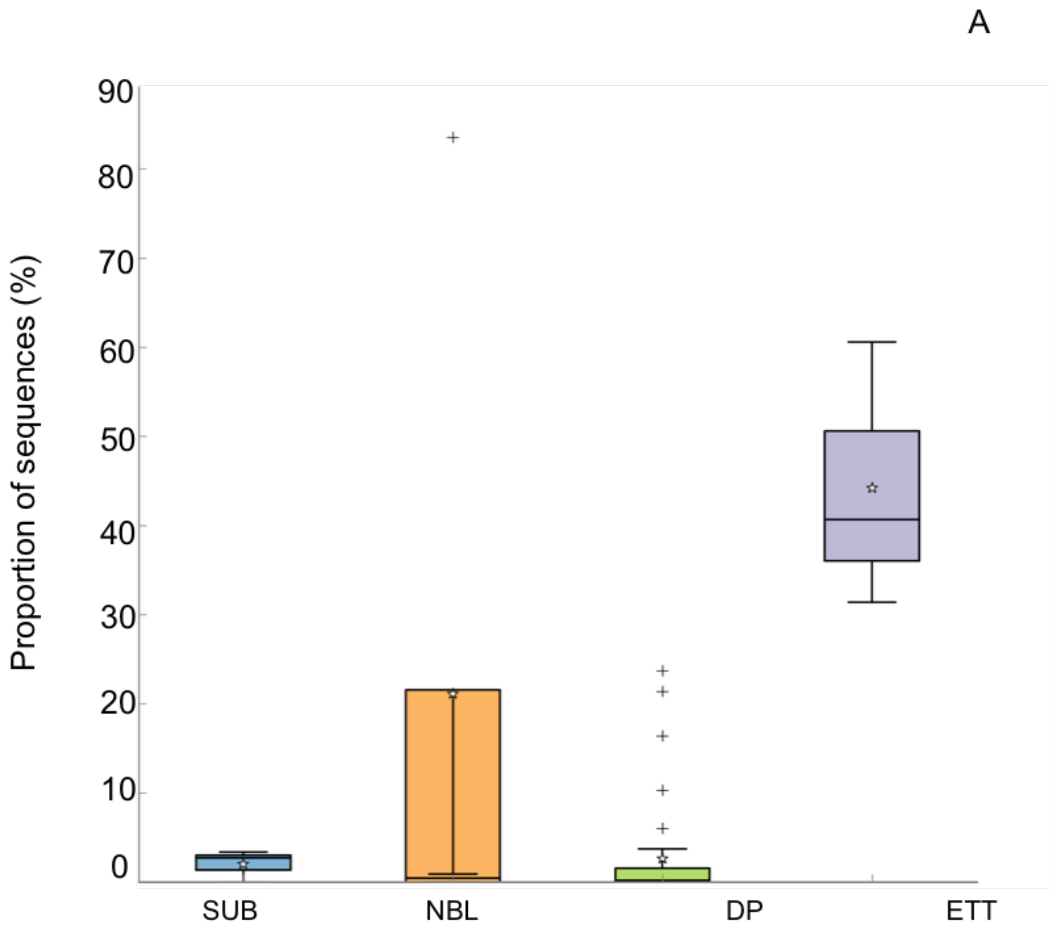

Figure 3.18A – Box and whisker plot comparing the percentage of dental plaque sequences for Staphylococcus between dental plaque, the lower airways and ETT biofilms. The addition of multiple testing corrections allows the testing of multiple hypotheses.

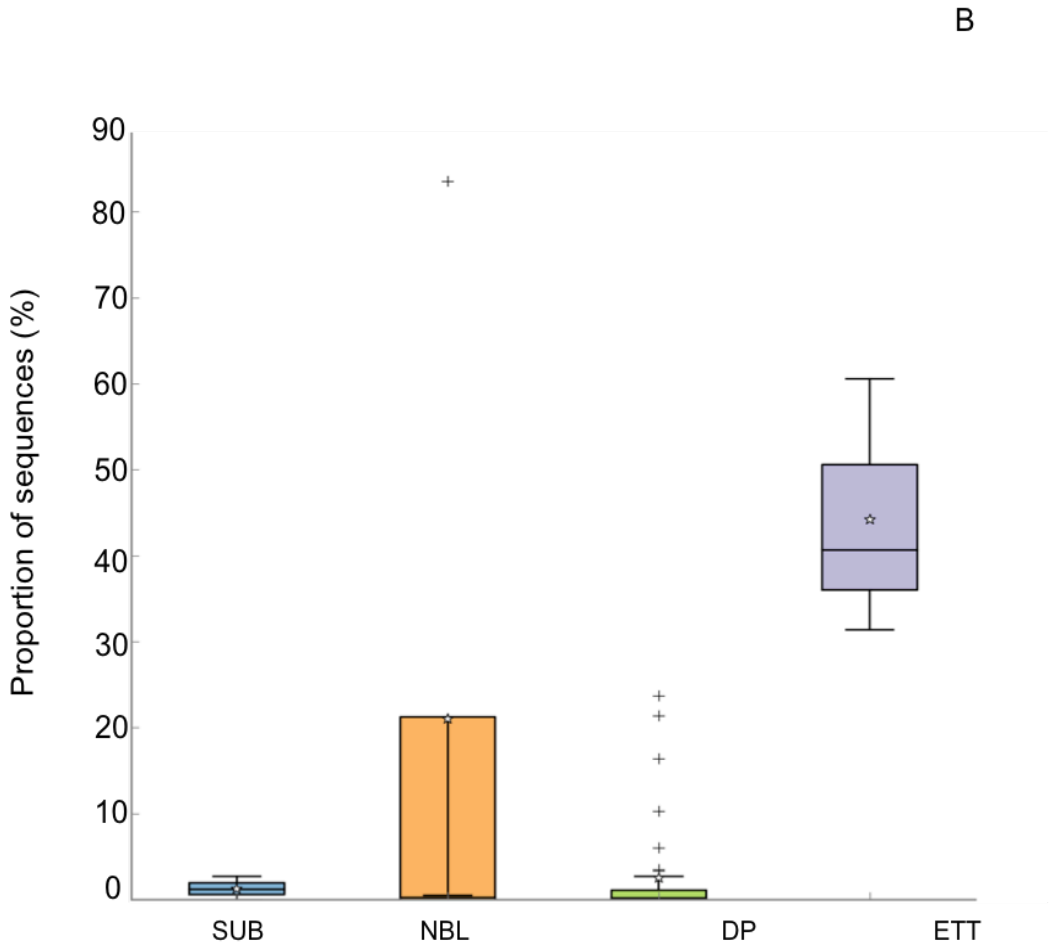

Figure 3.18B – Box and whisker plot comparing the percentage of dental plaque sequences for *Staphylococcus aureus* between dental plaque, the lower airways and ETT biofilms.

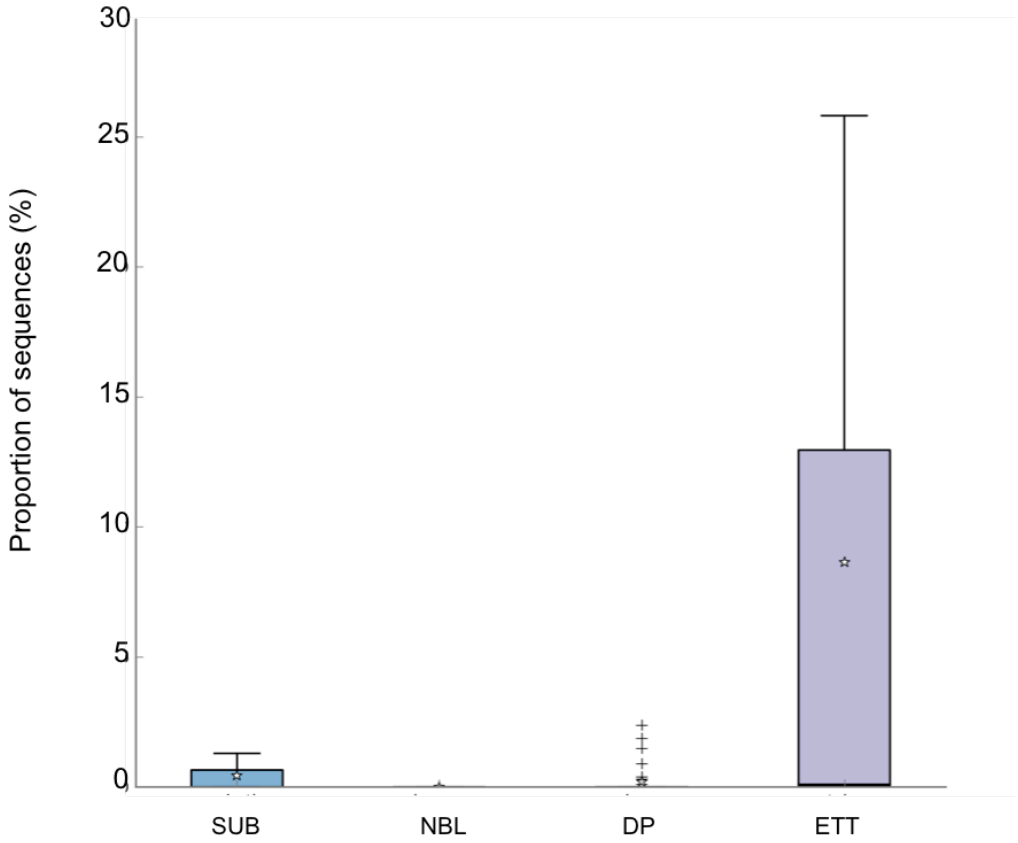

Figure 3.19 – Box and whisker plot at the species level for the oral organism *Prevotella nanceiensis. Prevotella nanceiensis* was detected in the subglottic aspirations, the dental plaque, and the ETT. ANOVA (with Benjamini-Hochber multiple correction testing) was performed and a p-value of 0.023 emphasised a statistically significant difference between *P. nanceiensis* detection amid clinical sites.

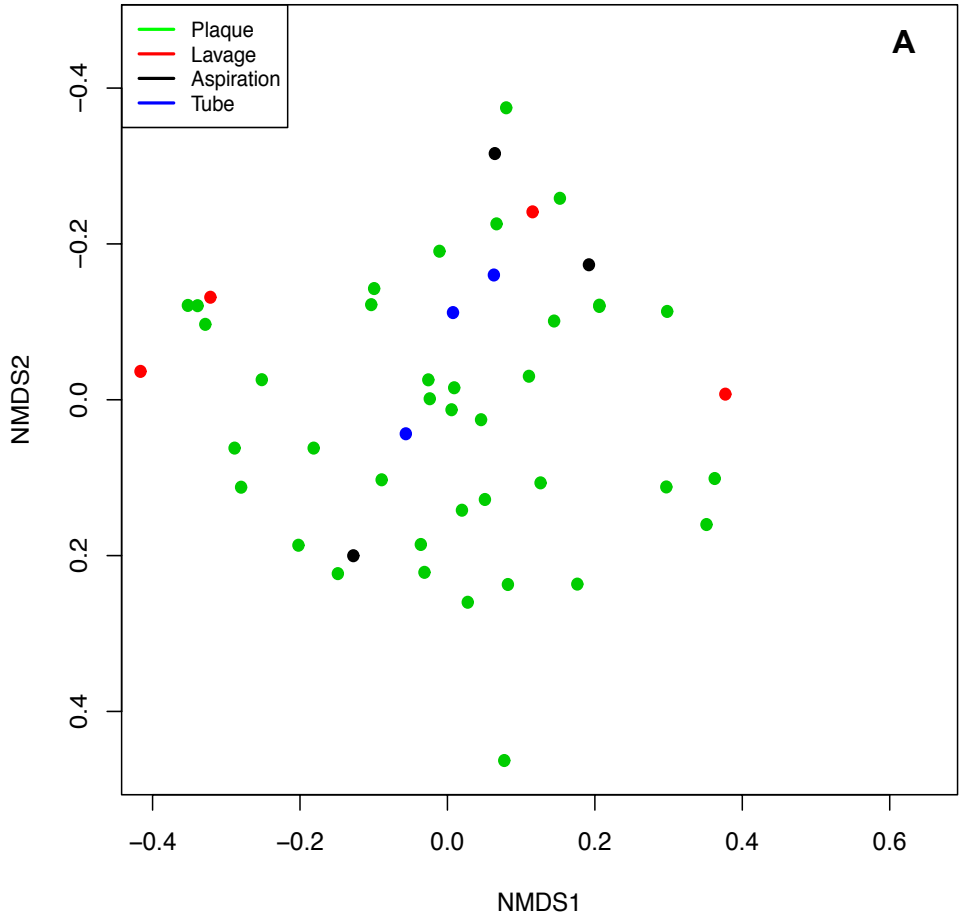

Figure 3.20A - NMDS analysis of microbial communities taken from all sample sites: dental plaque (green), NBL (red), subglottic aspiration (black) and ETTs (blue), based on the weighted distances.

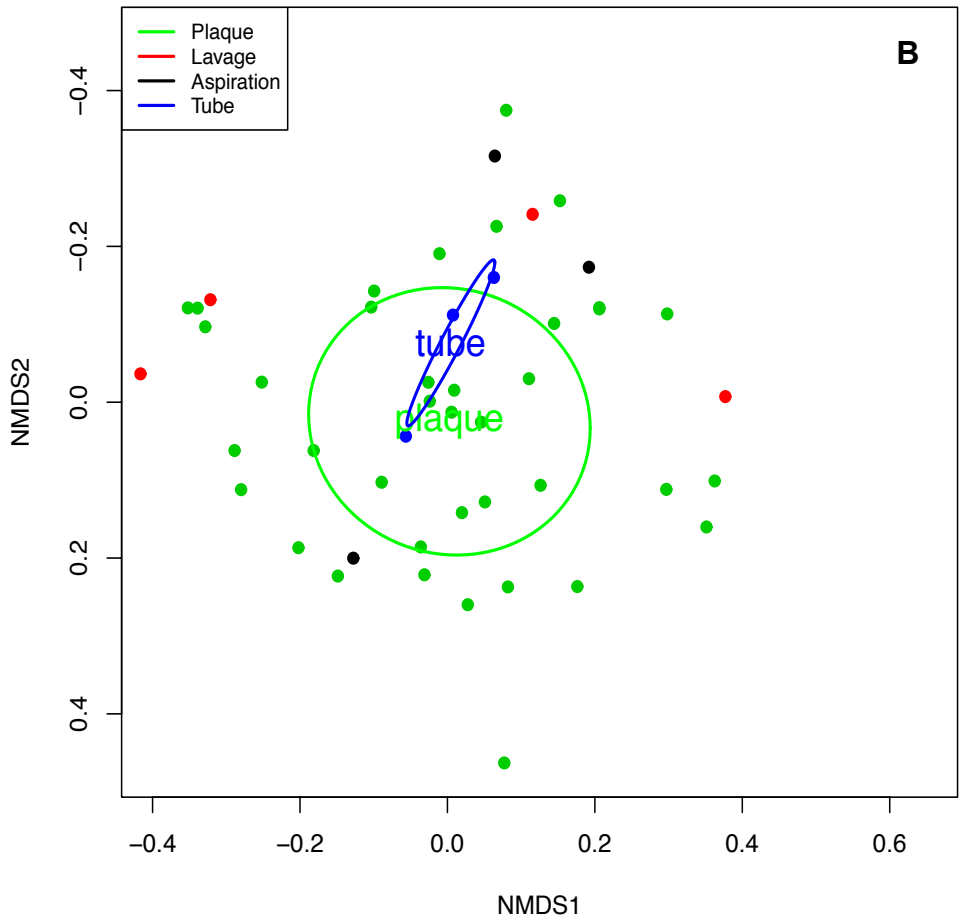

Figure 3.20B - NMDS analysis of microbial communities of dental plaque and ETT biofilms

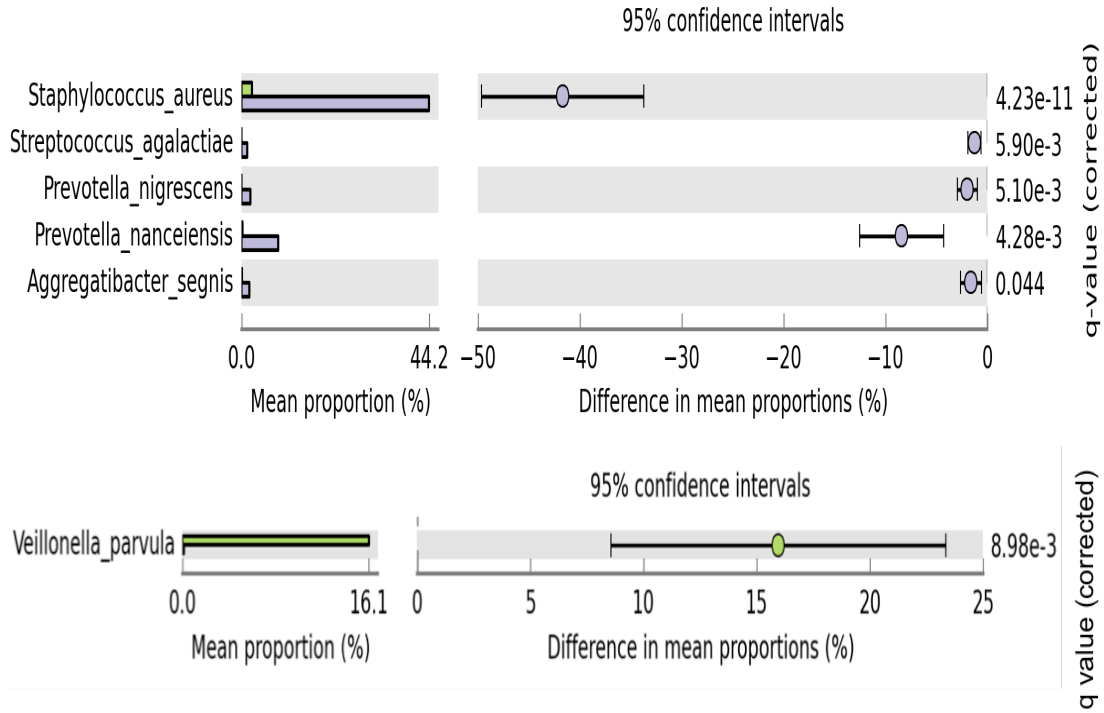

Figure 3.21 – Comparison of the isolation of six different microbial species from the dental plaque and the ETTs using t-tests. The first bar-row (green) represents dental plaque isolation, and the second bar-row (purple) represents the ETT. The mean proportion of sequence is situated on the left of the image, followed by the difference in mean proportions between the dental plaque and the ETTs. On the far right of figure 2 are the corresponding q-values for the t-tests.

3.4 Discussion

This is the first time that molecular community profiling has been used to characterise changes that occur within the microbial communities of dental plaque and the lower airways both during MV and post ETT-extubation. In addition, the microbiome of the lower airways and biofilms from extubated ETTs were analysed. Next-generation sequencing based technologies to enable microbial community profiling provide simultaneous identification of microorganisms as well as species quantitative data (Zaura, 2012).

During this study, MiSeq Illumina sequencing provided comprehensive microbial profiles for different clinical sites within mechanically ventilated patients, and highlighted significant increases in the detection of respiratory-relevant pathogens within dental plaque of mechanically ventilated patients. These findings confirmed and considerably extended those obtained from culture-based studies, that have previously demonstrated changes in microbial plaque composition which may facilitate the development of secondary respiratory infections such as VAP (Mori et al., 2006; Zuanazzi et al., 2010). In this context, it has been proposed that the placement of the ETT may be one of the drivers that changes oral environmental factors (Cairns et al., 2011; Lloyd et al., 2011). The results demonstrated that several 'non-oral' genera co-existed with oral microorganisms in the dynamic biofilm that is dental plaque. Physical alterations (such as the insertion of ETTs) may influence microbial displacement and translocation within the upper and lower respiratory system. The dynamics and influence of dental plaque may in fact be underestimated and the amount of dental plaque can be a significant contributing factor to several oral and systemic infections, such as VAP.

Respiratory pathogens were identified within the dental plaque during the early stages of MV, however a significant increase in pathogen abundance was demonstrated during MV (Table 3.1; figure 3.7). The result emphasises the potential of the mouth to act as a reservoir for pneumonia causing bacteria. Zuanazzi et al., 2010 performed similar community profiling analyses, in which the dental plaque and saliva of hospitalised patients was

analysed by microbial culture and culture-independent analysis. Zuanazzi et al., 2010, did not isolate *staphylococcus aureus,* however, coagulase negative staphylococci were present. In contrast, during the present microbiome study, *S. aureus* was the most abundant respiratory pathogen identified in ETT biofilms (Figure 3.18A, B) and was amongst the most abundant species of the dental plaque microbiome during MV (Figures 3.12 and 3.18). Performing high-throughput analysis alongside conventional cultivable studies is useful for revealing any differences in the methodologies and highlighting cases where respiratory pathogen detection was different between microbial culture and culture-independent analysis. Although such variation exists, the detection of respiratory pathogens in dental plaque and the lower airways reinforces results from previous studies using microbial culture analysis of dental plaque during MV.

Pseudomonas aeruginosa is considered a virulent and prominent species in the aetiology of VAP and other associated lower airways infections (Parker et al., 2008; Luyt et al., 2014). Interestingly, *Pseudomonas* species were rarely detected in the high-throughput analyses of the 13 mechanically ventilated patients. However, *P. aeruginosa* had previously been isolated in ~25% of dental plaque via microbial culture (Chapter 2 - 2.3.2). Previous studies have suggested that *P. aeruginosa* is a predominant coloniser of dental plaque during MV and a causative pathogen of VAP (Parker et al., 2008; Raad et al., 2011; Tarquinio et al., 2014). *Pseduomonas aeruginosa* is a frequent coloniser of both water and soil (Perkins et al., 2010). Evaluating the presence of *P. aeruginosa* in the water supply within the hospital could help detect one of the sources for this pathogen.

Current microbiological analysis relies on cost effective, readily available and reproducible microbial culture. *Pseudomonas aeruginosa* is a rapid coloniser on nutritional media and can effectively outcompete the growth of other microbial species and may therefore be misrepresented as a dominant species by culture. The composition of dental plaque is extremely diverse with many species not yet capable of being cultivated, reinforcing the potential for *P. aeruginosa*, to ‘out-compete’ and be over-represented on

microbiological media. Although, *P. aeruginosa* is considered an opportunistic pathogen of the oral cavity within mechanically ventilated patients, further high-throughput studies could elucidate the true extent of *P. aeruginosa* within dental plaque during MV. Future studies could investigate and compare commercially available total bacterial DNA extraction methods, as certain elution techniques could result in a low detection of Pseudomonas species. Further analysis concerning the detection of *P. aeruginosa* is warranted to determine whether *P. aeruginosa* should still be considered the predominant bacterium in this context, compared to *S. pseudopneumoniae, S. aureus* and Gram-negative species such as *E. coli.*

Dental plaque is known to be diverse and dynamic with its microbial constituents responding and adapting to their immediate microenvironment (Hojo et al., 2009; Robinson et al., 2006). Within this study, 5 major phyla and 40 genera were identified (Figure 3.4). These organisms ranged from frequently identified and studied genera such as streptococci*, Tannerella* species and *Oscillibacter* species; some of which lesser known genera have been revealed in similar community profiling studies (Galimanas et al., 2014).

Haemophilus influenzae, an organism associated with respiratory infection, was also detected within the dental plaque of mechanically ventilated patients. *Haemophilus influenzae,* can often colonise the upper and lower respiratory tract of healthy individuals and is considered a significant pathogen in respiratory infection (King, 2012).
Microbial changes occurred in a high proportion of mechanically ventilated patients, and at least one putative respiratory pathogen was detected in the dental plaque of all 13 patients analysed in this study. *Streptococcus pseudopneumoniae* has been suggested to be an early onset causative pathogen of VAP, alongside *S. aureus* and Gram-negative bacilli (Kalanuria et al., 2014). *Streptococcus pseudopneumoniae* was detected in 11 of the 13 mechanically ventilated patients (representing the larger cohort) during this study (Figure 3.16 and table 3.2). *Enterococcus faecalis,* an opportunistic

pathogen identified as being amongst the four most frequently occurring species in dental plaque during MV, has the ability to integrate into the polymicrobial dental oral biofilm *in situ* (Al-Ahmad et al*.,* 2010). *Enterococcus faecalis*, although not commonly regarded as a member of the oral microbiota in health, may be isolated from the oral cavity in some individuals and is interestingly frequently isolated from secondary endodontic infections (Al-Ahmad et al., 2009). Vancomycin-resistant enterococci are increasingly associated with hospital-acquired infections (HAIs), especially after the patient has been in receipt of antibiotics (Austin et al., 1999; Rengaraj et al., 2016). It may therefore not be surprising that *Enterococcus* colonisation persisted during MV, emphasising the vast changes that are occurring in the homeostasis mechanisms in dental plaque and host immunity.

Sachdev et al., 2013 reported that the amount of dental plaque in hospitalised and ventilated patients increases. Associating increases in gingival inflammation is one marker of host immune activity in response to such elevated plaque levels thus highlighting the importance of oral measures such as plaque scoring in critical care medicine (Wise & Williams, 2013). DMFT scores were recorded for all patients recruited to the observational cohort study (2.3.1.5). Correlation analysis between DMFT scores and total dental plaque abundance (OTU abundance average per patient) was performed to analyse microbial communities in relation to DMFT scores (Figure 3.15). Although not a measure of current health, DMFT scores can provide an indication of previous oral health status (Becker et al., 2007). Analysis (Figure 3.15) revealed there was no positive correlation between increased DMFT scores and mean dental plaque abundance, in fact a slight negative correlation was found. This may suggest that patients with previous good oral hygiene may be at risk of increasing dental plaque and furthermore, the introduction of respiratory pathogens during MV. Along with the DMFT score, future work could investigate if correlations exist between DMFTs, dental plaque and gingival index scores, and respiratory pathogen colonisation of dental plaque. Such correlation analyses on larger cohorts

could reveal whether patients admitted with poor pre-existing oral hygiene were at greater risk of respiratory pathogen colonisation of dental plaque in comparison to patients with previously good oral hygiene.

In addition to microbial profiling of dental plaque during MV, NBL, subglottic aspirations and ETT biofilms were characterised for microbial content during MV. A total of 10/48 samples processed for community profiling were non-dental plaque samples. Within these microbial profiles, both oral-indicator species including *Prevotella* species and respiratory pathogens were detected (Figures 3.17 and 3.19). These findings may support the theory of microbial translocation from the oral cavity towards the lower airways (Li Bassi et al. 2015; Cairns et al. 2011). NMDS analysis comparing the microbiome at different clinical sites (Figure 3.20A, B) analysed 3 patients' ETT biofilms and compared these to 38 dental plaque samples. Preliminary results indicated overlapping clusters, highlighting similarities between the microbiomes of dental plaque and ETT biofilms. This analysis therefore, although indicative of microbial similarities between sampling sites, in this instance did not hold true statistical power, due to incomplete and uneven sample size. A series of t-tests were performed on six different species, comparing the mean proportion of sequences detected from either dental plaque or ETT biofilms. *S. aureus, Streptococcus algalactiae* and interestingly *Prevotella* species were present in higher numbers in the ETT biofilm compared to dental plaque.

Analysis of the patients' dental plaque during their recovery and post extubation, revealed a decrease in detection of respiratory pathogens for many patients, and suggests that the microbial community begins to revert back to one that is predominantly comprised of healthy oral microbiota (Figure 3.13). Respiratory pathogens, although at a lower abundance, were detected from dental plaque of patients into their recovery period, suggesting that once these organisms proliferate into the biofilm, they may resist replacement whilst the patient is still immunocompromised. Future studies could analyse dental plaque with a larger sample size and over a longer time

period post recovery to statistically reflect the community and recovery period of critically ill patients

Conclusion

Microbial community profiling was successfully applied to characterise the microbiome of dental plaque in mechanically ventilated patients, both during MV and into the recovery period when the patient was liberated from mechanical ventilation. A significant 'microbial shift' in the composition of dental plaque was demonstrated for patients with potential respiratory pathogens including *S. aureus, S. pseudopneumoniae* and *E. coli* increasingly detected during MV. Interestingly, and in contrast to previous culture studies (Chapter 2), *P. aeruginosa* was not detected in this molecular analysis.

The lower airways and ETT biofilms were also subjected to characterisation via community profiling. The detection of oral species within the lower airways and strict oral anaerobic species including *Prevotella* within the ETT biofilm highlights their presence, and preventing the translocation of dental plaque may be important.

Fulfilling the chapter's aims, dental plaque was further characterised into the post-ETT extubation period whereby both the presence and abundance of respiratory pathogens was reduced following extubation. However, some of these microbial species could still be identified at reduced number within the community, and may therefore still represent risk factors for infection in the longer term. Both oral indicator species and potential respiratory pathogens were detected in oral and lower airways. This is the first comprehensive characterisation of the microbiomes of dental plaque and lower airways in this patient group. A better understanding of the compositional changes within dental plaque, and the extent of microbial translocation may inform interventional strategies to reduce incidence of VAP.

Chapter 4

A longitudinal study of saliva properties and protein composition during mechanical ventilation

4.1 Introduction

The oral cavity is unique, containing both hard surfaces, namely the teeth, and soft mucosal tissues, which are constantly subject to change both in health and in illness. In addition, the oral microbiota is complex with distinct microbial communities within different oral sites and currently over 1,000 bacterial species have been identified (Lazarevic et al., 2009; Wade, 2013b). Dental plaque, an archetypal biofilm upon tooth surfaces, harbours an estimated 500 different bacterial species indicating that the microbial structure is not uniform, more so ever-changing (Rosan & Lamont, 2000).

Streptococcus species are recognised as primary pioneer colonisers of teeth and therefore initiators of dental plaque development (Denepitiya & Kleinberg 1982). Early microbial colonisers within dental plaque, predominantly members of the Streptococci genera including Streptococcus *mitis* and *Streptococcus salivarius*, adhere to immobilised proteins within saliva such as statherin that are anchored on the enamel surface of the tooth (Chaudhuri et al., 2007; Rudney et al., 2003). Saliva therefore promotes positive bacterial selection thus contributing to the production of dental plaque and biofilm formation upon the tooth surface (Sekine et al., 2004).

Salivary mucins can bind to bacterial cells, ultimately maintaining adequate levels of bacterial adhesion to the tooth's enamel. The composition of saliva, in particular free calcium and phosphate form buffering systems helping to maintain the integrity of the tooth (Amerongen & Veerman, 2002; Lamkin & Oppenheim 1993). Salivary proteins extend to the inclusion of antibacterial peptides such as lysozyme and lacotransferrin serving to protect and defend against organisms and foreign debris entering the oral cavity (Gorr & Abdolhosseini, 2011; Silva et a., 2012).

In the past 10-15 years there have been numerous studies suggesting that changes occur to the composition of dental plaque in critically ill and

mechanically ventilated patients (Scannapieco et al., 1992; Fourrier, et al., 1998; Heo et al., 2008; Zuanazzi et al., 2010; Jones, Munro & Grap, 2011; Sachdev et al., 2013). The current study has documented the extent of dental plaque changes during MV by longitudinal microbial culture and microbiome community profiling (Chapters 2 and 3). Dental plaque was shown to become heavily colonised with respiratory pathogens including *S. aureus, E. coli, P. aeruginosa* and *S. pseudopneumoniae* during MV, species which in many cases are absent at the beginning of ventilation. These microbial changes will likely have been driven by alterations to the local and/or systemic environment. Potential variables that could drive microbial compositional changes within dental plaque include saliva flow, pH and proteomic composition.

Currently, there is little evidence of large studies investigating saliva properties and any subsequent role that saliva may have in dental plaque colonisation by respiratory pathogens. Dennesen et al., 2003, have however measured saliva flow of 44 hospitalised patients (24 during intubation and 20 elective surgery). They reported inadequate salivary flow in intubated intensive care patients, with a nearly absent stimulated salivary flow during intubation, and comparably, only a temporary reduced postoperative stimulated salivary flow for elective surgical patients (Dennesen et al., 2003). Most existing literature addressing salivary flow during MV, or assessing the benefits on the application of oral gels to intubated patients are primarily focused around oral decontamination, and oral moistening (O'Reilly, 2003; Scannapieco et al., 2009). In recent years there has been much emphasis on the value of saliva as a prognostic and diagnostic fluid with proteomic changes in saliva being used to assess and potentially predict certain disease progression (Schipper et al., 2007; Pfaffe et al., 2011). Use of saliva in this manner has the advantage of being a minimally invasive process.

The overall aim of the work presented in this chapter was to determine if there were detectable changes in the saliva of patients during and after mechanical ventilation and to relate any changes to the composition of dental plaque.

Specifically, the objectives were:

(1) - To determine whether there were changes in salivary parameters during MV, including volume, protein concentration and pH.

(2) – To analyse the proteome of saliva during MV and in the post-ETT extubation period.

(3) - To quantify pro-inflammatory cytokines in saliva, plasma and ETT fluid during MV and (saliva) into the post-ETT extubation period.

4.2 Materials and Method

4.2.1 Ethical approval

Ethical approval was obtained from National Research Ethics Service (NRES) within the Research Ethics Committee (REC), Wales (Ref: 13/WA/0039) for a single NHS organisation study on 20th June 2013 to evaluate dental plaque biofilms and salivary parameters during the course of MV (2.2.1). Mechanically ventilated patients at a single University Hospital Critical Care Unit were eligible for inclusion in the study if they were aged >18 years, had >8 original teeth, anticipated period of MV >24h, and an expected survival of >24 h. Informed consent for participation in the study was obtained from the next of kin by the responsible clinician (2.2.2).

Ethical approval was granted on April 2014 from the School of Dentistry, Cardiff University, for the collection of 5 saliva samples over a period of 10 d from 5 healthy volunteers, forming the control group. The inclusion criteria for the healthy volunteers was as follows: >18 years, >8 original teeth, and not in receipt of prescription medications. The collected saliva was anonymised and stored at -80°C following HTA guidelines for fluid containing human cells.

4.2.2 Saliva, plasma and ETT fluid from mechanically ventilated patients

Saliva and blood were collected three times during the first week of MV and weekly thereafter until ETT extubation. All collections were undertaken at the same time in the morning of the due date. Saliva was also collected weekly during the post ETT extubation recovery period, and for up to 3 months post-ICU discharge.

Whole saliva was obtained using Salivettes® (Sarstedt™), an effective and non-laborious device containing a sterile cotton plug, within polypropylene collection tubes. The cotton plug was placed and held securely in the oral cavity of the individual for 45 s and placed in the polypropylene tube (Topkas et al., 2012). Saliva was recovered by centrifugation at 1,000 × *g* for 2 min.

The supernatant (saliva) was transferred into a 1.5 ml microcentrifuge tube and the saliva stored at -80°C within 5 h of collection to prevent activity of proteases (Thomadaki et al., 2011). Where possible, fluid was also collected from the inner lumen of extubated ETTs using sterile swabs. The swab was then immersed in 1 ml of PBS and stored -80°C prior to analysis.

Whole blood was collected in an anticoagulant (EDTA) treated vial to prevent clotting. The tubes were inverted gently between 8-10 times to ensure even distribution of EDTA before centrifugation. Blood cells were separated via refrigerated centrifugation for 10 min at 1,000-2,000 × *g*. After blood components were separated, plasma was collected using a pipette, transferred to a 1.5 ml screw cap tube and stored at -80°C.

4.2.3 Measurement of salivary parameters: volume, pH and protein concentration

4.2.3.1 Measuring salivary volume and pH

The volume of collected saliva (obtained over 45 s) was measured using a pipette and a calibrated collection tube. A microelectrode pH meter (Fisher) attached to a pH reader (Eutech Instruments) was used to measure the pH.

4.2.3.2 Bicinchoninic acid assay (BCA) for total protein measurement

Whole saliva samples were diluted 1:10 in double distilled water (ddH_2O). For analysis of fluids obtained from the ETTs, a 1 cm section of the lumen was scraped with a cotton swab for 30 s and placed in 500 µl of Sputasol™ (Oxoid) and vortex mixed for a further 30 s. Cells were removed by centrifugation at 14,000 × *g* at 4°C for 20 min.

Serial dilutions of bovine serum albumin (BSA) from 1 ng/µl were prepared in ddH_2O in the wells of 96-well microtitre plates. These dilutions were used as concentration standards, and were prepared in triplicate for each assay. A 25-µl volume of the diluted sample was added to duplicate clean wells. A 200-µl volume of BCA solution (5 ml BCA to 100 µl reducing agent (copper

sulphate solution)) (Sigma) was added to all test and standard wells. The microtitre plate was incubated at 37°C for 30 min and the absorbance measured at 570nm. A standard curve was generated from the diluted BSA and used to quantify protein concentrations of test samples.

4.2.4 Gel based salivary proteomics

4.2.4.1 SDS-PAGE 1D electrophoresis

A total of 13 µl of 30-ng/µl protein (diluted in ddH_2O water, using protein concentration data obtained from 4.2.3.2), 2 µl of reducing agent (x10 NuPAGE) and 5 µl of SDS buffer (x4 NuPAGE) was loaded into each well of a microtitre plate. The samples were placed in a heating block at 70°C for 10 min before loading. An 800-ml volume of running buffer (NuPAGE SDS-Invitrogen) was prepared using 40 ml of 3-(N-morpholino) propanesulfonic acid (MOPS) × 20 buffer (NuPAGE) and 760 ml of ddH_2O. A 200ml volume of this solution was added to the cathode section of the tank (containing the gel), the remaining buffer was added to fill the tank. A 20-µl volume was added to each well (4-12% gel), including 20 µl of unstained molecular marker (Invitrogen). Electrophoretic conditions were 200 V/ cm^2 (120 mA, 25 W) for 55 min (plus 25 min for extra separation if required).

4.2.4.2 Staining electrophoretic protein gels

4.2.4.2.1 Colloidal Coomassie Blue staining

Electrophoretic gels were transferred to weighing boats and placed in fixing solution (40 ml ddH_2O, 50 ml methanol and 10 ml of glacial acetic acid) on a rocking platform at room temperature for 10 min. The fixing solution was discarded and replaced with 20 ml of staining solution A (55 ml of ddH_2O and 20 ml of methanol). The gel was returned to the rocking platform at room temperature for 10 min, and 5 ml of stainer B (Invitrogen) added to stain overnight. The staining solution was then removed and the gel washed and kept hydrated in 200 ml of ddH_2O prior to scanning using ImageScannerIII

(GE Healthcare). For long-term storage of the gel, a 1% acetic acid solution was used as a preservative.

4.2.4.2.2. Silver nitrate staining

For silver nitrate staining, the gel was transferred to a plastic weighing boat and placed on a rocking platform at room temperature. The procedure was as described by the PlusOne Silver Nitrate Staining Kit (GE Healthcare). The gel was placed in 250 ml of fixing solution (10% acetic acid and 40% ethanol) for 30 min. The fixing solution was discarded and replenished with 250 ml of sensitising solution (75 ml of ethanol, 17g of Na-acetate and 10 ml of 5% Na-thiosulphate) for 30 min. The solution was discarded and replaced with 250 ml of ddH_2O for a total of 3 separate 5 min washing steps. After the final ddH_2O was discarded, a 25-ml volume of 2.5% silver nitrate in 250 ml of ddH_2O was added for 20 min, followed by two 5 min washing steps in ddH_2O. After the ddH_2O was discarded, a 250-ml volume of developing solution (6.25 g of Na-carbonate and 100 µl of 37% formaldehyde) was added to the gel. This solution was left for 4 min, discarded and replaced with 250 ml of stopping solution (containing 3.65 g of EDTA) for 10 min. Following three final 5 min ddH_2O washes, the gel was scanned using the ImageScannerIII (GE Healthcare) and placed in a preservative solution of 500 ml of 1% acetic acid.

4.2.5 2D gel electrophoresis (2DE)

4.2.5.1 Protein precipitation for 2DE

The starting protein concentration needed for 2D gel electrophoresis was 150 µg/µl (with a maximum volume for the kit of 100 µl; using the '2D clean up kit' by GE Healthcare). A 300-µl volume of protein precipitant was added, the sample was vortex mixed for 5 s and incubated on ice for 15 min. A 300-µl volume of co-precipitant was added and the sample was vortex mixed for 5 s and centrifuged at 12,000 × *g* for 5 min. The supernatant was removed and a further 40-µl volume of co-precipitant was added on top of the pellet, and

placed on ice for 5 min. The samples were centrifuged at 12,000 × *g* for 5 min with the supernatant discarded, replenished with 25-µl of ddH_2O, and vortex mixed until the sample was dispersed into the liquid. A 1-ml volume of chilled wash buffer and 5 µl of wash additive (GE Healthcare) was added to the sample and incubated at -20°C for 30 min with a 30 s vortex mix every 10 min. The samples were centrifuged (12,000 × *g* for 5 min) and the pellet was left to dry for 5 min.

4.2.5.2 Isoelectric focusing (IEF)

The protein pellet (obtained from section 4.2.5.1) was resuspended in 116 µl of rehydration solution (7M urea, 2M thiourea and 2% (v/v) 3-[(3-cholamidopropyl) dimethylammonio]-1 propanesulfonate (CHAPS)) and vortex mixed for 5 s. A 1-µl volume of bromophenol blue (10 mg/mL), 2 µl of IPG Buffer (GE Healthcare) and 6.25 µl of dithiothreitol (DTT, 50 mM) were added to the resuspended protein pellet to create the rehydration solution.

A total volume of 125 µl was required to distribute evenly into the IPG gel strip (Immonbiline™ DryStrip pH 3-10 NL, 7cm) IPGphore coffin. The immobilised pH gradient (IPG) gel strip was inserted into the coffin gel. An 800-µl volume of DryStrip cover fluid (mineral oil, Fisher) was placed over the strip and the coffin was then placed on the IEF manifold for an initial rehydration period of 12 h at 20°C. IEF parameters were 50 microamps (µA)/strip at 20°C (step and hold 500 V 1 h, gradient 1000 V 2 h, step and hold 1,000 V 1 h, gradient 8,000 V 2 h and step and hold 8,000 V 8 h).

4.2.5.3 Equilibration of the IPG strip

The IPG strip was removed from the gel-coffin and placed in 5 ml of reducing solution (0.5 ml of NuPAGE sample reducing agent and 4.5 ml of 1× NuPAGE LDS buffer), which was gently rocked for 15 min. The IPG strip was then transferred to 5 ml of alkylating solution (116 mg of iodoacetamide and 5 ml of 1× NuPAGE LDS buffer) for 15 min on the rocking platform. The IPG strip was then transferred into the loading well of the Zoom™ gel (Invitrogen).

4.2.5.4 SDS-PAGE using Zoom™ gels

A 200ml volume of running buffer (prepared as previously described, 4.2.4.1) was added to the centre chamber, and the IPG strip was transferred to the loading well of the gel (NuPAGE 4-12% Bis-Tris Zoom Gel). A 5-µl volume of a 1:20 Mark 12 unstained molecular marker (Invitrogen) was added to the single well. The SDS-PAGE and protein staining was performed as previously described (sections 4.2.4.1 and 4.2.4.2).

4.2.5.5 Preparation of peptides from gel electrophoresis for Mass Spectrometry (MS)

4.2.5.5.1 Gel spots and de-staining

Using a manual spot cutting pipette tip, targeted spots were cut and transferred into an Ettan Disgestor 96 well plate (total gel volume 2.65mm^3). For quality control, one gel plug was cut from the protein marker (Invitrogen Unstained Protein Standard Mark12) – β-galactosidase (116.3kDa). Any remaining liquid in the wells was removed to prevent protein leeching from the bands into solution. A 50-µl volume of acetonitrile (50% acetonitrile (ACN) (v/v) in 0.1% (v/v) trifluoroacetic acid (TFA)) was added to the gel plugs for 5 min until the plugs turned opaque. To de-stain from coomassie blue, each gel plug was covered with 50 µl of 50% (v/v) ACN in 25 mM ammonium bicarbonate (NH_4HCO_3) and this was gently rocked for 1 h.

The supernatant was discarded and the protein plug washed ×3 times in 50 µl of 25 mM NH_4HCO_3. The gel plugs were dehydrated in 50 µl of 50% (v/v) ACN for 15 min followed by rehydration in 50 µl 25 mM NH_4HCO_3 for 10 min. This process was repeated twice and the spots were then incubated at 65ºC for 20 min.

4.2.5.5.2 Reducing and alkylation

Disulphide bonds of the proteins were irreversibly broken to achieve optimum unfolding of the tertiary structure for downstream protein identification. A 25-µl volume of 10 mM DTT in 25 mM NH_4HCO_3 was added to the 96-well plate, covered and incubated at 56ºC for 1 h. Once the plate had cooled for 15 min,

the supernatant was removed and replenished with 25 µl of 55mM iodoacetamide in 25 mM NH_4HCO_3, covered and incubated for 45 min at room temperature in the dark. The gel plugs were washed with 25 µl of 25 mM NH_4HCO_3 for 10 min and two dehydration steps were performed as described previously (section 4.2.5.5.1).

4.2.5.5.3 Trypsin digestion

Trypsin (V5111 Promega UK Ltd) was prepared to a final concentration of 12.5 ng/µl in 25 mM NH_4HCO_3. A 5-µl volume of trypsin was added to fully dehydrate the gel plug and the plate was sealed and incubated at 37ºC for 20 min. Once fully rehydrated, the plug was covered with 10 µl of 25 mM NH_4HCO_3 (to keep the gel wet during digestion) and incubated for 4 h at 37ºC.

4.2.5.6 Spotting peptides onto the mass spectrometry plate for Matrix-Assisted Laser Desorption Ionization (MALDI) analysis

Peptides were resuspended from the gel plug in 5 µl of 50 ACN % (v/v) in 0.1% TFA (v/v) and sealed for incubation at 60°C for 1 h. A 0.5-µl volume of peptide solution was spotted onto the mass spectrometry (MS) plate and left to air dry. After 10 min, a 0.5-µl volume of matrix (section 4.2.6.5) was added to each spot. Standards using pre-defined calibrations were also added to the MS plate (Applied Biosystems MALDI TOF/TOF analyser 4800). Peptide MS loading was devised and employed by qualified employees at The Henry Wellcome Building (Cardiff University).

4.2.6 Gel free proteomics: Liquid chromatography, MALDI MS & Mascot global protein identification (LC-MS/MS)

4.2.6.1 Protein extraction from extracellular saliva

Saliva was incubated on ice with a 500-µl volume of lysis buffer (1 ml of tergitol-type nonyl phenoxypolyethoxylethanol detergent (NP-40) (10% v/v), 200 µl of triethylammonium bicarbonate (1 M), 100 µl of protease inhibitor (20 mg/ml) and 8.7 ml of distilled water for 15 min. Saliva was centrifuged

(Allegra 21R, Beckman Coulter) at 14,000 × *g* for 5 min at 4°C and the protein levels were quantified using the protein assay as described in 4.2.3.2.

4.2.6.2 Trypsin digestion for iTRAQ™ labelling

Trypsin digestion was completed following sample reduction, denaturing and cysteine blocking. A-20 µl volume of dissolution buffer, 1 µl of denaturant reagent (Applied Biosystems) and 2 µl of reducing reagent (Applied Biosystems) were added to samples containing 100 µg of protein. After gentle vortex mixing the tubes were incubated at 60°C for 1 h. A 1-µl volume of cysteine blocking reagent (Sigma) was added to each sample, vortex mixed and incubated at room temperature for 10 min. A 2-µl volume of 0.8 µg of trypsin solution was added and gently vortex mixed. The samples were incubated overnight (12-16 h) at 37°C. Each tube was centrifuged for 1 min at 6,500 ×*g* to move the sample digest at the bottom of the tube for iTRAQ™ labeling.

4.2.6.3 iTRAQ™ labelling

iTRAQ™ reagent vials 114-117 (Sciex) were mixed with 70 µl of ethanol added and gently vortex mixed for 10 s. The contents of one iTRAQ™ reagent vial were mixed with extracted salivary proteins representing one saliva sample and incubated for 1 h.

4.2.6.4 Liquid chromatography (LC)

iTRAQ™ labeled peptides were fractionated using LC for the downstream application of MS/MS analysis to provide peptide sequences. The pump wash was filled with 50% isopropanol, with the transport solvent at 2% ACN in 0.05% TFA. Buffers A (2% ACN) and B (90% ACN) (flush and washing buffers respectively) were prepared for each run. Buffer A was flushed through the system for 20 min ensuring the buffer flowed to the needle of the Probot. For quality control measures, Cytochrome C (1:80 water) was run as an internal control to validate the performance of the LC. Two runs were performed to retrieve the optimum data and spot sets for MALDI MS plating,

the 1-D long run (210 min) and the short salt plug run (6×75 min), using a reverse phase column aiding the sample spotting onto the MALDI plate.

4.2.6.5 MALDI Matrix (α-cyano-4-hydrxycinnamic acid) preparation

Matrix facilitated the ion adsorption of sample matter from the plate into the MALDI workflow. A 1-ml volume of 70% ACN/0.1% TFA (v/v) was added to a prepared α-cyano-4-hydrxycinnamic acid vial (Sigma) producing 10 mg/ml. The matrix was further diluted to 2 mg/ml (800 µl to 3.2 ml of water) with 2 µl of 50 pmol/µl Glu-Fib spike. The matrix was placed in a syringe (1 ml) and loaded into the Probot mechanical spotter.

4.2.6.6 MALDI MS peptide analysis

Samples were spotted onto the MALDI plate using a Probot fraction collector running in conjunction with LC. Two additional calibration spots were loaded onto the plate (8% calibration mix and 92% matrix solution) as controls. Figure 4.1 is a schematic highlighting the gel-free proteomic workflow used.

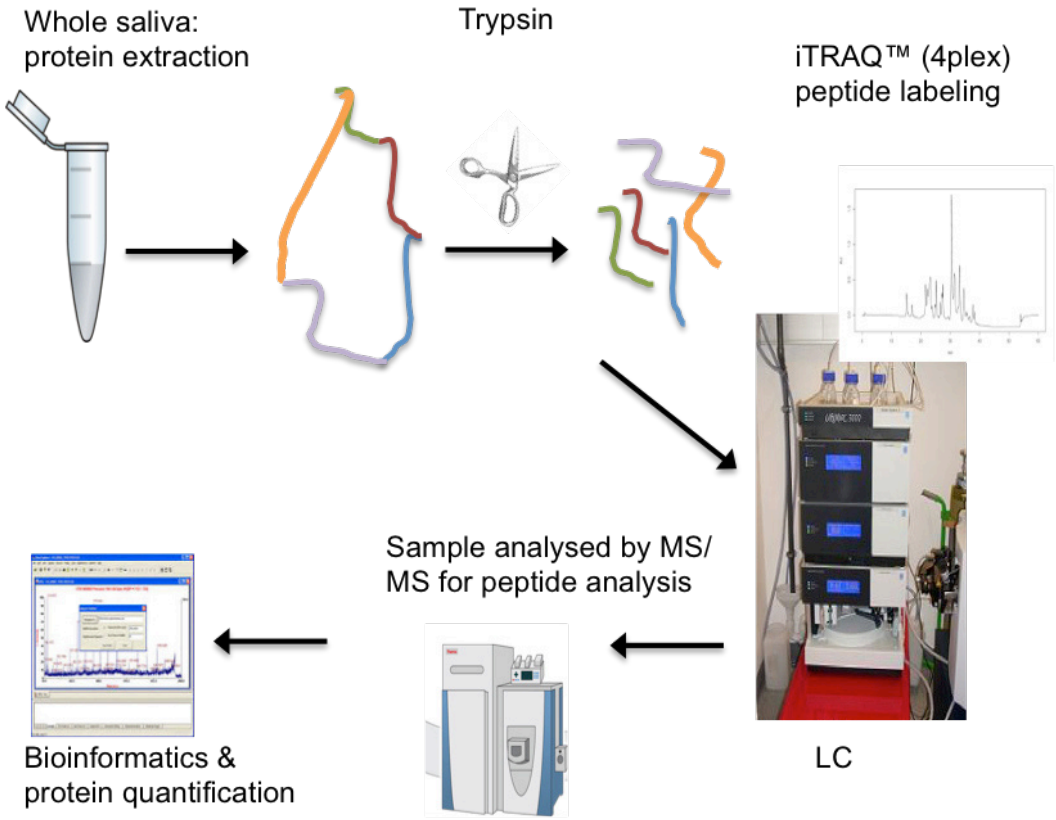

Figure 4.1 - Schematic illustrating the stages for global protein identification from whole saliva, adapted from Amado et al., 2013.

4.2.6.7 Bioinformatics: proteomic data analysis

During analyses, saliva from three time points during MV were analysed from one patient, whilst saliva from four time points (over a period of 10 d) was analysed from one healthy volunteer. Each proteomic workflow (LC-MS/MS of pooled saliva per individual) was repeated twice. Peptides were identified according to peptide sequence matches at a 95% confidence, 2+ peptide match level using search engines MASCOT and Paragon™ (applicable with downstream quantitative software Protein Pilot™).

Gel-based (SDS-PAGE and 2DE) protein identification analysis was performed using MS coupled with Mascot. For gel-free quantitative analysis, raw peptide sequences were input into Protein Pilot™ coupled with the Paragon algorithm to automatically optimise peptide identification. Paragon™ uses hybrid sequence tags and precursors to simultaneously identify and analyse protein modifications in large quantities. ProteinPilot™ transferred the proteomic data from MS (protein identification using Mascot search engine) to generate expression peaks. Pooled saliva samples were identified according to their iTRAQ label for differential protein expression (a relative measure of quantitation) comparing proteomic content of saliva between mechanically ventilated patients and healthy volunteers. Protein classification and biological function comparisons were performed using a protein-to-gene converter tool- neXtProt coupled with the Protein Analysis Through Evolutionary Relationships (PANTHER) classification system, using gene names (Vitorino et al., 2012).

4.2.7 IL-6 detection in plasma by ELISA - pilot assay to determine cytokine recovery

ELISA was used to detect IL-6 cytokine in plasma from mechanically ventilated patients. Fifty µl of capture antibody (1:250 anti-human IL-6 capture antibody eBioscience) were placed in the wells of a Nunc ELISA plate which was incubated with shaking (100 rev/min; 30 s) overnight at 4°C. The wells were aspirated, washed (×3; washing buffer – PBS, 0.05% Tween-

20) and 100 µl of blocking buffer (×5 assay diluent; eBioscience) was added to each well and incubated for 2 h at room temperature. After a further three washing steps, 50 µl of control standards were added, ranging between 200 pg/ml to 3.125 pg/ml. A 150-µl volume of sample was added to triplicate wells, and the plate was incubated overnight at 4°C. The wells were washed (×6) and a 100-µl volume of detection antibody (IL-6 anti-human detection antibody 1:250, eBioscience) was added to each well, and incubated at room temperature for 1 h. The plate was washed (×6) and 50 µl of horseradish peroxidase (HRP) (eBioscience) added at room temperature for 30 min. After washing (×6), 50 µl of tetramethylbenzidine (TMB) ELISA substrate (×1; eBioscience) was added, and the plate placed in the dark for 30 min for colour development. The reaction was stopped with 50 µl of stopping solution (ELISA stopping solution; eBioscience), and the absorbance read at 450_{nm} wavelength.

4.2.8 Cytometric bead array (CBA) analysis of inflammatory cytokines in saliva, plasma and fluid from ETTs

4.2.8.1 Preparation of standards: human inflammatory cytokines

All reagents unless otherwise stated were from the Cytometric Bead Array (CBA) Human Inflammatory Cytokines Kit (BD Biosciences). Standards were reconstituted as described by the manufacturer.

4.2.8.2 Preparation of cytokine beads

Each capture bead preparation was vigorously vortex mixed and 5-µl transferred into single tube for each sample. For plasma analysis, beads were centrifuged (200 × *g* for 5 min), the supernatant was removed and replaced with an equal volume of serum enhancement buffer before vortex mixing. The mixed capture beads were incubated for 30 min at room temperature in the dark.

4.2.8.3 ETT preparation for cytokine analysis

The lumen of the 1 cm ETT section was scraped with a cotton swab for 1 min, and the swab was then placed into 1 ml of Sputasol™ (Oxoid) in a microcentrifuge tube. The preparation was centrifuged (16,000×*g*, 1 min) and the supernatant removed and used for cytokine analysis.

4.2.8.4 Cytokine bead assay for saliva and ETT fluid

Saliva samples were diluted based on total protein concentration (determined in 4.2.3). ETT fluids were initially added undiluted from the preparation described above. A 25-µl volume of the diluted samples (and all standards and controls) was transferred into a labeled microcentrifuge tube (StarLab), and a further 25 µl of the vortex mixed pooled capture beads added to all samples. A 25-µl volume of pro-inflammatory cytokine PE detection reagent was added to all assay tubes and gently mixed. All assay tubes were incubated in the dark at room temperature for 3 h. A 500-µl volume of wash buffer was added to each assay tube and centrifuged (200×*g* for 5 min). The supernatant was discarded, leaving the cytokine beads, which were re-suspended in 150-µl of wash buffer and transferred into a flow acquisition tube (BD Falcon^{TM}) for flow cytometry multiplex analysis.

4.2.8.5 Cytokine bead array for plasma samples

A 25-µl volume of undiluted sample was transferred into a labeled microcentrifuge tube (StarLab), and a further 25 µl of the vortex mixed pooled capture beads added. All assay tubes were incubated in the dark at room temperature for 90 min. A 500-µl volume of wash buffer was added to each assay tube and centrifuged (200 × *g* for 5 min). The supernatant was discarded, leaving 50 µl of liquid above the pellet and a 25-µl volume of human inflammatory cytokine PE detection reagent was added to all assay tubes, and gently mixed. Tubes were incubated away from direct light exposure at room temperature for 90 min. The washing step before bead analysis was repeated as with the saliva samples (4.2.8.4).

4.2.8.6 Cytokine FACS analysis

For the multiplex analysis there were 6 different cytokine parameters with different measured fluorescent intensities, ultimately allowing the collection of six different clusters. A sandwich complex (capture bead and analyte (cytokine) and detection reagent) was measured using the flow cytometer.

The Flow Cytometric Analysis Program (FCAP) array™ software was used to analyse raw data collected from the cytometer, assigning each bead (cytokine) to one of six clusters and creating a standard curve for each detected cytokine bead. Fluorescence-activated cell sorting (FACS) was performed and the standard curves used to measure the mean fluorescence intensity and thus cytokine concentration in each test sample.

4.2.9 Statistical analysis

Mechanically ventilated patients were grouped in relation to respiratory pathogen colonisation of dental plaque based on previous microbiological results (Chapters 2 and 3). A series of t-tests were performed analysing salivary volume, pH (untransformed hydrogen ion concentration data) and total protein concentration during MV in relation to respiratory colonisation of dental plaque. All statistical analysis was performed using IBM SPSS v20.

Non-parametric analysis was performed upon cytokine analysis using one-way ANOVA (Kruskal- Wallis) (Rogers, 2013). In addition, correlation analysis between cytokine expression and previous microbiological findings (respiratory pathogen colonisation of dental plaque) was also undertaken using R script programming and STAMP software (3.2.6).

4.3 Results

4.3.1 Salivette® processing

Saliva samples were collected from 107 mechanically ventilated patients during MV and where possible into the post-ETT extubation period. A total of 488 Salivettes® were processed, yielding 136 saliva samples for analysis.

Over the course of 10 d, 5 saliva samples were collected from 5 healthy volunteers (n=25), representing a control group. The control group age range was between 23-35 years, 3 males and 2 females.

4.3.2 volume of saliva during mechanical ventilation

Of 107 patients, 36 failed to produce saliva at a detectable level either during MV or into the post-ETT extubation period. A further third of patients (n=37) only produced sufficient saliva only at the commencement of MV, with no detectable saliva produced during MV (Table 4.1). The dataset of measured salivary parameters (volume, pH, protein concentration) for individual saliva samples and each mechanically ventilated patient is presented in Appendix III. For analysis of salivary volume, patients were grouped according to previous microbiological culture and next-generation sequencing findings (Chapters 2 and 3) dependent on whether respiratory pathogens were isolated within dental plaque during MV.

Saliva production decreased for 52 mechanically ventilated patients during ETT intubation. Whilst there was an average reduction in the salivary volume during MV for those patients exhibiting respiratory pathogen colonisation within dental plaque (Figure 4.2), the reduction was not statistically significant for these patients in comparison to those patients not colonised with target respiratory pathogens (*S. aureus* and *P. aeruginosa*).

4.3.3 pH of Saliva during mechanical ventilation

The pH of saliva is shown in table 4.1 and figure 4.3. A full list of salivary pH measurements per patient can be found in appendix III. In comparison to the

mean pooled salivary pH of the healthy volunteers, (pH 6.98; table 4.2), the greatest decrease in pH is observed around the commencement of MV, for both groups (pH 6.57 for patients exhibiting normal oral microbiota and pH 6.44 for patients exhibiting respiratory pathogens within their dental plaque).

Salivary pH was slightly acidic (mean pH 6.51) for all mechanically ventilated patients, in comparison with the mean pH values obtained from saliva collected from healthy volunteers (pH 6.98). Importantly, at midpoint of MV (mean time of 2-4 d) there was a significant reduction (p=0.009) in pH for those patients exhibiting respiratory pathogens in their dental plaque (Figure 4.3) in comparison with those patients exhibiting predominantly oral microbiota.

Table 4.1 - A summary of saliva volume production and pH from mechanically ventilated patients.

Saliva measurement	**Number of patients**
No detectable saliva during intubation	36
Respiratory pathogen colonisation and no detectable saliva during intubation	6
>2 saliva samples	34
Respiratory pathogen colonisation and decrease in saliva volume	8
Saliva volume reduced during MV	52
Acidic pH <6.2 developed	20
Saliva pH reduced by 1 unit	8
Respiratory pathogen colonisation and decrease in saliva volume and pH	12

MV, mechanical ventilation

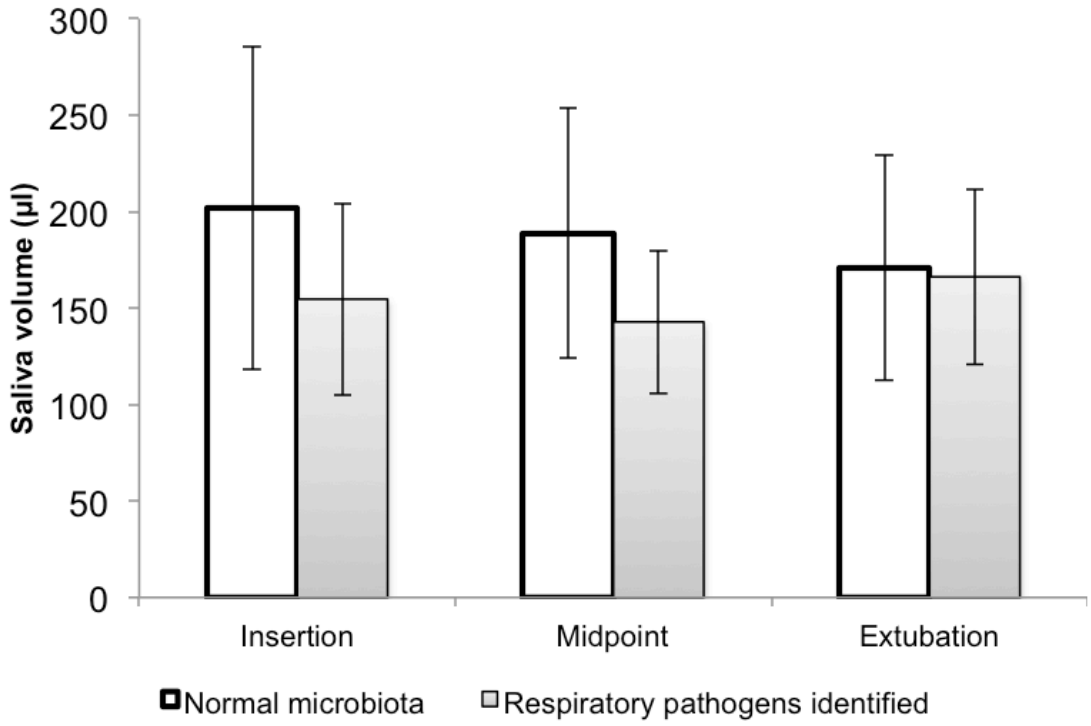

Figure 4.2 – Salivary volume produced (over 45 s) for patients during MV in relation to respiratory pathogen presence in dental plaque.

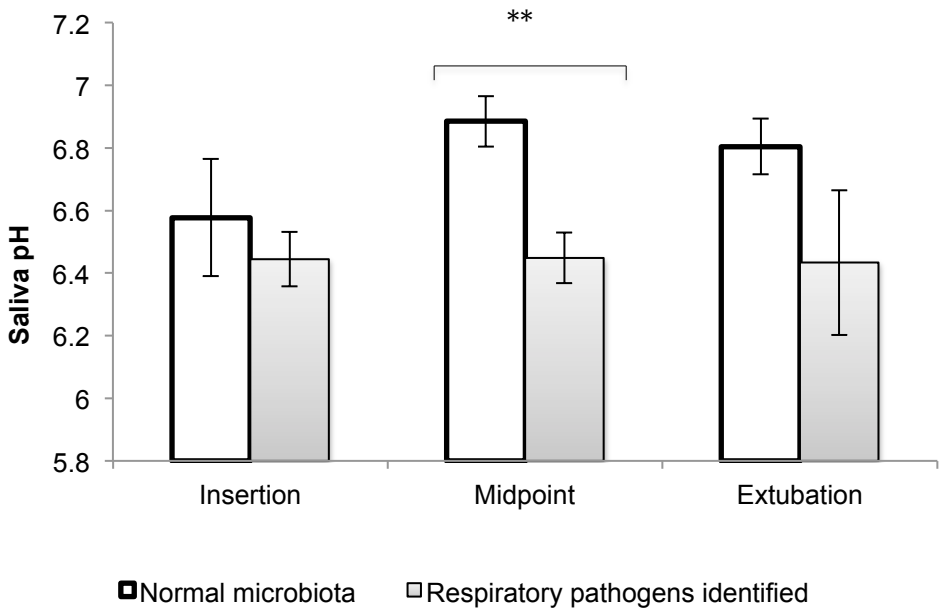

Figure 4.3 – The pH of saliva during MV for patients exhibiting predominantly normal oral microbiota, and patients colonised with respiratory pathogens.

4.3.4 Protein concentration of saliva during mechanical ventilation

Total protein concentration was determined for saliva, primarily to inform downstream applications. For 128/136 samples there was sufficient volume to complete a protein concentration assay. The protein concentration range of saliva from healthy volunteers (Table 4.2) was 1.5 - 4.95 µg/µl. Protein concentration levels in healthy saliva were relatively stable during a 10 d period (collected every 2-3d), however they were significantly lower than those of mechanically ventilated patients ($p<0.035$). Furthermore, there was less variation in protein concentration over time for saliva from healthy volunteers compared to mechanically ventilated patients.

On average, at the start of MV, salivary protein concentration was ~15 µg/µl, compared to ~10 µg/µl during the remainder of MV and post extubation

(Figure 4.4). Patients exhibiting respiratory pathogen colonisation of dental plaque had greater variations in salivary protein concentration during MV, with an average concentration >10 µg/µl.

Table 4.2 – Salivary volume, protein concentration and pH for 5 healthy volunteers over a 10 d period.

Volunteer	Sample	Protein (mg/µl)	Volume (µl)	pH
	1	2.55	750	7.1
	2	1.65	700	6.97
A	3	1.50	800	7.15
	4	1.80	700	7.15
	5	2.40	900	6.89
	Average	**1.98**	**770**	**7.05**
	SD	**0.50**	**83.7**	**0.1**
	1	3.3	850	7.09
	2	2.7	1000	7.01
B	3	3.75	900	7.00
	4	3.45	1200	6.81
	5	3.3	1000	7.00
	Average	**3.3**	**990**	**6.98**
	SD	**0.4**	**134.2**	**0.1**
	1	1.8	750	7.03
	2	2.4	700	7.04
C	3	1.8	800	7.04
	4	2.4	800	7.02
	5	4.8	750	6.78
	Average	**2.64**	**760**	**6.98**
	SD	**1.2**	**41.8**	**0.1**
	1	2.25	1200	6.68
	2	1.35	1300	6.96
D	3	3.3	900	6.91
	4	1.8	1000	7.01
	Average	**2.175**	**1100**	**6.89**
	SD	**0.8**	**182.6**	**0.1**
	1	3.75	600	7.02
	2	3.6	550	7.04
E	3	4.05	650	6.99
	4	3.3	600	7.10
	5	4.95	700	6.91
	Average	**3.93**	**620**	**7.01**
	SD	**0.6**	**57.0**	**0.1**

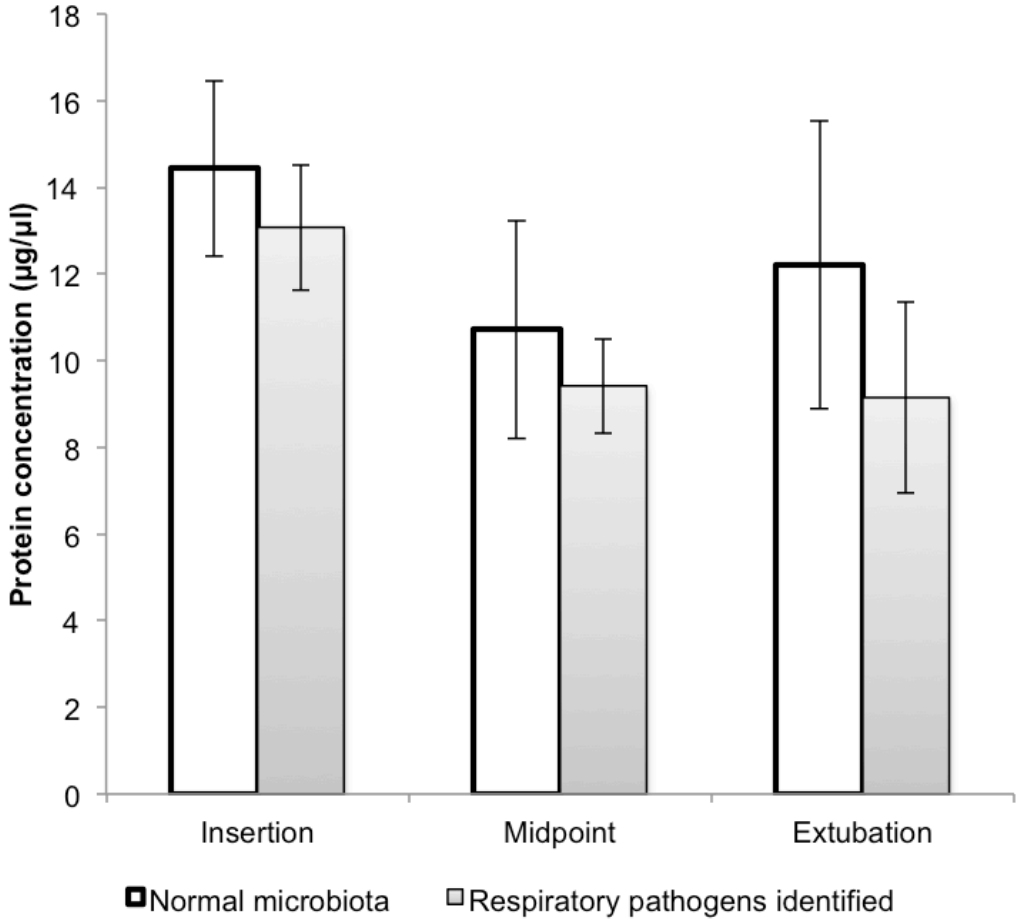

Figure 4.4 – Total saliva protein concentration (µg/µl) obtained during the course of MV and in relation to respiratory pathogen presence in dental plaque.

4.3.5 Gel based salivary proteomics

4.3.5.1 SDS-PAGE

Saliva from 10 patients was analysed by SDS-PAGE for salivary protein composition over MV. SDS-PAGE compared 5 saliva samples from 5 different time points during MV and into the post-ETT extubation/recovery period (Figure 4.5, gel A). These were further compared to 5 saliva samples from a single healthy volunteer over 10 d (Figure 4.5, gel B).

During MV, numerous protein compositional and abundance changes were evident in saliva (Figure 4.5 gel A). In contrast, very similar protein profiles were evident from the healthy volunteer over a period of 10 d (Figure 4.5, gel B).

SDS-PAGE analysis of saliva from 3 mechanically ventilated patients was compared to the proteomic profile of fluid collected from extubated-ETTs (Figure 4.6). Peptides of low and high molecular weight were isolated from saliva and ETTs. There were multiple differences in protein profiles between saliva samples and fluids from ETTs over the course of MV (Figure 4.6).

Peptides were stained with colloidal coomassie blue (Figures 4.5 and 4.6). Figure 4.7 illustrates peptide staining using two techniques, colloidal coomassie blue and silver nitrate. The silver staining was deemed more sensitive, revealing numerous additional protein bands, not detected using colloidal coomassie blue (Figure 4.7 gel B). Proteins of higher abundance within the separation were found to cross over on occasion leading to blurring (Figure 4.7), and this was particularly evident for proteins within the range of 116.3 kDa to 36.5 kDa. There were likely to be multiple proteins within these overlapped bands.

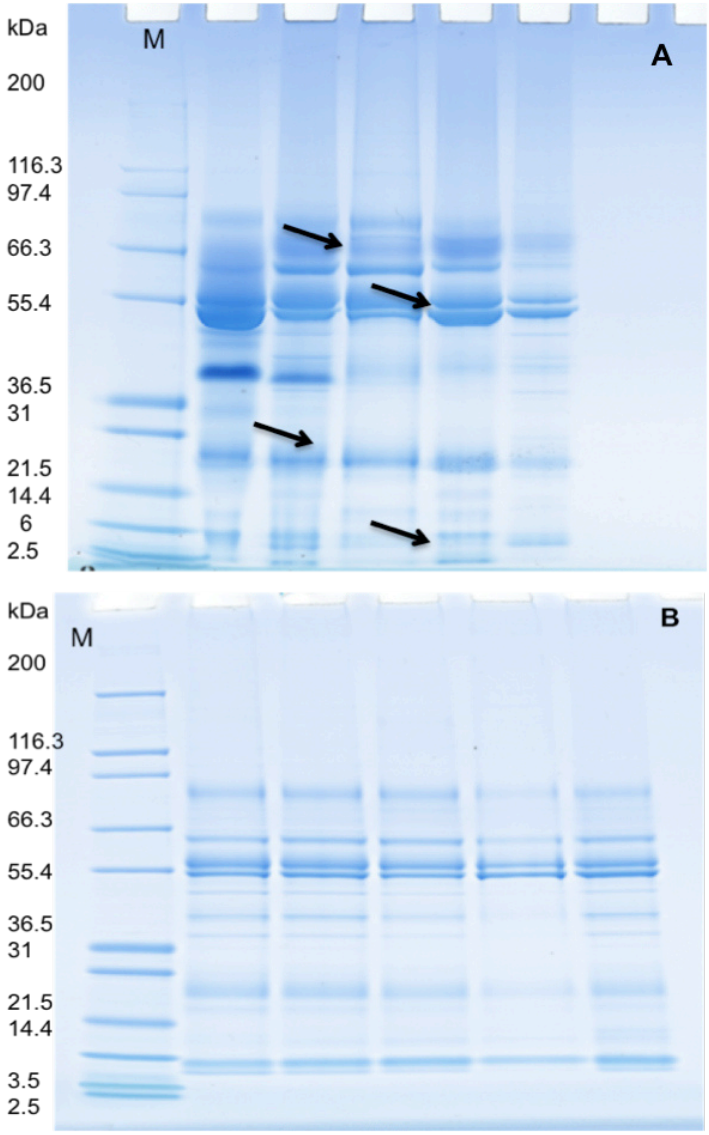

Figure 4.5 – SDS-PAGE analysis A; Mechanically ventilated patient colonised with respiratory pathogens during the course of MV (peptide differences shown by the arrows); B, 5 saliva samples within a 10 d period from a healthy volunteer. M protein marker.

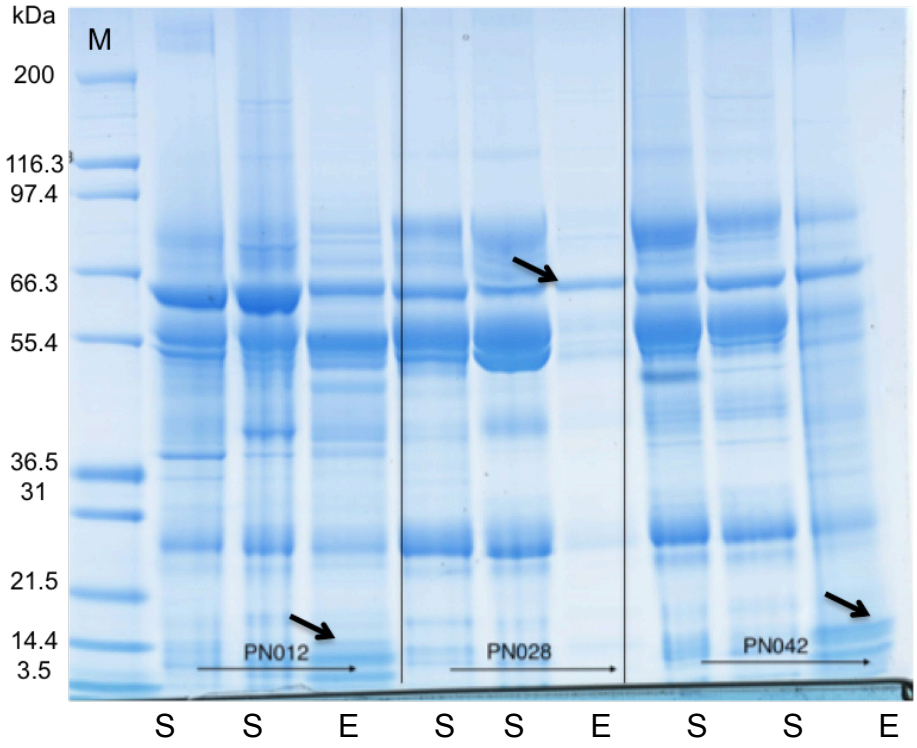

Figure 4.6 - SDS-PAGE of saliva from 3 patients during MV whose dental plaque was colonised with respiratory pathogens. A total of 2 saliva samples (S) and the fluid from 1 ETT (E) was analysed for each patient. Peptides were detected from both saliva and ETT fluid, as shown by the arrows. M, protein marker.

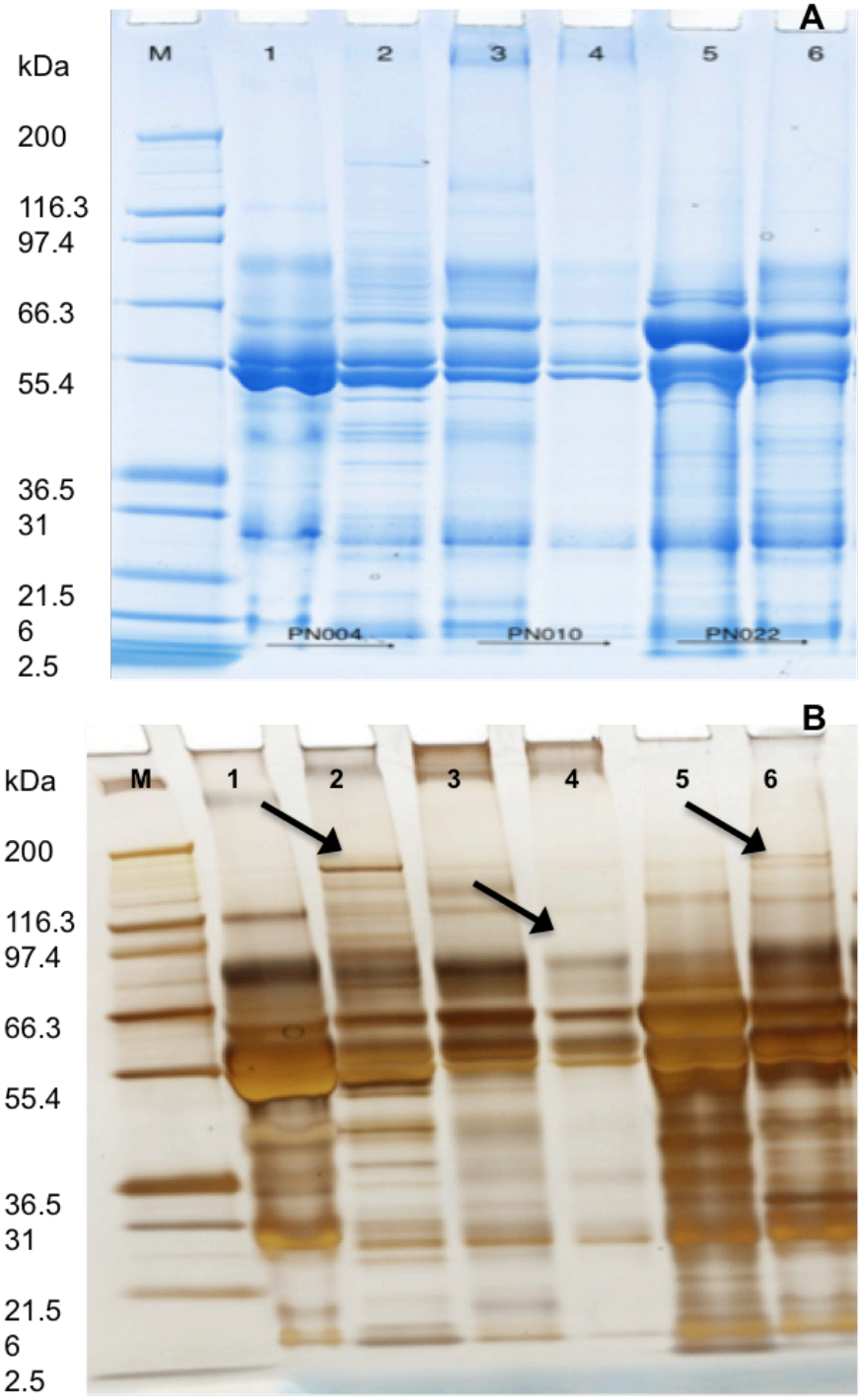

Figure 4.7 - SDS-PAGE of saliva from 3 mechanically ventilated patients (2 saliva samples per patient). A; stained with coomassie blue; B stained with silver nitrate, with additional peptide bands detected (arrowed). M, protein marker.

4.3.5.2 2DE analysis of saliva

Based on results from the SDS-PAGE, saliva was further analysed by 2DE. During 2DE, one saliva sample can be processed per gel, yielding higher quantities of data (Figures 4.8, 4.9 and 4.10).

Saliva from two time points during MV of a patient was analysed via 2DE (Figure 4.8). A series of defined protein spots (Figure 4.8) were subject to protein identification using MALDI-MS. Protein identifications from peptide gel plugs (Figure 4.8) are present in Table 4.3. Extremely low e-values were evident highlighting high quality and confidence of identification (Table 4.3). Proteins frequently isolated from healthy saliva were identified using MALDI-MS including α-amylase, serotransferrin and cystatins. At 3 different locations within the gel (Figure 4.8), α-amylase was identified indicating potential complex formation.

Silver nitrate staining (Figure 4.9) of the same salivary samples was performed. As highlighted by arrows in gel B, there were numerous proteins absent in Figure 4.9 gel A, and by coomassie blue staining (Figure 4.8).

2DE was also performed on two separate saliva samples from a healthy volunteer. There were areas within the gel that highlighted compositional differences over time (Figure 4.10). Fewer peptides and compositional changes were detected over time for the healthy saliva, compared with saliva from a mechanically ventilated patient.

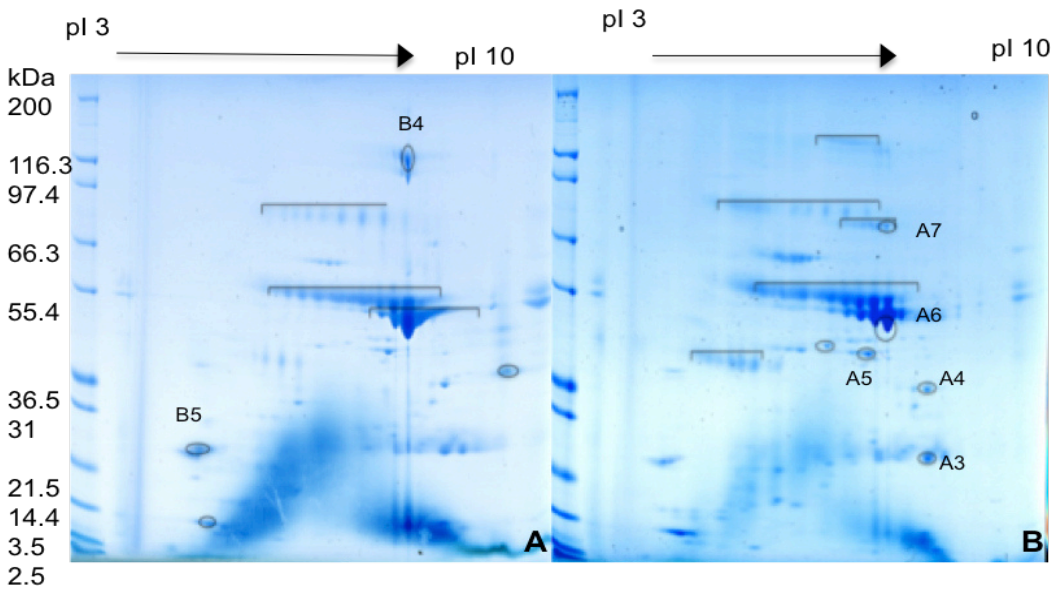

Figure 4.8 - 2DE analysis of two saliva samples during MV. A; commencement of MV and B; midpoint (day 3) MV.

Table 4.3 – Protein Identification (with 2 peptides[8]) for gel-plugs A3-A7, B4-B5 (gel, figure 4.9).

Sample spot	Protein Name	Peptide(s)	e value
A3	**Ig kappa chain C**	SGTASVVCLLNNFYPR	1.20E-12
A4	**Alpha-amylase 2B**	EVTINPDTTCGNDWVCEHR	2.20E-14
		TGSGDIENYNDATQVR	8.70E-12
A5	**Serum albumin**	FQNALLVR	7.80E-05
		KVPQVSTPTLVEVSR	2.60E-04
A6	**Alpha-amylase 1**	NVVDGQPFTNWYDNGSNQVAFGR	6.20E-18
		EVTINPDTTCGNDWVCEHR	6.60E-16
A7	**Serotransferrin**	FDEFFSEGCAPGSK	1.00E-07
		EGYYGYTGAFR	6.00E-05
B4	**Alpha-amylase 1**	EVTINPDTTCGNDWVCEHR	1.10E-14
		TGSGDIENYNDATQVR	1.00E-13
B5	**Cystatin-S**	SQPNLDTCAFHEQPELQK	4.70E-07
		IIPGGIYDADLNDEWVQR	1.80E-06

[8] Identification at a 95% confidence level

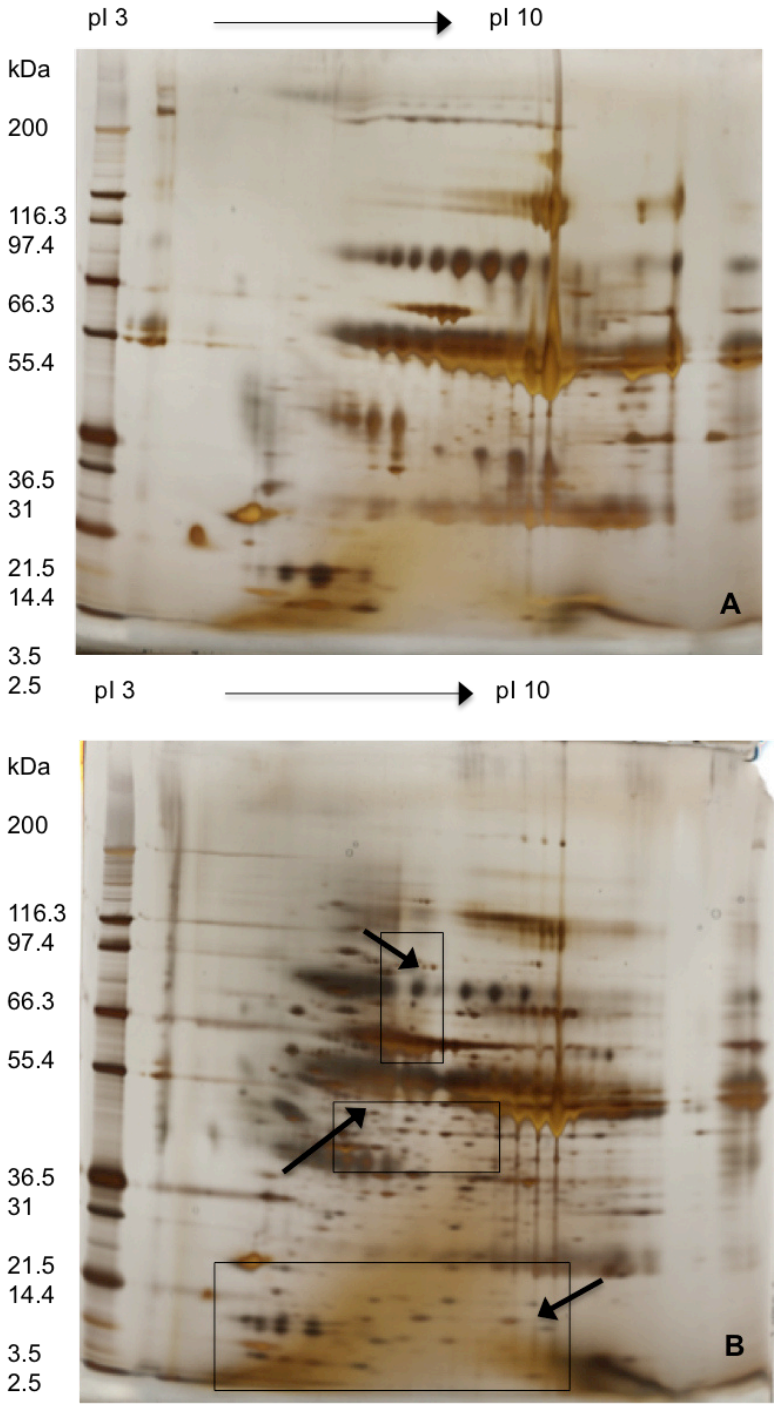

Figure 4.9 - 2DE of 2 saliva samples from a patient during MV stained with silver nitrate (repeat saliva samples as analysed in figure 4.7) A; start of MV, B; day 3, midpoint MV. Arrows (B) highlight additional differences in protein spots/clusters identified during MV.

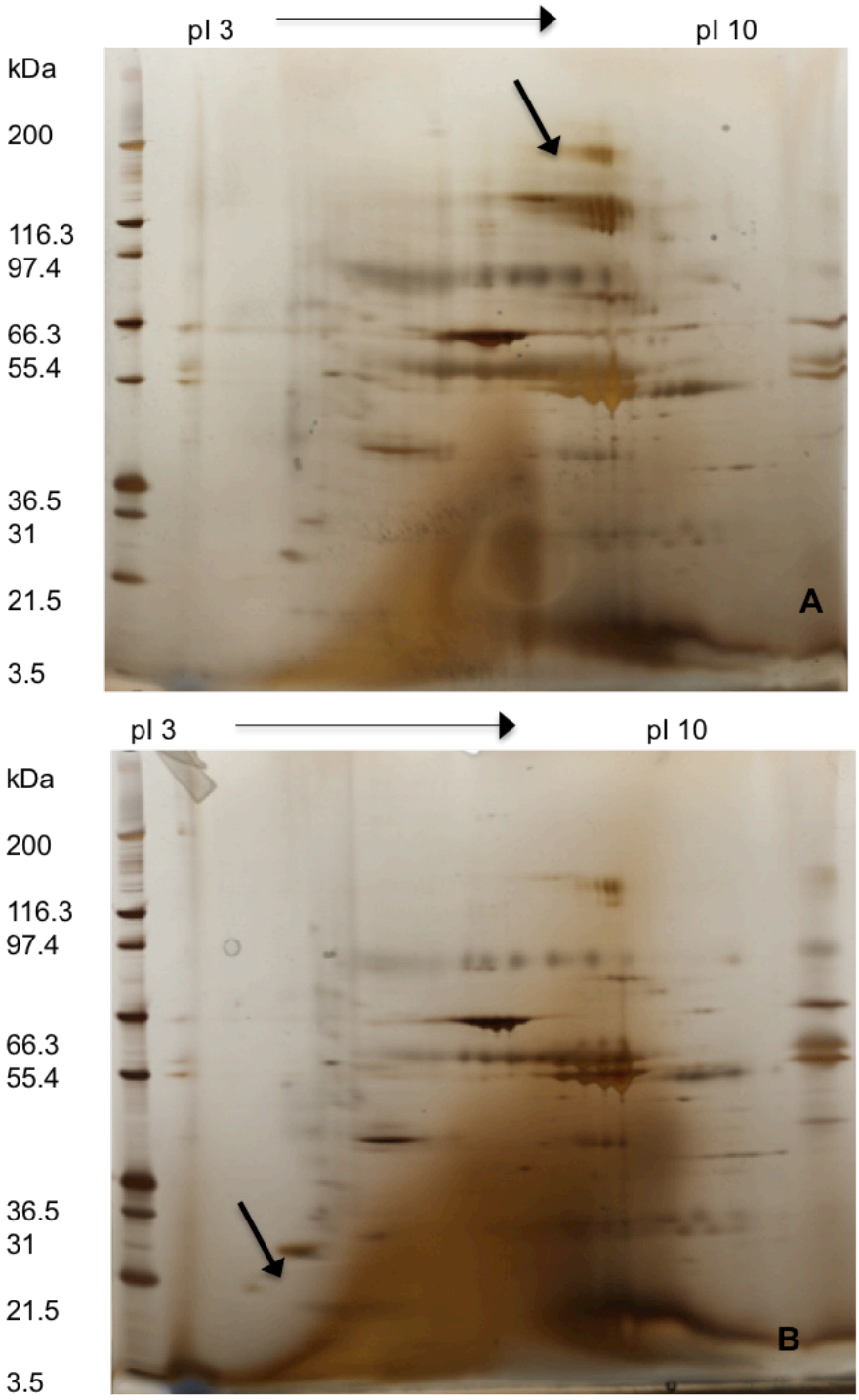

Figure 4.10 – 2DE analysis performed on two saliva samples from a healthy volunteer. A; collected on day 1 and B; collected on d 5. Arrows highlight areas of potential peptide compositional differences over time.

4.3.6 Gel-free proteomics

4.3.6.1 Global-scale protein identification

To complete gel-free proteomic analysis, based on results from gel-based proteomics, whole saliva was initially separated according to peptides using liquid chromatography (LC). The complex peptide mixture was further analysed using MS/MS coupled with databases (MASCOT and Uniprot) for global peptide identification/ quantification. Proteins were identified according to peptide sequences and were grouped according to their protein class and biological function (4.3.6.2). Relative protein expression was quantified during MV (and within a 10 d period for 'healthy saliva') using iTRAQ labeling (4.3.7).

Total number of proteins identified from pooled salivary samples collected from a healthy individual and a mechanically ventilated patient are presented in table 4.4. For fresh saliva a total of 178 and 141 proteins were identified (at a 95% CI level) for the healthy volunteer and the mechanically ventilated patient, respectively. At the 2+peptide parameter (proteins identified with >2 matching peptides and the >95% CI), 116 and 98 proteins were identified for saliva from the healthy volunteer and the mechanically ventilated patient, respectively. The number of peptides identified during the frozen repeat was 141 and 146 proteins (at a 95% CI level), and using the 2+matching peptide for identification parameter >90 proteins were identified for both healthy saliva and saliva collected during MV (Table 4.4).

Haemoglobin, complement factors B and C4-B, and neutrophil elastase and neutrophil defensin were identified in saliva from a mechanically ventilated patient (Table 4.5). These proteins were absent from saliva obtained from the healthy individual. Identified peptides were grouped according to protein class and biological function.

Table 4.4 - Peptides identified with high confidence during the LC- MS/MS workflow[9].

LC-MS/MS work flow	Identification criteria	Healthy volunteer	Mechanically ventilated patient
FRESH RUN	2+ peptides	116	98
	>95% CI	178	141
FROZEN RUN	2+ peptides	92	90
	>95% CI	141	146

Two parameters for identification criteria were used: 2+ peptides –proteins identified according to 2+ matching peptides, and a protein identification scoring at >95% confidence interval (CI), relating to high confidence in the protein ion score.

Table 4.5 - Proteins identified in saliva from a mechanically ventilated patient, but absent in healthy saliva.

Protein Name	Accession number	Protein MW	Protein PI	Peptide Count
Haptoglobin	HPT_HUMAN	45728	6.13	13
Complement factor B	CFAB_HUMAN	86582	6.67	8
Protein S100-A8	S10A8_HUMAN	10874	6.51	8
Haemoglobin subunit alpha	HBA_HUMAN	15294	8.72	4
Lysozyme C	LYSC_HUMAN	16894	9.38	4
Complement C4-B	CO4B_HUMAN	193872	6.89	3
Neutrophil elastase	ELANE_HUMAN	29006	9.71	2
Neutrophil defensin 3	DEF3_HUMAN	10514	5.71	2

MW – theoretical molecular weight, PI – isoelectric point, peptide count – peptide identifiers

[9] Table 4.4 represents a summary of pooled salivary samples from both a healthy control, and a mechanically ventilated patient respectively.

4.3.6.2 Protein classification into protein class and biological function

Protein classes for pooled saliva from the healthy volunteer (control) and mechanically ventilated patient were compared (Figure 4.11 and figure 4.12). Protein classes ranged from calcium-binding proteins, to defense/immunity proteins, proteases and transporter proteins. Generally, protein classes identified for the healthy individual and mechanically ventilated patient were similar. Certain proteins, including cell junction and transcription proteins were identified in saliva from the mechanically ventilated patient but were absent from the saliva of the healthy individual. Figures 4.13 and 4.14 illustrate the identified proteins (genes) according to biological processes.

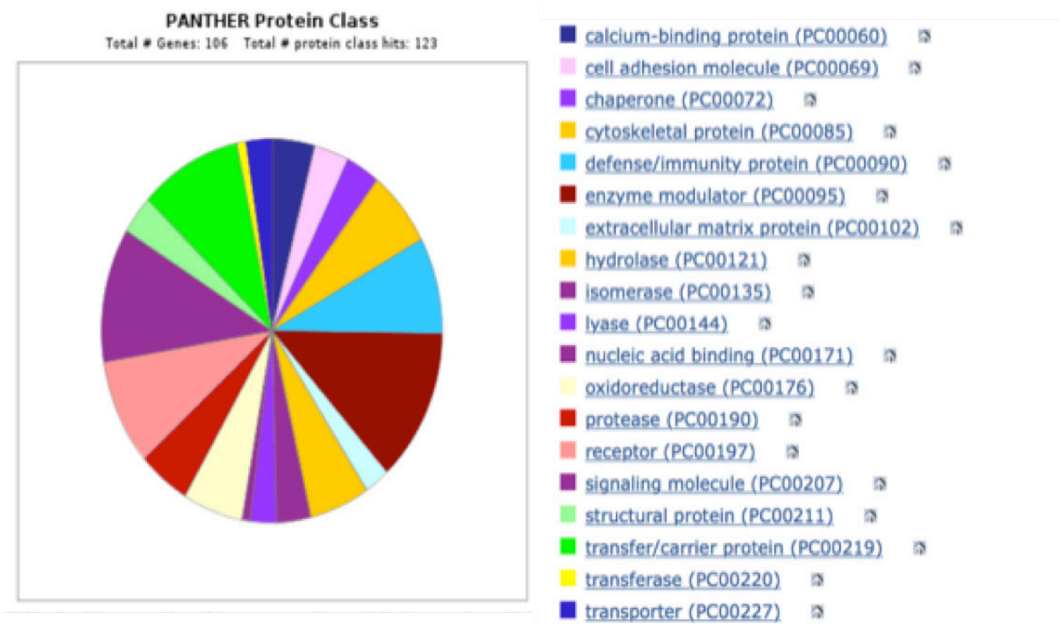

Figure 4.11 – Grouping a total of 116 identified peptides within saliva collected from a healthy volunteer according to protein class (PC).

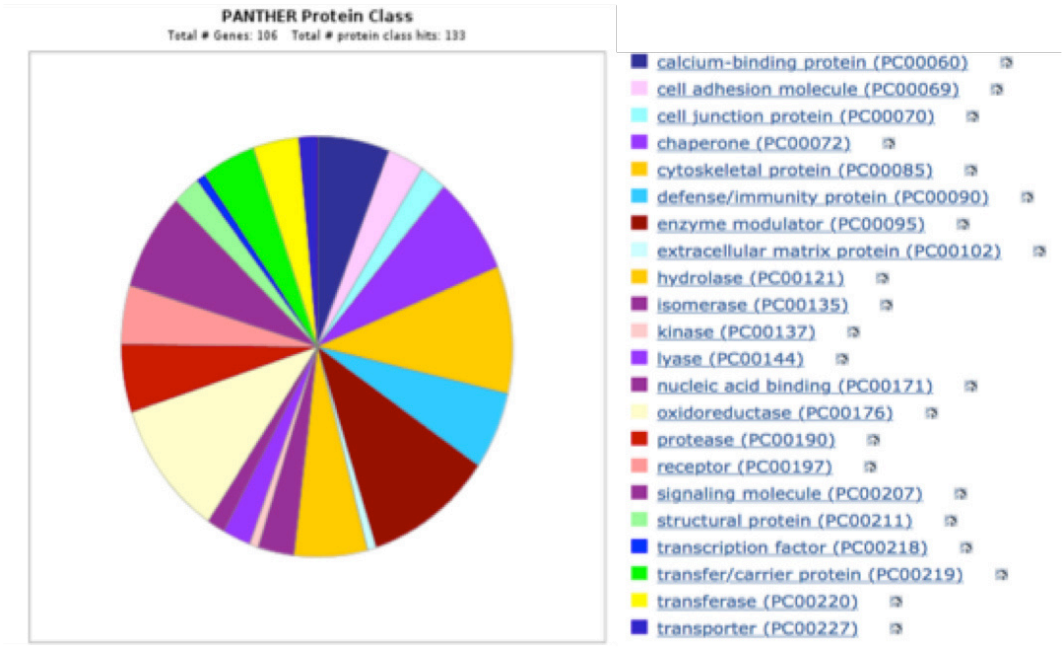

Figure 4.12 – Grouping 98 identified peptides from a mechanically ventilated patient over a period of time according to protein class (PC).

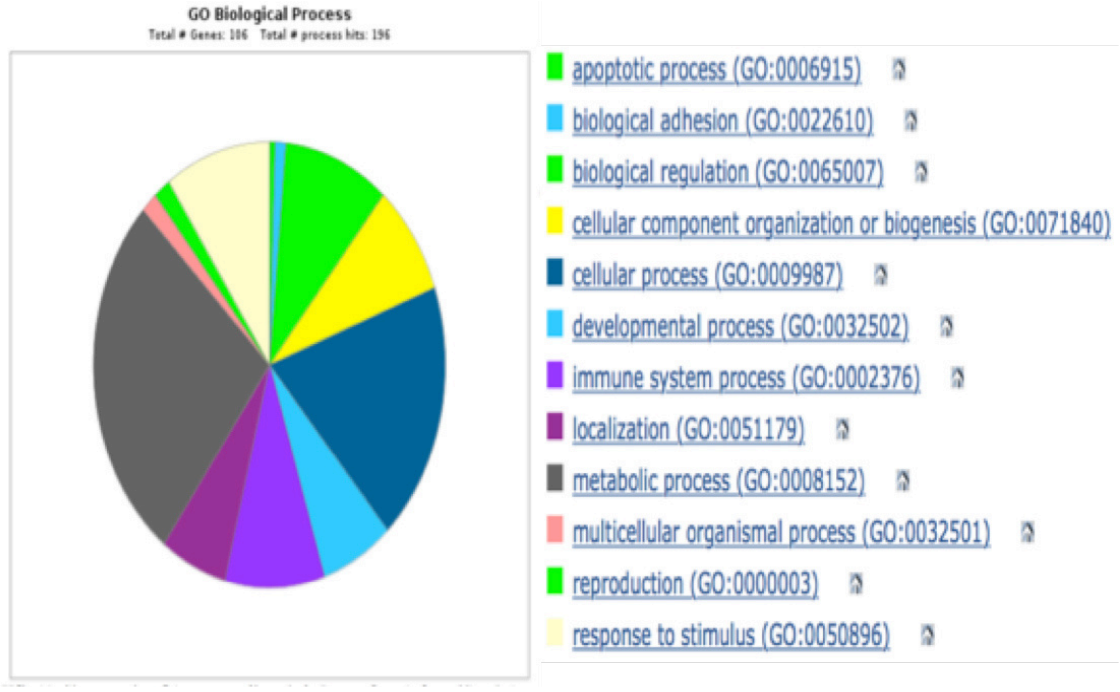

Figure 4.13 – Comparison of biological function from 116 peptides identified from saliva collected from a healthy volunteer. GO – gene ontology. Gene

ontology (GO) is a bioinformatics initiative aiming to unify protein and gene classification.

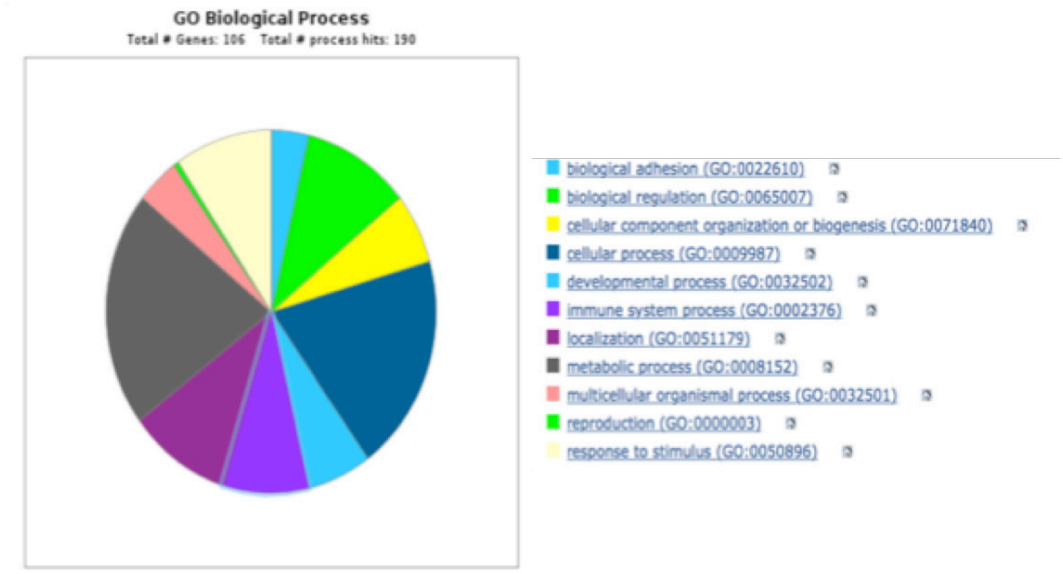

Figure 4.14 – Comparison of biological processes of identified peptides from a mechanically ventilated patient. Gene ontology (GO) is a bioinformatics initiative aiming to unify protein and gene classification.

4.3.6.3 Bacterial and fungal peptides identified in saliva

All bacterial and fungi originating peptides isolated within saliva were identified with a maximum of 1 peptide match, all with a CI score >95%, representing a confident identification (Table 4.6). The protein pI representing the relative pH of the peptide suggests both acidic and basic peptides were present ranging from a theoretical pH of 4.45 to 10.28 from both the healthy individual and the mechanically ventilated patient analysed.

Peptides originating from *C. albicans,* lactobacilli, *Campylobacter concisus* and anaerobic bacteria such as *Bacteroides fragilis* were identified from pooled healthy saliva. Similarly, peptides from oral species such as lactobacilli and *Candida* species were identified from saliva from a mechanically ventilated patient. Interestingly, a peptide from the potential respiratory pathogen *Escherichia coli* was detected within saliva during MV. This mechanically ventilated patient (PN046) exhibited respiratory pathogen colonisation within their dental plaque during MV.

Table 4.6 – Bacterial and fungal peptides detected in saliva from a mechanically ventilated patient, and a healthy volunteer. MW – molecular weight, PI – isoelectric point, peptide count – peptide matches

Saliva	Species	Peptide (Protein name)	Accession number	Protein MW	Protein PI	Ion Score C.I. %	Peptide Count
Mechanically ventilated patient	Bacteria	Trigger factor *Lactobacillus reuteri* (strain DSM 20016)	TIG_LACRD	48717.24	4.45	99.63	1
		Fimbrial protein MS11-D1, Neisseria gonorrhoeae	FMD1_NEIGO	23236.61	7.6	99.60	1
		CDP-diacylglycerol--glycerol-3-phosphate 3-phosphatidyltransferase, *Escherichia coli* O1:K1	PGSA_ECOK1	20733.05	8.89	99.53	1
		GMP synthase *Lactobacillus acidophilus* strain ATCC 700396	GUAA_LACAC	57822.82	5.26	99.30	1
		Lipoyl synthase, *Micrococcus luteus* (strain ATCC 4698 / DSM 20030)	LIPA_MICLC	37935.15	6.18	99.29	1
	Fungi	Actin, *Candida dubliniensis* (strain CD36)	ACT_CANDC	41895.78	5.45	100	4
		Histone H2A.1, *Candida albicans* (strain SC5314)	H2A1_CANAL	14000.69	10.28	99.92	1
		6-phosphogluconate dehydrogenase, decarboxylating, *Candida albicans*	6PGD_CANAX	57118.07	6.14	99.31	1
Healthy volunteer	Bacteria	3-oxoacyl synthase 3, *Agrobacterium vitis* (strain S4 / ATCC BAA-846)	FABH_AGRVS	34343.62	5.4	99.47	1
		DNA replication and repair protein RecF, *Bacteroides fragilis* (YCH46)	RECF_BACFR	43035.94	6.09	99.46	1
		50S ribosomal protein L9, Pelagibacter ubique (strain HTCC1062)	RL9_PELUB	17317.60	9.22	99.44	1
		Sec-independent protein translocase protein TatA,	TATA_PHEZH	7593.99	8.15	99.42	1
		Glutamate--tRNA ligase, *Synechococcus* sp. (strain CC9605)	SYE_SYNSC	53552.12	5.15	99.31	1
		Homoserine kinase, *Campylobacter concisus* (strain 13826)	KHSE_CAMC1	32350.40	6.92	99.28	1
		Demethylmenaquinone methyltransferase, *Lactococcus lactis*	UBIE_LACLA	28614.61	9.06	99.07	1
	Fungi	Phosphoglycerate kinase, *Candida glabrata* (strain ATCC 2001)	PGK_CANGA	44681.56	6.37	99.86	1
		Autophagy-related protein 34, *Saccharomyces cerevisiae* (ATCC 204508)	ATG34_YEAST	46947.49	5.33	99.51	1
		Protein SDS23, *Phaeosphaeria nodorum* (strain SN15 / ATCC MYA-4574)	SD23_PHANO	57184.72	8.99	99.45	1
		Ubiquitin-like modifier-activating ATG7, *Candida glabrata* (ATCC 2001)	ATG7_CANGA	71298.68	5.48	99.41	1
		RNA polymerase II transcription 21, *Candida albicans* (strain SC5314)	MED21_CANAL	20368.28	4.54	99.18	1

4.3.7 Quantitative analysis: Protein expression during mechanical ventilation

A total of 4 salivary samples from a healthy volunteer were labeled with a different iTRAQ™ label and subsequently pooled for LC-MS analysis. This process was repeated independently for 3 samples collected from a patient during MV and post-ETT extubation recovery. Raw peptide sequences were input into Protein Pilot™ for initial identification using Paragon™ software coupled with downstream quantification analysis. Identified peptides were quantified by expression-fold differences over time, for both healthy saliva and saliva collected from a mechanically ventilated patient, (Figure 4.15).

Healthy saliva was compared at 3 time points over 10 d to assess protein compositional differences. Twenty-one proteins exhibited an expression change >50% from a total of 200 proteins that were identified (Table 4.7). Protein expression of serum albumin and cystatins noticeably increased over the 10 d period. Expression of Peregrin and Tripeptidyl-peptidase 2 also increased between 50-85 fold.

For the majority of identified proteins, expression and relative quantification over the tested period were not noticeably different. Appendix III provides a full list of proteins identified from saliva of a healthy volunteer over the 10 d period.

Differences in protein expression during MV were assessed (Table 4.8). Within Protein Pilot™ identified peptides (n=156) were displayed at a CI of 95%. Proteins including complement C3, serotransferrin, antibody peptides, neutrophil elastase and hemoglobin were highly expressed at the time of intubation. Proteins including complement C4, S-100, and antibody peptides increased during MV (Table 4.8).

There were several proteins with noticeably increased expression during the post extubation recovery period *e.g.* antimicrobial proteins such as lactotransferrin, lysozyme and lactoperoxidase increased in expression by up to 10-fold. Relative expression of proteins involved in the scavenging of toxins and pH homeostasis, (*e.g.* lipocalin and carbonic anhydrase, respectively), increased during the post-ETT extubation period. In addition, expression of α-amylase increased >25-fold in the post –ETT extubation recovery period. Finally, proteins including cystatin-B, thioredoxin, cystatin-SN, involucrin, and mucin-5B were expressed at similar levels to that encountered in saliva from the healthy individual during both MV and into the recovery period.

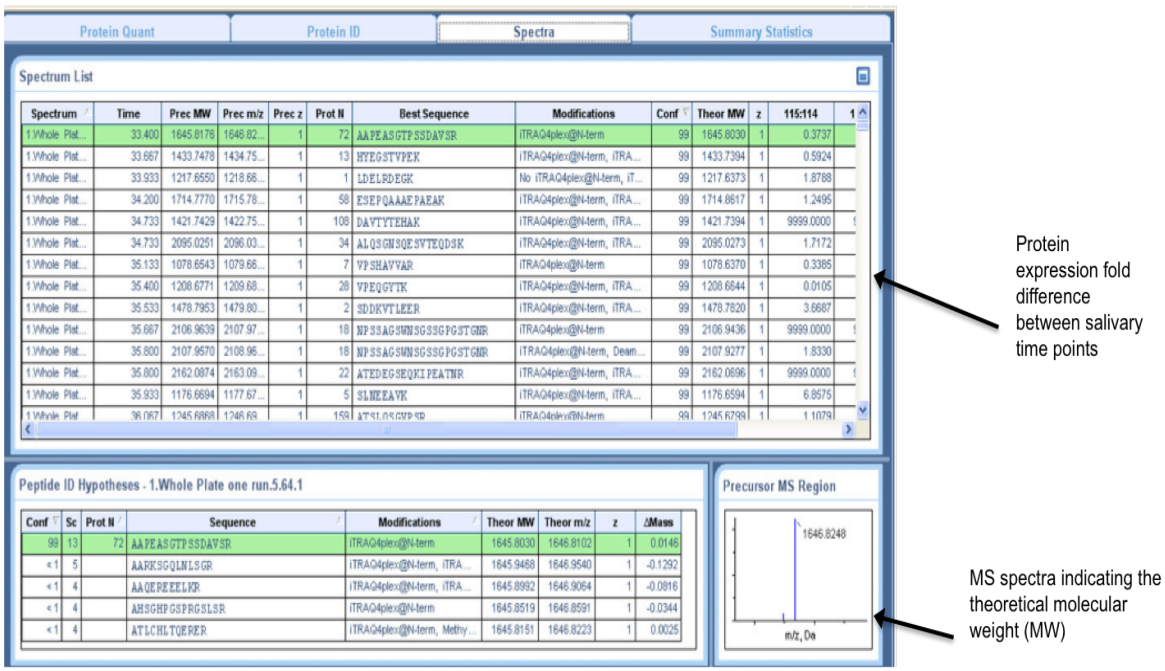

Figure 4.15 – Screen shot of Protein Pilot™ software during protein expression and quantification analysis using iTRAQ™ multi-plex labels for saliva samples during mechanical ventilation (115, 116, and 117) compared to pooled saliva collected at multiple time points (days) from a healthy volunteer.

Table 4.7 – Peptides with expression differences >50% from the first time point, within a 10 d period, from healthy saliva.

Name	Accession number	Peptides (95%)	d1	d4	d8
Serum albumin	sp|P02768|ALBU_HUMAN	50	1.00	1.03	1.51
Cystatin-S	sp|P01036|CYTS_HUMAN	28	1.00	1.34	1.66
Cystatin-SN	sp|P01037|CYTN_HUMAN	28	1.00	1.24	1.87
Ig lambda-2 chain C regions	sp|P0CG05|LAC2_HUMAN	17	1.00	1.15	0.47
Cystatin-SA	sp|P09228|CYTT_HUMAN	16	1.00	1.47	1.71
Prolactin-inducible protein	sp|P12273|PIP_HUMAN	7	1.00	1.40	1.94
Cystatin-D	sp|P28325|CYTD_HUMAN	7	1.00	1.12	2.19
Keratin, type I cytoskeletal 10	sp|P13645|K1C10_HUMAN	5	1.00	1.00	0.49
Beta-2-microglobulin	sp|P61769|B2MG_HUMAN	5	1.00	0.97	1.58
Ig kappa chain V-IV region JI	sp|P06313|KV403_HUMAN	3	1.00	1.34	2.52
Keratin, type II cytoskeletal 5	sp|P13647|K2C5_HUMAN	2	1.00	0.96	0.00
Peregrin	sp|P55201|BRPF1_HUMAN	1	1.00	0.05	82.3
Tripeptidyl-peptidase 2	sp|P29144|TPP2_HUMAN	1	1.00	1.00	52.7
Filamin-A	sp|P21333|FLNA_HUMAN	1	1.00	2.01	0.14
Tropomyosin alpha-3 chain	sp|P06753|TPM3_HUMAN	1	1.00	2.82	5.86
EF-hand domain- protein D2	sp|Q96C19|EFHD2_HUMAN	1	1.00	0.75	1.72
Proline-rich protein 27	sp|Q6MZM9|PRR27_HUMAN	1	1.00	2.16	2.07
Nucleobindin-2	sp|P80303|NUCB2_HUMAN	1	1.00	2.00	8.44
Cytidine deaminase	sp|P32320|CDD_HUMAN	1	1.00	0.22	0.93
Cathelicidin antimicrobial peptide	sp|P49913|CAMP_HUMAN	1	1.00	0.66	1.62

Table 4.8 – Changes in salivary protein expression during mechanical ventilation (MV). Peptides highlighted with expression differences >50% from start of MV.

Protein Name	Accession number	Peptides 95%	Start MV	Midpoint MV	Extubation MV
Serum albumin	sp|P02768|ALBU_HUMAN	141	1.00	0.49	0.51
Complement C3	sp|P01024|CO3_HUMAN	51	1.00	1.03	0.27
Serotransferrin	sp|P02787|TRFE_HUMAN	47	1.00	0.47	0.25
Alpha-amylase 1	sp|P04745|AMY1_HUMAN	51	1.00	1.00	26.79
Alpha-2-macroglobulin	sp|P01023|A2MG_HUMAN	31	1.00	0.61	0.72
Alpha-1-antitrypsin	sp|P01009|A1AT_HUMAN	37	1.00	1.03	0.52
Lactotransferrin	sp|P02788|TRFL_HUMAN	29	1.00	3.79	3.45
Polymeric immunoglobulin receptor	sp|P01833|PIGR_HUMAN	28	1.00	1.45	13.68
Fibrinogen beta chain	sp|P02675|FIBB_HUMAN	28	1.00	1.33	0.21
Apolipoprotein A-I	sp|P02647|APOA1_HUMAN	23	1.00	0.16	0.13
Zinc-alpha-2-glycoprotein	sp|P25311|ZA2G_HUMAN	22	1.00	1.31	15.04
Ig gamma-2 chain C region	sp|P01859|IGHG2_HUMAN	20	1.00	0.44	0.47
Haptoglobin	sp|P00738|HPT_HUMAN	20	1.00	13.13	1.02
Myeloperoxidase	sp|P05164|PERM_HUMAN	13	1.00	1.36	0.36
Alpha-1-antichymotrypsin	sp|P01011|AACT_HUMAN	15	1.00	1.55	0.98
Actin, cytoplasmic 2	sp|P63261|ACTG_HUMAN	17	1.00	1.81	0.81
Ig alpha-1 chain C region	sp|P01876|IGHA1_HUMAN	22	1.00	0.88	3.32
Fibrinogen alpha chain	sp|P02671|FIBA_HUMAN	15	1.00	1.59	0.33
Ceruloplasmin	sp|P00450|CERU_HUMAN	11	1.00	0.58	0.42
Fibrinogen gamma chain	sp|P02679|FIBG_HUMAN	13	1.00	1.49	0.11
Complement factor B	sp|P00751|CFAB_HUMAN	11	1.00	0.85	0.21
Antithrombin-III	sp|P01008|ANT3_HUMAN	9	1.00	0.53	0.47
Ig mu chain C region	sp|P01871|IGHM_HUMAN	12	1.00	0.64	0.27
Leucine-rich alpha-2-glycoprotein	sp|P02750|A2GL_HUMAN	9	1.00	1.09	1.43
Hemoglobin subunit beta	sp|P68871|HBB_HUMAN	12	1.00	0.82	0.11
Protein S100-A8	sp|P05109|S10A8_HUMAN	15	1.00	3.77	0.23
Plastin-2	sp|P13796|PLSL_HUMAN	10	1.00	2.77	1.12
Small proline-rich protein 3	sp|Q9UBC9|SPRR3_HUMAN	9	1.01	4.36	25.80
Lipocalin-1	sp|P31025|LCN1_HUMAN	9	1.00	5.10	73.40
Alpha-1-acid glycoprotein 1	sp|P02763|A1AG1_HUMAN	12	1.00	0.70	0.97
Fibronectin	sp|P02751|FINC_HUMAN	7	1.00	0.47	0.29
Leukocyte elastase inhibitor	sp|P30740|ILEU_HUMAN	7	1.00	1.94	1.85
Hemopexin	sp|P02790|HEMO_HUMAN	7	1.00	0.82	0.87
Ig kappa chain C region	sp|P01834|IGKC_HUMAN	16	1.00	0.40	1.22
Cystatin-S	sp|P01036|CYTS_HUMAN	11	1.00	1.00	4.00
Vitronectin	sp|P04004|VTNC_HUMAN	6	1.00	0.41	0.39
Matrix metalloproteinase-9	sp|P14780|MMP9_HUMAN	6	1.00	3.53	1.02
Prolactin-inducible protein	sp|P12273|PIP_HUMAN	6	1.00	0.83	24.98

Apolipoprotein B-100	sp|P04114|APOB_HUMAN	5	1.00	0.48	0.40
Cystatin-B	sp|P04080|CYTB_HUMAN	6	1.00	1.25	2.00
Complement C4-A	sp|P0C0L4|CO4A_HUMAN	5	1.00	1.15	0.47
Protein S100-A9	sp|P06702|S10A9_HUMAN	12	1.00	4.37	0.27
Lysozyme C	sp|P61626|LYSC_HUMAN	9	1.00	3.52	33.97
Ig gamma-1 chain C region	sp|P01857|IGHG1_HUMAN	17	1.00	0.37	0.41
Inter-alpha-trypsin inhibitor	sp|Q14624|ITIH4_HUMAN	6	1.00	0.77	0.73
Heat shock 70 kDa protein 1A/1B	sp|P08107|HSP71_HUMAN	6	1.00	2.69	1.37
Vitamin D-binding protein	sp|P02774|VTDB_HUMAN	6	1.00	0.79	0.32
Immunoglobulin lambda-like polypeptide	sp|B9A064|IGLL5_HUMAN	12	1.00	0.56	1.10
Gelsolin	sp|P06396|GELS_HUMAN	5	1.00	1.63	0.71
Alpha-2-HS-glycoprotein	sp|P02765|FETUA_HUMAN	5	1.00	0.38	1.48
Alpha-enolase	sp|P06733|ENOA_HUMAN	5	1.00	2.25	0.56
Keratin, type II cytoskeletal 1	sp|P04264|K2C1_HUMAN	5	1.00	0.77	2.39
Alpha-1B-glycoprotein	sp|P04217|A1BG_HUMAN	5	1.00	0.71	0.34
Plasma protease C1 inhibitor	sp|P05155|IC1_HUMAN	6	1.00	0.61	0.59
6-phosphogluconate dehydrogenase	sp|P52209|6PGD_HUMAN	5	1.00	4.18	0.52
Cystatin-A	sp|P01040|CYTA_HUMAN	5	1.00	1.13	8.63
Transthyretin	sp|P02766|TTHY_HUMAN	5	1.00	0.46	0.93
Brain acid soluble protein 1	sp|P80723|BASP1_HUMAN	5	1.00	5.30	2.74
14-3-3 protein zeta/delta	sp|P63104|1433Z_HUMAN	5	1.00	1.00	0.49
Immunoglobulin J chain	sp|P69905|HBA_HUMAN	6	1.00	1.44	7.44
Hemoglobin subunit alpha	sp|P26038|MOES_HUMAN	7	1.00	0.80	0.15
Moesin	sp|P22079|PERL_HUMAN	4	1.00	2.86	0.91
Lactoperoxidase	sp|P13645|K1C10_HUMAN	4	1.00	1.42	12.79
Keratin, type I cytoskeletal 10	sp|Q01518|CAP1_HUMAN	3	1.00	0.43	1.78
Adenylyl cyclase-associated protein 1	sp|P37837|TALDO_HUMAN	4	1.00	4.12	0.91
Transaldolase	sp|P10599|THIO_HUMAN	4	1.00	4.65	3.10
Thioredoxin	sp|P01037|CYTN_HUMAN	4	1.00	1.50	3.25
Cystatin-SN	sp|P02652|APOA2_HUMAN	7	1.00	0.67	1.00
Apolipoprotein A-II	sp|P60174|TPIS_HUMAN	3	1.00	0.16	0.12
Triosephosphate isomerase	sp|A8K2U0|A2ML1_HUMAN	3	1.00	2.56	1.00
Alpha-2-macroglobulin-like protein 1	sp|P31146|COR1A_HUMAN	4	1.00	1.00	2.05
Coronin-1A	sp|P07476|INVO_HUMAN	3	1.00	2.52	0.96
Involucrin	sp|P08603|CFAH_HUMAN	5	1.00	2.33	4.00
Complement factor H	sp|P04075|ALDOA_HUMAN	3	1.00	0.69	0.39
Fructose-bisphosphate aldolase A	sp|P14618|KPYM_HUMAN	3	1.00	1.78	1.10
Pyruvate kinase PKM	sp|P01042|KNG1_HUMAN	3	1.00	1.31	0.77
Kininogen-1	sp|Q6P5S2|LEG1H_HUMAN	3	1.00	0.75	0.44
Protein LEG1 homolog	sp|P01772|HV311_HUMAN	3	1.00	1.13	2.34
Ig gamma-4 chain C region	sp|P01861|IGHG4_HUMAN	19	1.00	0.45	0.98
Cystatin-SA	sp|P09228|CYTT_HUMAN	8	1.00	0.60	0.00
Apolipoprotein E	sp|P02649|APOE_HUMAN	3	1.00	0.35	0.33
Neutrophil gelatinase- lipocalin	sp|P80188|NGAL_HUMAN	3	1.00	2.57	9.87
Beta-2-glycoprotein 1	sp|P02749|APOH_HUMAN	3	1.00	0.63	0.83

Ig heavy chain V-III region VH26	sp|P01764|HV303_HUMAN	3	1.00	0.48	0.94
Ig heavy chain V-I region V35	sp|P23083|HV103_HUMAN	5	1.00	0.54	1.34
Olfactomedin-4	sp|Q6UX06|OLFM4_HUMAN	3	1.00	1.81	1.10
Deleted in malignant brain tumors 1	sp|Q9UGM3|DMBT1_HUMAN	2	1.00	2.70	17.30
Ig lambda-2 chain C regions	sp|P0CG05|LAC2_HUMAN	11	1.00	0.5	1.0
Ig heavy chain V-III region	sp|P01767|HV306_HUMAN	2	1.00	0.8	0.8
Ig kappa chain V-III region	sp|P18136|KV313_HUMAN	4	1.19	0.9	1.2
Alpha-actinin-1	sp|P12814|ACTN1_HUMAN	2	1.00	2.4	1.0
Mucin-5B	sp|Q9HC84|MUC5B_HUMAN	1	1.00	1.8	1.7
Cysteine-rich secretory protein 3	sp|P54108|CRIS3_HUMAN	2	1.00	1.09	4.76
Superoxide dismutase [Cu-Zn]	sp|P00441|SODC_HUMAN	2	1.00	1.48	4.25
Ig kappa chain V-I region AG	sp|P01593|KV101_HUMAN	3	1.00	0.67	0.80
Inter-alpha-trypsin inhibitor	sp|P19827|ITIH1_HUMAN	2	1.00	0.66	0.64
Phosphoglycerate kinase 1	sp|P00558|PGK1_HUMAN	2	1.00	2.07	0.89
Histone H2A.J	sp|Q9BTM1|H2AJ_HUMAN	2	1.00	1.48	1.00
Alpha-1-acid glycoprotein 2	sp|P19652|A1AG2_HUMAN	6	1.00	0.77	1.16
Glucose-6-phosphate isomerase	sp|P06744|G6PI_HUMAN	2	1.00	1.70	0.75
Ig kappa chain V-III region CLL	sp|P04207|KV308_HUMAN	2	1.00	0.70	1.06
Peptidyl-prolyl cis-trans isomerase A	sp|P62937|PPIA_HUMAN	2	1.00	5.52	0.54
Transketolase	sp|P29401|TKT_HUMAN	2	1.00	3.73	0
Polyubiquitin-C	sp|P0CG48|UBC_HUMAN	2	1.00	1.28	1.14
L-lactate dehydrogenase A chain	sp|P00338|LDHA_HUMAN	2	1.00	2.92	1.51
Histone H4	sp|P62805|H4_HUMAN	3	1.00	1.37	0.14
Neutrophil defensin 3	sp|P59666|DEF3_HUMAN	2	1.00	0.86	0.94
Protein S100-A11	sp|P31949|S10AB_HUMAN	2	1.00	4.47	2.71
Protein S100-P	sp|P25815|S100P_HUMAN	2	1.67	2.36	0.99
Carbonic anhydrase 6	sp|P23280|CAH6_HUMAN	2	1.00	1.08	10.41
Neutrophil elastase	sp|P08246|ELNE_HUMAN	2	1.00	0.71	0.61
C-reactive protein	sp|P02741|CRP_HUMAN	2	1.00	0.70	0.56
Ig kappa chain V-I region HK102	sp|P01602|KV110_HUMAN	2	1.00	0.65	1.15
Keratin, type II cytoskeletal 6A	sp|P02538|K2C6A_HUMAN	3	1.00	0.44	0.87
Ig lambda chain V-I region WAH	sp|P04208|LV106_HUMAN	2	1.00	0.53	0.64
Ig gamma-3 chain C region	sp|P01860|IGHG3_HUMAN	17	1.00	0.51	0.24
Rho GDP-dissociation inhibitor 2	sp|P52566|GDIR2_HUMAN	2	1.00	1.96	0.56
Ig lambda chain V-III region LOI	sp|P80748|LV302_HUMAN	2	0.60	0.28	0.73
Ig heavy chain V-III region GAL	sp|P01781|HV320_HUMAN	2	1.00	0.86	1.55
14-3-3 protein sigma	sp|P31947|1433S_HUMAN	3	1.00	1.10	1.00
Ig kappa chain V-II region RPMI 6410	sp|P06310|KV206_HUMAN	2	1.00	0.50	0.92
Ig heavy chain V-III region TEI	sp|P01777|HV316_HUMAN	2	1.00	0.55	0.99
Keratin, type I cytoskeletal 13	sp|P13646|K1C13_HUMAN	2	1.00	0.13	0.27
Ig alpha-2 chain C region	sp|P01877|IGHA2_HUMAN	19	1.00	1.05	4.70
14-3-3 protein beta/alpha	sp|P31946|1433B_HUMAN	3	1.00	2.59	0
Putative V-set and immunoglobulin	sp|A6NJ16|IV4F8_HUMAN	2	1.00	0.47	1.32
Histone H2B type 1-A	sp|Q96A08|H2B1A_HUMAN	2	1.00	1.33	2.86
Peroxiredoxin-6	sp|P30041|PRDX6_HUMAN	1	1.00	1.38	0.44

Protein AMBP	sp|P02760|AMBP_HUMAN	1	1.00	0.68	1.34
Ig lambda chain V-II region BUR	sp|P01708|LV205_HUMAN	1	1.00	0.85	1.37
BPI fold-containing family A member 2	sp|Q96DR5|BPIA2_HUMAN	1	1.00	0.55	1.09
Golgi membrane protein 1	sp|Q8NBJ4|GOLM1_HUMAN	1	1.00	1.53	12.46
Protein S100-A12	sp|P80511|S10AC_HUMAN	1	1.00	3.40	0.75
Actin-related protein 2/3 subunit 4	sp|P59998|ARPC4_HUMAN	1	1.00	3.38	0.42
Serum amyloid A-4 protein	sp|P35542|SAA4_HUMAN	1	1.00	0.26	0.56
Protein S100-A4	sp|P26447|S10A4_HUMAN	1	1.00	4.47	1.10
Cornifin-B	sp|P22528|SPR1B_HUMAN	1	1.00	1.38	7.88
Glucose-6-phosphate 1-dehydrogenase	sp|P11413|G6PD_HUMAN	1	1.00	1.51	0.80
Apolipoprotein A-IV	sp|P06727|APOA4_HUMAN	1	1.00	0.58	0.19
Serum amyloid P-component	sp|P02743|SAMP_HUMAN	1	1.00	0.88	1.20
Ig lambda chain V-IV region Hil	sp|P01717|LV403_HUMAN	1	1.00	0.59	0.97
Ig kappa chain V-I region Wes	sp|P01611|KV119_HUMAN	1	1.00	0.48	1.27
Ig heavy chain V-I region WOL	sp|P01760|HV105_HUMAN	1	1.00	0.73	0.79
Fatty acid-binding protein, epidermal	sp|Q01469|FABP5_HUMAN	1	1.00	1.46	1.00
Keratin, type I cytoskeletal	sp|P02533|K1C14_HUMAN	1	1.00	0.30	0.83
E3 ubiquitin-protein ligase Arkadia	sp|Q6ZNA4|RN111_HUMAN	1	1.00	0.80	2.56
Proline-rich protein 27	sp|Q6MZM9|PRR27_HUMAN	1	1.00	0	36.8
Carbonic anhydrase 1	sp|P00915|CAH1_HUMAN	1	1.00	0.70	0.68
F-actin-capping protein subunit alpha-1	sp|P52907|CAZA1_HUMAN	1	1.00	0.95	0
Alpha-actinin-4	sp|O43707|ACTN4_HUMAN	1	1.00	1.06	0.87
Myosin-13	sp|Q9UKX3|MYH13_HUMAN	1	1.00	0.16	0.23
Cytospin-A	sp|Q69YQ0|CYTSA_HUMAN	1	1.00	0.49	0.28
CAP-Gly domain-containing linker 2	sp|Q9UDT6|CLIP2_HUMAN	1	1.00	4.16	0.29
Adiponectin receptor protein 2	sp|Q86V24|ADR2_HUMAN	1	1.00	0.61	0.38
Lysyl oxidase homolog 4	sp|Q96JB6|LOXL4_HUMAN	1	1.00	0.82	0.55
				Decrease	Decrease
				Increase	Increase

4.3.8 Detection of IL-6 from plasma using ELISA

ELISA was performed on 24 plasma samples from 8 mechanically ventilated patients to detect IL-6. The cytokine IL-6 was detected in 14 plasma samples during MV, with the highest levels recorded at the start and midpoint of MV (Table 4.9).

Table 4.9 – IL-6 detection from plasma during MV.

	IL-6 pg/ ml in plasma during MV		
Patient Number	**Start MV**	**Midpoint MV**	**ETT extubation**
PN001	15.42	7.81	23.67
PN003	64.90	16.21	5.75
PN026	93.10	38.27	9.41
PN031	0.00	0.00	0.00
PN040	0.00	0.00	1.17
PN077	4.54	0.00	0.00
PN088	46.32	804.90	8.11
PN109	0.00	0.00	0.00
Mean IL-6	28.04	108.40	6.01

IL-6 was detected at all stages during MV. IL-6 ELISA was performed on 24 plasma samples from 8 mechanically ventilated patients.

4.3.9 Cytokine quantification during MV using the cytometric bead array analysis

4.3.9.1 Salivary cytokine analysis from healthy volunteers

Nine saliva samples from 2 healthy volunteers were analysed for cytokine presence over 10 d (Figure 4.16). Levels of cytokines ranged between 0.1 and 100-pg/ml (Table 4.10). Highest cytokine levels in healthy saliva were for IL-8 and IL-1β. There was a variation of ~50 pg/ml over 10 d.

4.3.9.2 Plasma cytokine analysis during mechanical ventilation

Plasma (n=105) was analysed for cytokines from 35 mechanically ventilated patients. The cytokines, IL-8 and IL-6 were present at >200 pg/ml, with all other cytokines (IL-1β, TNFα, IL-10 and IL-12p70) detected at <10 pg/ml. Figure 4.17 presents the detection of IL-8, IL-1β and IL-6 during MV. At the time of intubation the highest cytokine levels were for IL-6. There were no significant differences in the concentrations of IL-8 and IL-6 during MV (Figure 4.17). For IL-1β there was a significant increase post-ETT extubation period (p-<0.001). All individual cytokine quantifications are in appendix III.

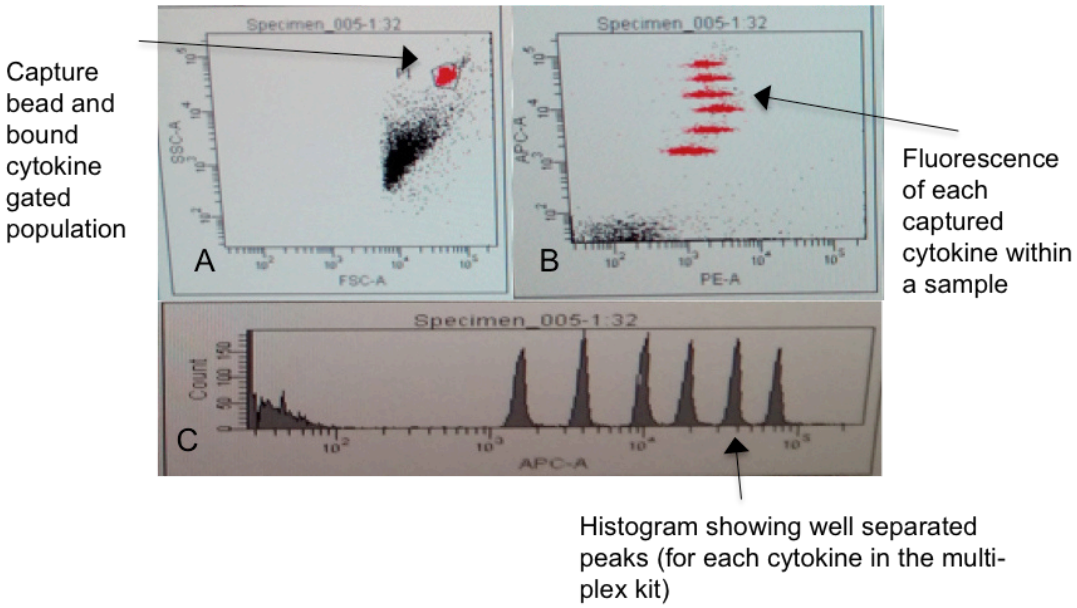

Figure 4.16 - The CBA acquisition template for flow cytometry analysis of samples conjugated with cytokine beads. A; Gated population of cells, B; Detection of each cytokine via captured fluorescence and C; Histogram showing the separation peak of each pro-inflammatory cytokine.

Table 4.10 – Cytokine detection over 10 d in saliva from two healthy volunteers[10].

		Cytokine Concentration (pg/mL)					
		IL-8	**IL-1β**	**IL-6**	**IL-10**	**TNFα**	**IL-12p70**
Volunteer 1	d1	199.38	98.01	19.93	11.13	0	13.4
	d3	99.05	134.72	20.76	0	14.61	0
	d8	63.5	45.31	20.76	0	0	16.16
	d9	13.31	30.48	0	0	0	0
	d10	133.58	77.46	11.72	0	18.34	17.71
Volunteer 2	d1	100.18	121.63	0	0	0	13.38
	d8	56.39	112.39	14.84	0	0	0
	d9	53.81	96.18	24.92	18.75	0	20.29
	d10	160.58	166.32	19.93	0	0	29.23
	Mean	**97.75**	**98.06**	**14.76**	**3.32**	**3.66**	**12.24**
	SD	**58.64**	**42.63**	**9.18**	**6.86**	**7.32**	**10.31**
	SEM	**19.55**	**14.21**	**3.06**	**2.29**	**2.44**	**3.44**

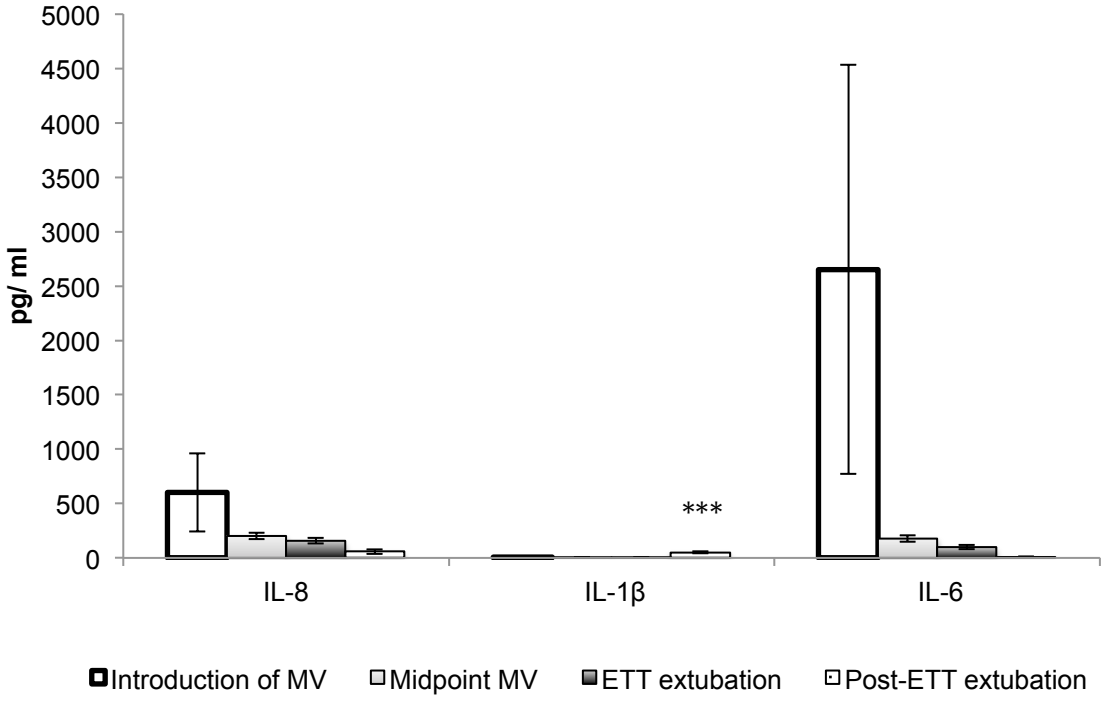

Figure 4.17 - Plasma cytokine concentrations during MV.

[10] d – day of mechanical ventilation

4.3.9.3 Endotracheal tube (ETT) fluid analysis

The fluid from 22 ETTs from 15 mechanically ventilated patients was analysed for cytokine presence. Cytokine's were detected in all of the ETT fluids analysed. IL-8 was detected at >20,000 pg/ml in patients who had respiratory pathogens isolated from their dental plaque, and IL-8 was noticeably lower at <14,000 pg/ml for patients exhibiting predominantly normal oral microbiota during MV. There was no significant difference in cytokine levels for these two patient groups (IL-8 $p=0.374$, IL-1β $p=0.214$, IL-6 $p=0.678$, IL-10 $p=0.069$, TNFα $p=0.237$ and IL-12p70 $p=0.340$).

One-way ANOVA compared pro-inflammatory cytokine concentrations within saliva and ETT fluids. Concentrations of IL8, IL-1β, IL6, IL10 and TNFα were similar for both sample types. The level of IL12p70 for mechanically ventilated patients was significantly lower ($p<0.001$) in ETT fluids compared to saliva. Half of the ETT fluids analysed (n=11) did not contain detectable levels of IL12p70.

4.3.9.4 Salivary cytokine levels during MV

A total of 68 saliva samples from 22 mechanically ventilated patients were analysed for pro-inflammatory cytokine concentration during MV (Figure 4.18). The salivary concentration of IL-8 was over 100-fold higher (up to 100,000 pg/ml) than in plasma. Concentrations of IL-10, TNFα and IL-12p70 were <500 pg/ml.

There were no significant differences ($p>0.387$) in salivary cytokine levels during MV. An increase in IL-8 levels was noted at ETT extubation (100,000 pg/ml relative to 70,000-pg/ml at start of MV). For IL-8 and IL-6, salivary levels were highest at the start of MV and were significantly reduced ($p<0.001$) post extubation. No significant difference in the levels of IL-10, IL12p70 and TNFα for saliva collected from mechanically ventilated patients and healthy volunteers ($p >0.255$) was found.

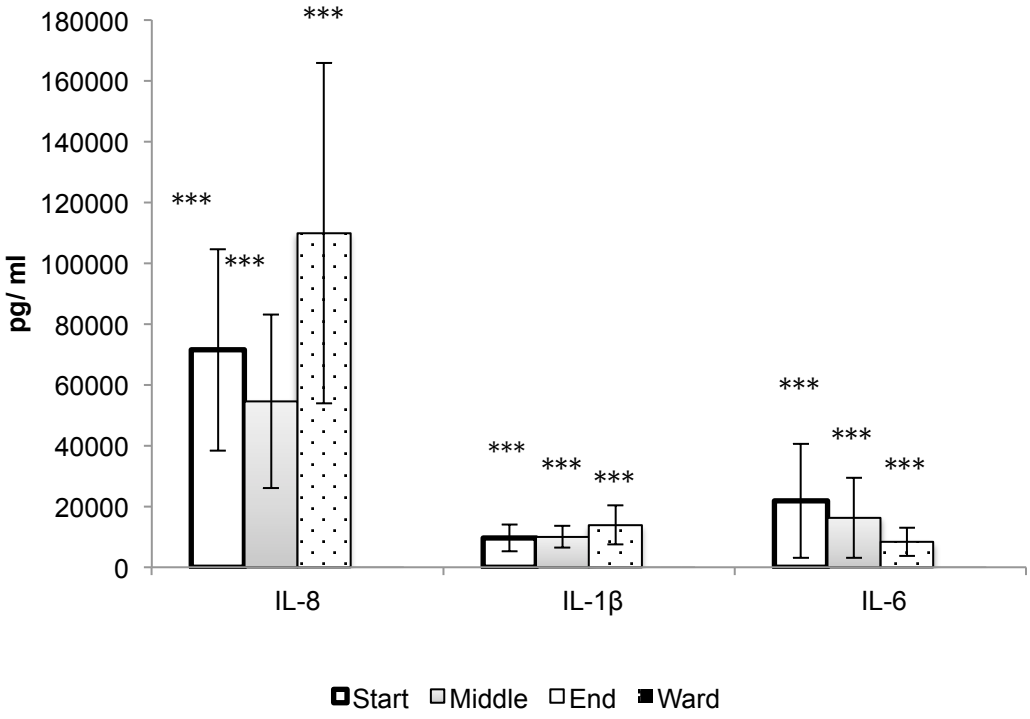

Figure 4.18 – Detection of IL-8, IL-1β and IL-6 in saliva during mechanical ventilation. *Denotes significant difference between cytokine concentration during MV and post-ETT extubation.

4.3.9.5 Cytokine levels and occurrence of respiratory pathogens in dental plaque of mechanically ventilated patients

Patients were divided into two groups based on presence or absence of respiratory pathogens in dental plaque during MV (Chapters 2 and 3). Figure 4.19 shows the cytokine levels in saliva relative to respiratory pathogens colonisation of dental plaque.

The distribution of IL-6 (Figures 4.20), and IL-1β (Figure 4.22) were noticeably increased in saliva of patients colonised with respiratory pathogens (namely *S. aureus* and/or *P. aeruginosa*) in dental plaque. In the case of IL-1β there was a significantly (p=0.035) higher variation in cytokine concentration for patients with respiratory pathogens in their dental plaque compared to other patients. The concentration of IL-8 (Figure 4.21) was however both lower in patients' dental plaque colonised with respiratory pathogens and more varied (as indicated with the larger whiskers in the box plot).

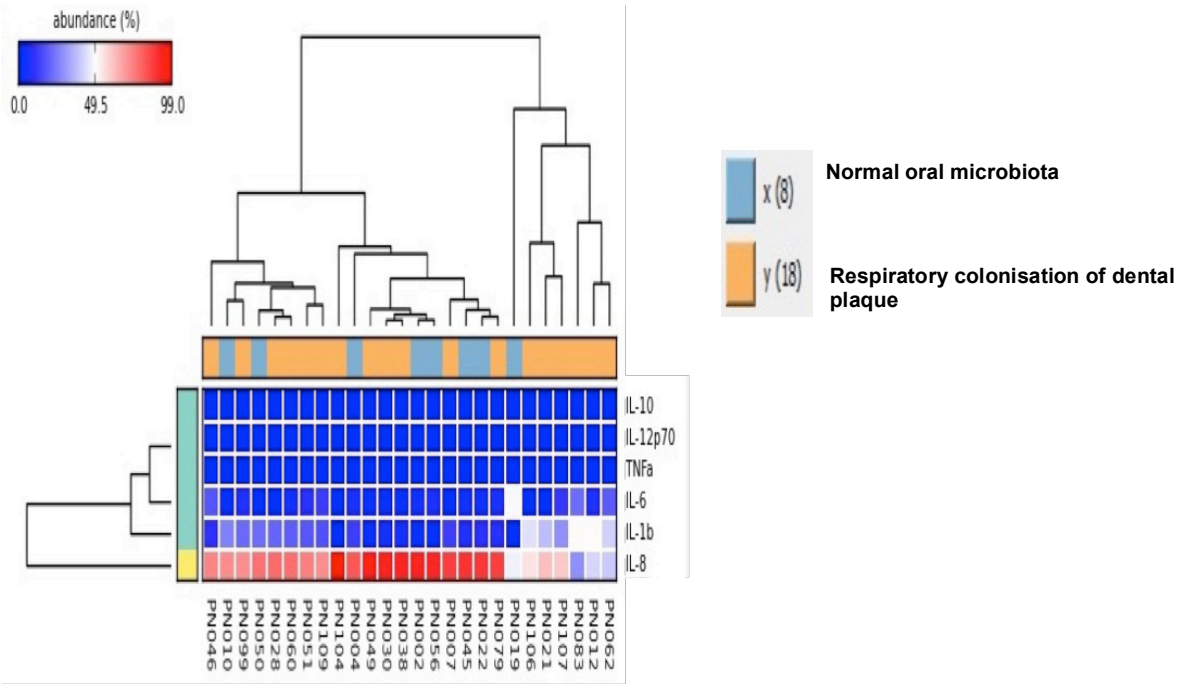

Figure 4.19 – Heat map and dendrogram showing the relationship between mechanically ventilated patients and relative distribution of cytokines. The abundance of each cytokine for each mechanically ventilated patient analysed is represented by a colour (blue-red).

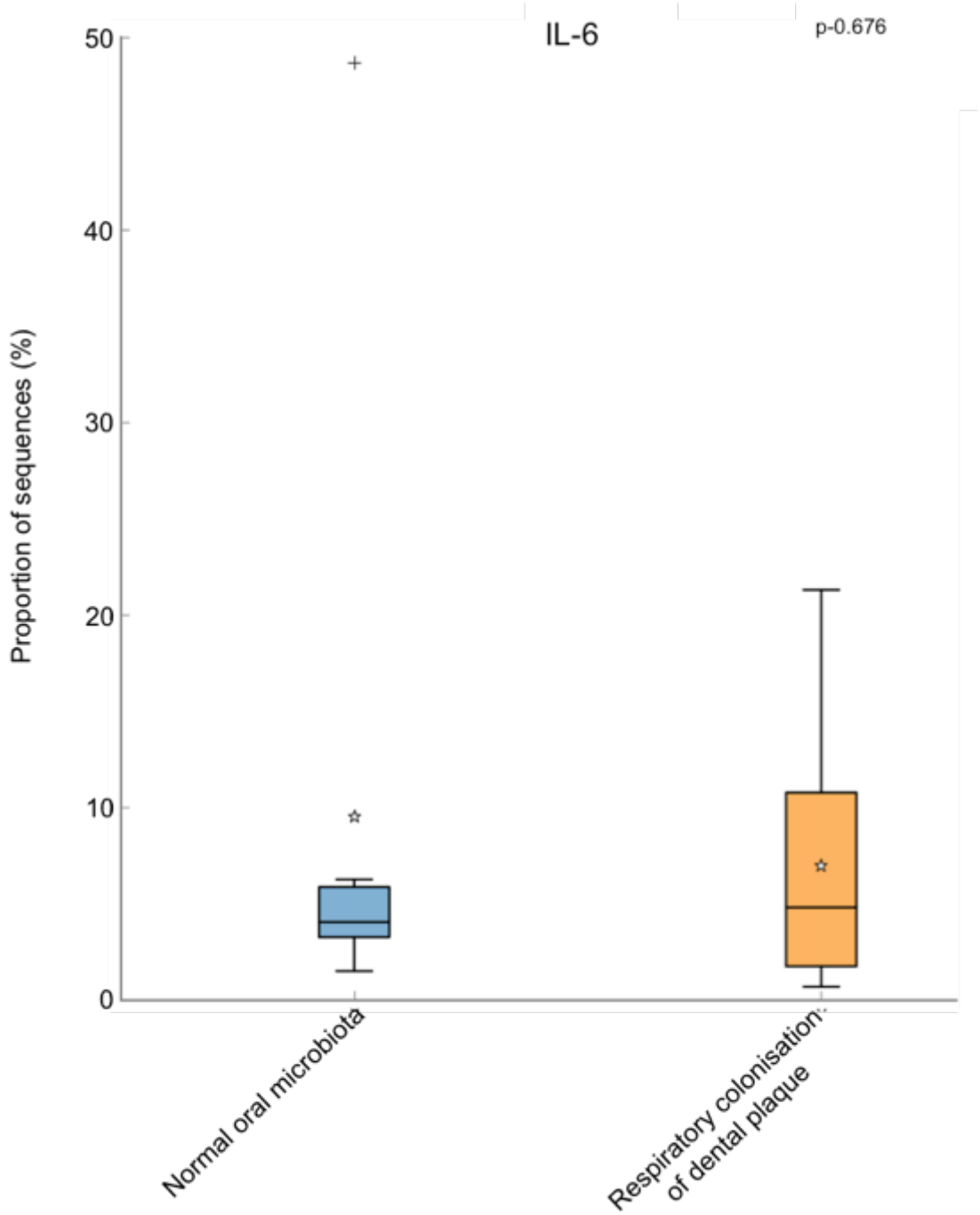

Figure 4.20 - Box and whisker plot comparing distribution of IL-6 concentrations between mechanically ventilated patients exhibiting normal oral and those exhibiting respiratory pathogens in dental plaque during mechanical ventilation[11].

[11] Within box and whisker plots, star values represent the mean value for each group of patients, with any noticeable outliers represented by a cross. The line within the box of the

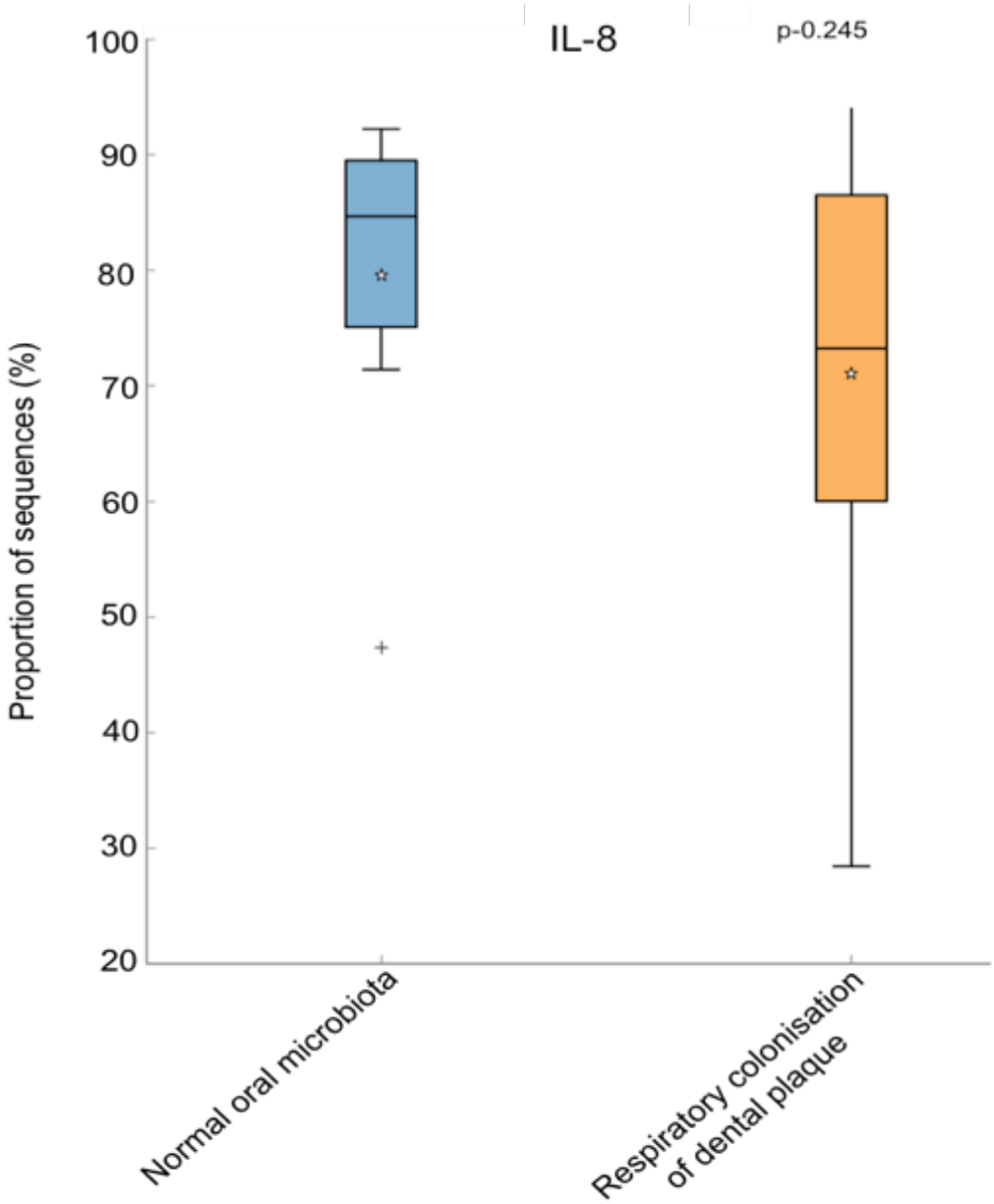

Figure 4.21 - Box and whisker plot comparing distribution of IL-8 concentrations for two patient groups; those exhibiting colonisation of dental plaque with potential respiratory pathogens and those in whom the plaque was not colonised with potential respiratory pathogens.

IQR represents the median value and the whiskers relate to the most extreme values when comparing the cytokine levels.

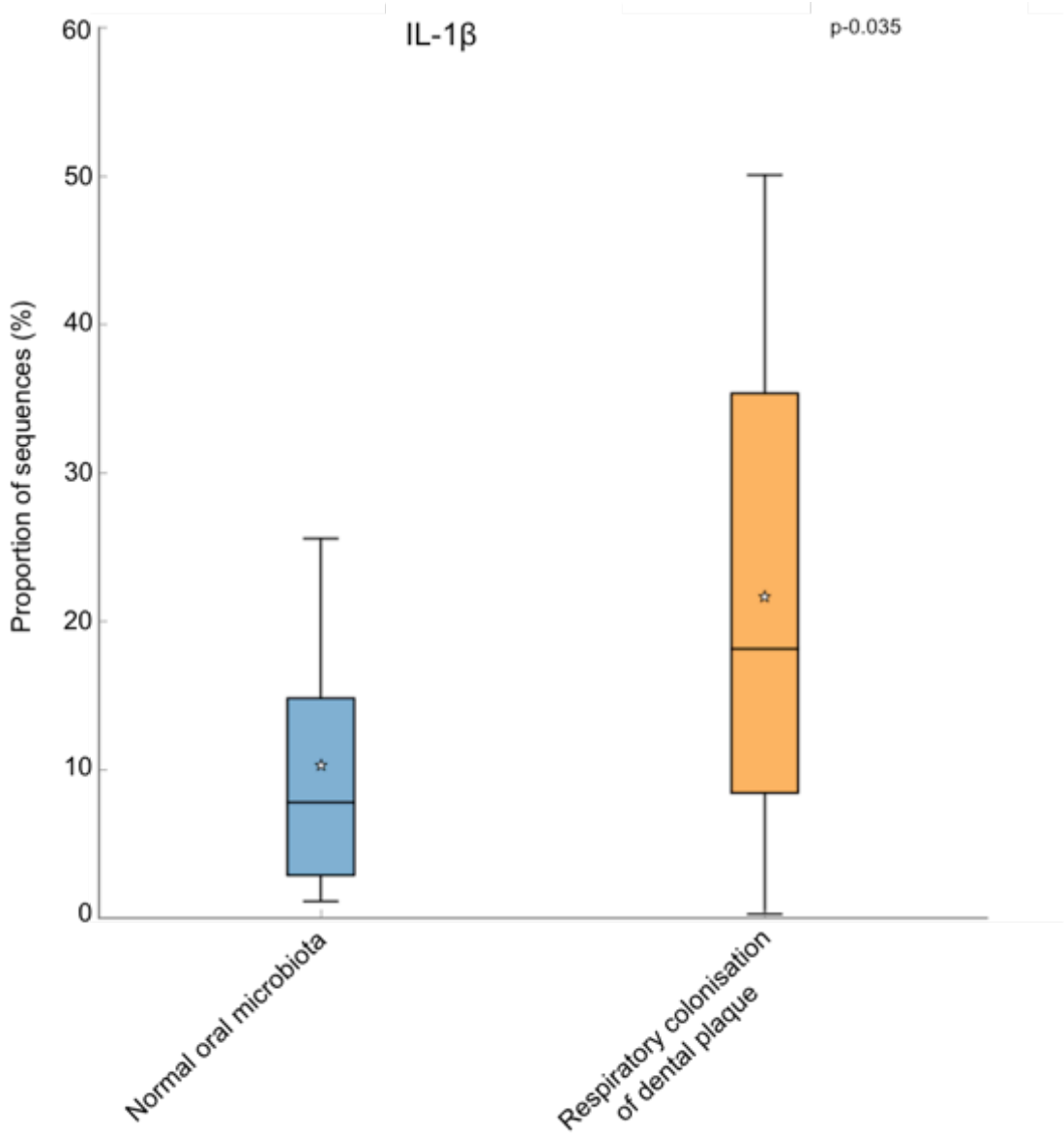

Figure 4.22 – Box and whisker plot comparing distribution of IL-1β concentrations for two patient groups; those exhibiting colonisation of dental plaque with potential respiratory pathogens and those in whom the plaque was not colonised with potential respiratory pathogens.

4.4 Discussion

Saliva is a complex fluid performing multiple functions, and contains several different antimicrobial peptides offering protection within the oral cavity (1.1). Although previous studies have reported reductions in salivary flow during hospitalisation, for example Dennesen et al., 2003 investigated salivary flow in intubated intensive care patients, the relationship between dental plaque and salivary parameters, including volume, pH and composition during mechanical ventilation (MV) is relatively unexplored. The main aim of this chapter was to evaluate whether changes in saliva volume, pH and composition occur both during MV and after ETT extubation, and whether any changes can be associated with the respiratory pathogen colonisation of dental plaque.

Reduced salivary volumes were evident for mechanically ventilated patients (Figure 4.2), both during MV and into the recovery period. Dryness of the mouth (xerostomia) is intimately linked to lower salivary flow (O'Reilly, 2003; Fenoll-Palomares et al., 2004). Salivary secretion in healthy individuals follows natural circadian rhythm (Flink et al., 2005). Critically ill and mechanically ventilated patients may mirror a state of sleep, and as a consequence, the observed salivary flow reduction could reflect natural circadian rhythm patterns (Thie et al., 2002). A total of 65 of the 107 recruited patients were >50 years of age (2.3.2, table 2.3) and salivary flow in healthy individuals has been shown to decrease with age (Dodds et al., 2005), perhaps increasing likelihood of patients in this age group having lower saliva production.

Dehydration of hospitalised patients, often indicated with high levels of sodium (hypernatraemia) (Adrogue & Madias, 2000), has been linked to increased length of hospital stay (Welte et al., 2012) and may further contribute to reduced salivary flow (Ship & Fischer, 1997), especially when coupled with decreased neural input from taste and chewing for mechanically

ventilated patients in critical care (normal activators of the salivary reflex) ((Bardow et al., 2001). Due to decreased salivary flow during MV, cleansing and clearance actions may also be impaired, which may influence the abundance and composition of dental plaque (Humphrey & Williamson, 2001).

Salivary flow is often affected by medications, including those frequently administered to mechanically ventilated patients. Prasanthi et al., 2014 investigated the effects of diuretics on salivary flow and oral health status. Diuretics are used to treat a variety of conditions including renal failure and congestive cardiac failure, and Prasanthi et al., 2014 reported significant reductions in salivary flow rates. Medications can cause xerostomia through a number of mechanisms. Certain drugs (including antihistamines) compete with acetylcholine release at the parasympathetic effector junction, leading to a decreased saliva production. Diuretics however produce alterations in electrolyte and fluid balance, leading to fluctuations and reductions in stimulated whole saliva (Vinayak et al., 2013).

Decreased production of saliva was evident in patients during MV and this would affect buffering within the oral cavity and may influence local pH levels (Fenoll-Palomares et al., 2004). When saliva is stimulated, for example after food, an increase in bicarbonate ions occurs leading to higher (more alkaline) salivary pH (Diaz-Arnold & Marek, 2002). Mechanically ventilated patients are obviously ‘nil by mouth’ and often receive a consortia of medications that decrease salivary flow and may therefore contribute to changes in salivary pH. For example, diuretics frequently used in critical care medicine, for controlling fluid balance (Tibor, 2007), can alter salivary buffering capability, again serving to reduce overall pH (Prasanthi et al., 2014).

Acidic end products of saccharolytic and cariogenic bacteria may in turn decrease salivary pH. *Streptococcus mutans* can ferment carbohydrates to acidic end products and is tolerant of acidic conditions as low as pH 4.5.

Under such conditions, aciduric bacteria will have a selective advantage over less acid tolerant species and will out-compete these species in dental plaque (Ilie & Picioreanu, 2012). Dental plaque containing *S. mutans* are more resilient to acid shock tests, suggesting increased resistance to an environment with a lower pH (Cvitkovitch, 2010).

Xerostomia during MV can contribute to the development of oral candidiasis, with *C. albicans* exploiting changes in the microenvironment of the oral cavity to grow at higher levels (Torres et al., 2002; Thein et al., 2007). Studies of microbial co-aggregation have demonstrated synergistic relationships between *C. albicans* and oral streptococci (Metwalli et al., 2013). Low salivary pH can influence hyphae growth of *C. albicans* by increasing the yeast-hyphae transition as a survival strategy and contribute to biofilm development (Xu et al., 2014). *Candida albicans* may also promote colonisation of *P. aeruginosa.* Azoulay et al., 2006 reported a positive correlation between *C. albicans* colonisation of the airways and *P. aeruginosa* causing VAP. The complex relationship between *C. albicans* and *P. aeruginosa* is further explored in biofilm *in vitro* development in chapter 5 (5.3.2.2). Changes in the oral microbiome arising from lowered pH could have been a factor in colonisation of plaque by *S. aureus* and *P. aeruginosa.* In this study, *S. aureus* was isolated from the plaque of 43 mechanically ventilated patients. This species can thrive in microenvironments with a pH as low as 5.5 (Korting et al., 1992; Baudoux et al., 2007; Lemaire et al., 2011). During this study, it was at the midpoint of MV when salivary pH was at its lowest (Figure 4.3). In addition, at midpoint of MV, respiratory pathogen colonisation of dental plaque was highest (demonstrated by microbial culture (Chapter 2) and high-throughput sequencing (Chapter 3)).

For collection of the salivary proteome, Salivettes® were used. Previous studies have shown increased proteolytic enzyme recovery, but a reduced mucin recovery using Salivettes® compared with passive collection (Lundy & Lamey, 1995; Amado et al., 2013). Collection of saliva using passive drool is

often considered the ‘gold standard’ method (Topkas et al., 2012), however in the mechanically ventilated patient cohort in this study, such an approach was not practical. In addition, one advantage of lower mucin levels is that the resulting saliva is less viscous and this can improve resolution of proteins during separation and peptide identification (Amado et al., 2013).

Compositional changes in the salivary proteome during MV may be a driver of microbial changes, or could be a result of such changes. Proteomic changes in saliva were analysed during MV using gel-based and high-throughput techniques. Analysis of whole saliva by SDS-PAGE revealed large proteomic differences during MV in saliva from mechanically ventilated patients compared with relatively stable longitudinal protein profiles observed in saliva from healthy individuals over 10 d (Figure 4.5 B). The saliva proteome is known to alter rapidly during episodes of physiological stress, which would be inevitably experienced by mechanically ventilated patients (Humphrey & Williamson, 2001).

Although informative, there are several limitations to SDS-PAGE. The complexity of the salivary proteome limits separation on a single dimension. Protein identification from bands resolved by SDS-PAGE is problematic as such bands are likely to consist of multiple proteins due to co-migration. Consequently, 2-dimensonal separation is the current gel-based gold-standard (Schwartz, 1995; Lamy et al., 2012; Amado et al., 2013).

Given the occurrence of post-translational modifications (PTM) and protein complexes within a proteinaceous fluid such as saliva (Gharahdaghi et al., 1999; Mata-Gómez et al., 2012), gel-based proteomics cannot truly reflect the salivary proteome. PTMs and protein complexes may lead to the same proteins being located at different positions within a single gel. Indeed, this was evident in this study using 2DE and colloidal coomassie blue staining and protein identification from gel spots by MALDI-MS coupled with MASCOT database searches. MASCOT provides calculated values for

molecular weight and pI (isoelectric point, pH) and therefore these are theoretical. Such calculations are for unmodified versions of peptides and do not take into account PTM. Identification of α-amylase at multiple locations within one saliva sample (Table 4.3) indicates that salivary proteins formed PTMs and complexes, highlighting the difficulties encountered using gel-based approaches. The enzyme α-amylase is primarily known as a digesting enzyme for carbohydrates. However during MV, patients were nil-by mouth suggesting that α-amylase, although constitutively produced independent of food intake, might have more than one biological function (common attribute of saliva proteins). For example, α-amylase has previously been identified in the enamel pellicle layer, and may have an important role in microbial adhesion and dental plaque formation by acting as a receptor and binding with oral *Streptococcus* species (Scannapieco et al., 1993).

To reveal the extent of compositional changes using a gel-based approach, both a colloidal coomassie blue and a silver nitrate stain were undertaken on the same saliva samples. Silver nitrate staining is more sensitive compared with colloidal coomassie blue (Gharahdaghi et al., 1999). However, when downstream analysis is to be undertaken, such as MALDI-MS, colloidal coomassie blue is often preferred. This is because silver nitrate in stained gels will affect any subsequent trypsin cleavage resulting in poor protein identification (Chevalier, 2010). For 2DE analysis, two saliva samples collected during MV were assessed (Figures 4.9 and 4.10). Differences in protein composition were evident during MV compared with saliva collected from a healthy individual (Figure 4.10). Results using silver nitrate staining, revealed that the second salivary sample collected midpoint MV had >2-dozen additional protein spots compared with the equivalent sample at the start of MV (Figure 4.9).

LC-MS/MS is a ‘shotgun’ bottom-up proteomic approach that can identify proteins in complex mixtures through a combination of chromatography and mass spectrometry (Lamy et al., 2012). Protein composition of saliva is

dynamic often changing according to age, gender, diet and status of oral hygiene (Fleissig et al., 2010). Identified proteins were grouped according to protein class and overall, were similar for both saliva collected from a healthy volunteer and a mechanically ventilated patient (Figures 4.11 and 4.12 respectively). However, a number of transcription proteins were shown to be present in saliva from a mechanically ventilated patient, that were absent in the saliva from a healthy individual.

Analysis of the salivary proteome during MV revealed multiple differences when compared to that of healthy saliva. For example complement proteins, and proteins associated with neutrophil recruitment were identified during MV (Table 4.5), but were absent from saliva at all time points for the healthy individual. This may suggest an initiation of innate immune responses and the recruitment of leukocytes for localised defense in the mechanically ventilated patient. Protein S-100 was identified during MV and has biological functions including promoting oral homeostasis in addition to regulatory roles for the recruitment of neutrophils and macrophages (Donato et al., 2012). The presence of peptides from hemoglobin in saliva collected during MV, may suggest an increase in localised inflammation and oral mucosal tissue damage during MV, perhaps as a result of dental plaque accumulation.
Several bacterial and fungal proteins were identified in saliva from the healthy volunteer and a mechanically ventilated patient (Table 4.6). Proteins from lactobacilli, anaerobic bacteria, *Candida,* and *Campylobacter concisus* were identified from healthy saliva. Although *C. concisus* has been associated with gingival inflammation, there have been reports of *C. concisus* isolation in healthy individuals (Zhang et al., 2010). Alongside proteins from members of the normal oral microbiota, *E. coli* peptides were detected in saliva during MV. Interestingly, *E. coli* was also identified within dental plaque during MV (3.3.3.4, table 3.1 and figure 3.10).

Quantitative analysis of proteins using iTRAQ™ reagents, compared protein expression over the course of MV. Previous studies using iTRAQ™ labeling

have reported success with the identification of proteins from a diverse abundance, isoelectric point (or pH) and molecular weight (Aggarwal et al., 2006). It was necessary to determine relative expression changes of proteins in healthy saliva before quantifying changes during MV. Quantitative analysis on healthy volunteer saliva over 10 d allowed assessment of potential protein variance prior to examining changes over the course of MV (Table 4.7). Limited changes in salivary proteins occurred over the 10 d period for the healthy volunteer saliva. Out of more than 100 identified proteins, noticeable increases were evident only with peregrin and tripeptidyl-peptidase 2, the latter a multipurpose peptidase involved in metabolic pathways (Tomkinson & Lindås, 2005), which increased up to 50 fold.

Proteomic analysis of saliva from a mechanically ventilated patient revealed noticeable increases in the expression of several proteins, particularly those associated with inflammatory responses (Table 4.8). Proteins such as neutrophil gelatinase, lactotransferrin, and haptoglobin were all elevated during MV, suggesting that the oral mucosa and gingival tissue may be responding to external stimuli and stress, including microbial changes and underlying patient condition. Protein expression was set to 1.0 at the start of MV, allowing calculation of the relative change in expression during MV. Although informative of fold-differences during MV, one such limitation of this approach is the investigation of highly expressed proteins at the beginning of MV. A drop in expression of a particular protein at midpoint of MV would however suggest an increased expression at intubation.

Furthermore, noticeably increased levels of lysozyme and lactoperoxidase were evident in saliva obtained after ETT-extubation compared to during MV. These findings could reflect diminished expression during MV, and a return to normal levels of this antibacterial peptide. Lipocalin-1, is a protein associated with scavenging of toxic molecules (Vitorino et al., 2004; Jessie et al., 2010), and this was highly expressed after ETT extubation, compared with levels at the commencement and during MV. Saliva levels of carbonic

anhydrase (associated with pH homeostasis) were noticeably higher in the post-ETT extubation (Table 4.8). Increased concentrations of carbonic anhydrase VI in saliva enhances the buffering capacity of saliva (Kimoto et al., 2006), and could therefore have a role in increasing the salivary pH following discontinuation of MV. Carbonic anhydrase VI has been reported as a potential anti-caries protein, neutralising dental plaque acid (Kimoto et al., 2006).

Oral mucosa is comprised of stratified squamous epithelial cells, offering immediate continuous multi-layered protection, and cytokine production to recruit neutrophils to infected surfaces (Abram et al., 2000; Krisanaprakornkit et al., 2000; Sandros et al., 2000). Innate inflammatory responses are highly complex, involving combinations of mediator cells and signal cascades (Medzhitov, 2007; Feller et al., 2013). Cytokines are glycoproteins that regulate the immune responses by initiating and coordinating cell communication (Degré, 1996; Gemmell & Seymour, 1998; R.-Q et al., 2014). Interleukins (IL) facilitate communication between blood cells (including neutrophils and macrophages), chemokines and interferons (Kleiner et al., 2013). Targeted cytokine levels (IL-8, IL-1β and IL-6) in saliva were significantly elevated during MV (Figure 4.18), with similar concentrations detected in fluid collected from the inner lumen of ETTs, which subsequently decreased post-ETT extubation to reflect levels exhibited in the saliva of healthy volunteers (Table 4.10).

IL-8 is a pro-inflammatory chemokine involved in recruiting neutrophils and mediating inflammatory responses within gingival tissues. This chemokine is localised in increasing numbers in the gingival tissue of patients with periodontitis (Yumoto et al., 1999; Sandros et al., 2000; Winkler et al., 2001). IL-8 production in surrounding epithelial tissue may be a response to an acute accumulation of dental plaque (Jenkinson & Lamont, 2005; Sandros et al., 2000). The elevated levels of IL-8 during MV were coupled with higher levels of IL-6 (Figures 4.21, 4.20). IL-6 has been shown to increase during

episodes of oral disease and inflammation (Gemmell & Seymour, 1998; Peyyala et al., 2012), which is not at all surprising during MV.

IL-1β is a pro-inflammatory cytokine generated by macrophages after exposure to microbial components, particularly lipopolysaccharide (LPS). An increase in IL-1β suggests the presence of inflammatory factors and perhaps an increase in Gram-negative bacteria within dental plaque (Reimann et al., 1994). Increases in pro-inflammatory proteins such as cytokines can promote local tissue destruction (Ginsburg et al., 2012; Rogers H, 2013). A study by Wilkinson et al., 2010 has shown increased IL-8 and IL-1β within alveolar tissues of patients with VAP. Interestingly, the distribution of IL-1β was significantly ($p=0.035$) increased in patients in whom respiratory pathogen colonisation of dental plaque occurred (Figure 4.22). After extubation, a significant reduction in all pro-inflammatory cytokines to levels of those evident in the saliva of healthy individuals was apparent.

Due to high levels of potential respiratory pathogens colonising dental plaque, oral health is an important unexplored marker for pneumonia, as changes to the oral microbiome may precede the translocation of potential pathogens into the lungs. Future work should include a clinical study investigating the role of cytokines circulating within saliva during MV to act as biomarkers for the prognosis and diagnosis of VAP. A series of adhesion molecules (CD62L, CD54, CD83), neutrophil elastase (Wilkinson et al., 2012; Hellyer et al., 2015) neutrophil receptors CD35/CR1 (Nuutila, et al., 2006) and cytokines (IL-6, IL-8, IL-1β and IL-12) (Conway Morris et al. 2010) could be quantified from saliva during MV and correlated to the respiratory pathogen colonisation of dental plaque.

Conclusion

During MV, there was reduced salivary flow and pH, which was particularly evident at the midpoint of MV, and for patients in whom respiratory pathogens were shown to be colonising dental plaque.

A series of gel-based proteomic analyses were performed to provide an overall picture of salivary proteins. Compared to a relatively stable saliva proteome expressed from a healthy individual over the course of 10 d, gel-based proteomics revealed major compositional changes occurring during MV.

LC-MS/MS analysis identified and quantified protein expression during MV in a high throughput manner. Initial studies indicated there were multiple protein changes occurring during MV, including proteins associated with inflammatory responses, such as lysozyme. Neutrophil elastase and lysozyme were among proteins identified from a mechanically ventilated patient with dental plaque colonisation of respiratory pathogens. Significant increases in salivary cytokine profiles during MV could have been instigators of associated microbial changes or be reflective of the changes themselves.

Further studies targeting proteins such as neutrophil elastase, proteases and proteins involved in initiating the immune response could determine their involvement in the dynamics of the oral microbiome during MV. There is also the potential to explore the potential of salivary proteins and respiratory colonisation of dental plaque, as a non-invasive prognostic and diagnostic tool for VAP.

Chapter 5

Effect of oral microorganisms and chlorhexidine on respiratory pathogen presence in *in vitro* biofilms

5.1 Introduction

Oral hygiene is an essential facet of care for critically ill patients, with the aim of reducing the risk of secondary infections such as Ventilator-Associated Pneumonia (VAP), as well as aiding comfort and oral lubrication (O'Reilly, 2003; Munro et al., 2009; Johnson et al., 2012). Typically, oral wash interventions such as chlorhexidine are administered to enhance the physical removal of dental plaque (Wise et al., 2008; Ames, 2011; Gu et al., 2012). Before studies can accurately investigate the effectiveness of oral interventions, there is a need to define an optimal and universal strategy for measuring oral hygiene in critically ill patients via dental plaque scoring for example (Wise & Williams, 2013).

Chlorhexidine (CHX) is an antiseptic with broad antimicrobial activity (1.7.5.5) and is administered widely in critical care units (Jones et al. 2004; Munro & Grap 2004; Pineda et al., 2006; Rello et al., 2007; Munro et al., 2009; Needleman et al., 2011). Depending on the outcome of oral assessments, CHX may be administered up to four times a day (Scannapieco et al. 2009; Needleman et al. 2011). Current literature shows conflicting evidence as to whether oral antiseptics such as CHX are effective at controlling dental plaque and consequently reducing VAP (Jones, 1997; Pineda et al., 2006 Bellissimo-Rodrigues et al., 2009). Studies indicate that CHX concentrations as low as 0.12% (v/v) are effective against oral microbial species, especially when administered in conjunction with tooth brushing (Zanatta et al., 2007). A clinical trial by Scannapieco et al., 2009, involving 175 intubated patients, studied the effectiveness of 0.12% CHX in reducing the overall colonisation of dental plaque by oral and non-oral pathogens. Scannapieco et al., 2009 documented the ability of CHX to reduce *S. aureus* in dental plaque, however, there was no equivalent reduction in the numbers of other respiratory pathogens including *Pseudomonas* and *Acinetobacter* species.

Changes in the microbial population during dental plaque formation and further colonisation by pathogenic species may influence the pH of saliva and dental plaque (Jakubovics & Kolenbrander, 2010). It is known that for the majority of critically ill patients, the level of oral hygiene frequently deteriorates during mechanical ventilation (MV; Fourrier et al., 1998; Sachdev et al., 2013), ultimately leading to accumulation of dental plaque and gingival inflammation (Wood et al., 2002). Decreases in local environmental pH can be an external pressure leading to the survival and growth of aciduric organisms such as *S. mutans* (Jakubovics & Kolenbrander, 2010). Increases in the abundance of aciduric and acidogenic streptococci will inevitably increase acidic by-products from fermentation of carbohydrates such as sucrose, perhaps serving to reduce the biofilm pH further and influence the surrounding salivary pH. Opportunistic pathogens such as *S. aureus, P. aeruginosa* and *Streptococcus pseudopneumoniae* can be predominant colonisers of dental plaque during MV. These respiratory pathogens may therefore exploit local environmental changes such as pH or oral microbial changes in dental plaque to subsequently colonise and possibly outcompete normal oral bacteria. *Staphylococcus aureus*, for example, will thrive under acidic conditions (as low as pH 5; Cotter & Hill, 2003; Baudoux et al., 2007).

The primary aim of the work presented in this chapter was to develop single and mixed species biofilm models which could be used to examine firstly, the interactions between selected species and secondly, the antimicrobial chlorhexidine and effects of different saliva pHs.

More specifically the objectives were as follows-

(1). To assess whether *S. mutans* or *C. albicans* would promote *S. aureus* and *P. aeruginosa* colonisation of biofilms on endotracheal tube (ETT) surfaces. This aim was assessed by both culture and imaging.

(2). To investigate the effect of specific oral microorganisms and oral hygiene interventions (as a result from the clinical investigation, chapters 2, 3 and 4) as drivers for respiratory pathogen colonisation of dental plaque. To assess this aim:

i. MIC values were obtained for antimicrobial mouthwashes including chlorhexidine (CHX) for planktonic and biofilm organisms.

ii. Dental plaque from healthy individuals was used to validate the constant depth film fermenter (CDFF) model (1.4.7) to produce oral biofilms for subsequent challenge experiments.

iii. Effect of CHX (using MICs determined in 1) challenge on oral biofilms upon respiratory pathogen colonisation was assessed.

iv. Effect of saliva at various different pHs upon the degree of respiratory pathogen viability/infiltration of dental plaque (previously formed in the CDFF) was assessed.

5.2 Materials and Methods

5.2.1. Identification of reference strains by 16S rRNA sequencing for *in vitro* analysis

5.2.1.1. Microbial culture and DNA extraction

Microorganisms (Table 5.1) were cultured on 5% (v/v) blood agar (Oxoid) from stock isolates and incubated at 37°C for 48 h. An overnight culture was prepared in Brain Heart Infusion (BHI) broth (Oxoid) and incubated aerobically at 37°C for 24-48 h for DNA extraction. DNA extractions were performed according to section 2.2.3.3 (*C. albicans* DNA extraction was as described for Gram-positive bacteria), incubated overnight at 15-25°C with gently shaking, followed by a centrifugation of 3 min at 16,000 × *g*.

Table 5.1 – Microbial reference strains used for *in vitro* analysis

Species	Source	Strain reference
Candida albicans	American Type of Culture Collection (ATCC)	90028
Streptococcus mutans	German collection of Microorganisms (GSM)	2053^{t}
Staphylococcus aureus	National Collection of Industrial Bacteria (NCIB)	9518
Pseudomonas aeruginosa	American Type of Culture Collection (ATCC)	25668

5.2.1.2 Bacterial 16S rRNA PCR and gel electrophoresis

The primers used to identify *P. aeruginosa, S. mutans* and *S. aureus* were: 1492r (CACGGATCCTACGGGTACCTTGTTACGACTT) and 27f (GTGCTGCAGAGAGTTTGATCCTGGCTCAG) (Eurofins MWG Operon) Dalwai et al., 2007; Zuanazzi et al., 2010) (sections 3.2.2.4-3.2.2.5). The total volume of PCR reagents was 50 µl (25 µl PCR master mix, 2 µl DNA

template, 0.5 µl reverse primer, 0.5 µl forward primer, and 22 µl of nuclease water). PCR thermal cycling parameters consisted of 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). A final single cycle extension step of 72°C for 15 min was also included. Gel electrophoresis was performed (1.0% (w/v) agarose gel) at 70/Vcm^2 for 50 min, in a 0.5× Tris Borate EDTA (TBE) buffer with 10% SafeView™ (NBS Biologicals NBS-SV1) for detection of nucleic acids alongside a 1 Kbp molecular weight ladder (Promega). A gel doc system was used to determine DNA presence pre-sequencing (Gel-DocIT_UVP; Biorad).

5.2.1.3 *Candida albicans* PCR

To amplify DNA, rDNA sequences of *C. albicans,* ITS1 (TCCGTAGGTGAACCTGCGG) and ITS2 (TCCTCCGCTTATTGATATGC) (Eurofins MWG Operon) primers were used (Fujita et al., 2001). PCR thermal cycling (G-Storm Thermocycler) consisted of an initial denaturation step of 4 min at 95°C, and then 35 cycles of denaturation for 30 s at 95°C, annealing for 1 min at 62°C, and elongation for 90 s at 72°C. A final extension step was set at 72°C for 5 min. Gel electrophoresis was performed as described in section 5.2.1.2.

5.2.1.4 DNA Sequencing

Amplified DNA was purified for DNA sequencing using the QIAquick PCR purification kit (Qiagen). DNA was added to a spin column with a 1:5 ratio of PB buffer (binding buffer) and centrifuged at 17,900 ×*g* for 1 min. The DNA was bound to the membrane and eluted into a microcentrifuge tube using nuclease free water. DNA was quantified using a Nanodrop (GE Healthcare Bio-Sciences) and sequenced using the BigDye® Terminator v3.1 kit (Life Technologies) coupled with the platform 3730xl DNA Analyzer (DNA Sequencing Core, Molecular Biology Unit, Bio-sciences, Cardiff University). The primers used in the original PCR were also supplied to the sequencing unit at 2 pmol/µl in a 15-µl volume.

5.2.2 Effect of oral microorganisms on *in vitro* colonisation of ETT biofilms by respiratory pathogen

5.2.2.1 Biofilm development on sections of ETT

Microorganisms were cultured on selective agar and incubated as previously described (2.2.3). Broth cultures were prepared by inoculation of a single colony in 20 ml of BHI, with *P. aeruginosa, S. aureus* and *C. albicans* incubated aerobically at 37°C for 24 h, and *Streptococcus mutans* was incubated in 5°C carbon dioxide for 48 h. A 0.5 McFarland standard (1x 10^8 CFU/ml) preparation of microorganisms was prepared from broth cultures to inoculate ETT sections. For single species biofilms, 1 ml of the 0.5 McFarland preparations was added to 9 ml of BHI. For polymicrobial biofilms, 1 ml of each organism was combined and a 1-ml volume added to 9ml of BHI and vortex mixed. For positive controls, serial dilutions of a 0.5 McFarland standard suspension were prepared for each species using a spiral-plater (Whitley automatic) and incubated at 37°C for 24 h for bacterial enumeration.

Using a sterile scalpel, a 0.5 cm length of ETT (TaperGuard™ Evac Oral Tracheal Tube 8.0 mm, ID 11.8 mm OD) was cut and added aseptically to the universal tube. For each individual inoculum, triplicate replicates were prepared. The universal tubes were incubated aerobically for a total of 10 d at 37°C. Every 24 h, the media was decanted and replenished with 10 ml of fresh BHI without agitation to the biofilm. The surface area of the 0.5 cm section of ETT was calculated to allow results of colonisation to be expressed as CFU per cm^2 (Figure 5.1).

ID – Inner diameter = 8.00mm

OD – Outer diameter = 11.8mm

r = .4cm

R = .59cm

h = .5cm

$= 2\pi rh + 2\pi Rh + 2(\pi R^2 - \pi r^2)$

$= 2\pi \ .4x.5 + 2\pi \ .59 \ x.5 + 2(\pi.59^2 - \pi.4^2)$

$= 1.25 + 1.85 + 1.68$

$= \mathbf{5.265 cm^2}$

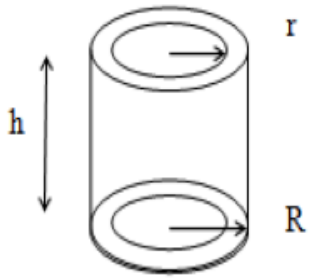

Figure 5.1 – The total surface area of the 0.5 cm length of ETT included the area of the internal surface, the area of the external surface and the area of the top and base edges. Surface area calculation of the ETT section to allow expression of results as colony forming units (CFU) per cm^2.

5.2.2.2 ETT biofilm enumeration

After 10 d, the BHI medium was discarded and 1 ml of PBS added and the mixture gently rocked on a platform for 2-5 min. The PBS was discarded, replenished and this process was repeated three times. After the final 1 ml of PBS was removed, a sterile cotton swab was used to remove the biofilm from the ETT tube, covering all surfaces for 20 s. The cotton swab was then resuspended in 5 ml of PBS and vortex mixed for 20 s. Serial dilutions were then prepared using PBS and these were spirally plated (Don Whitley) on selective agars and CFU/ml determined following culture for 24 h. Colony counts were then extrapolated to CFU/cm^2 of ETT section.

5.2.2.3 ETT section preparation for microscopic imaging: Scanning Electron Microscopy (SEM)

ETT biofilms were washed in PBS and sections placed in 2 ml of 10% formalin, and left for at least 24 h.

After processing in pathology for wax embedded sections, a series of 20 µm sections were transferred onto microscope slides (Superfrost, Fisher). To de-wax the sections for imaging, 3 incubation periods of 10 min each in xylene was undertaken, followed by a wash with 100% ethanol. The slides were air-dried and sputter coated with gold for 8 min in readiness for scanning electron microscopy (SEM). Biofilms were viewed in a JEOL 840A SEM at 5kV and digital images captured using SIS software (School of Medicine, Cardiff University).

5.2.3 Minimum Inhibitory Concentration (MICs) determination of antimicrobial mouthwashes

5.2.3.1 MIC of planktonic single and mixed species

Staphylococcus aureus, P. aeruginosa, S. mutans and C. albicans were cultured on selective agar and incubated appropriately (section 2.2.3.4). In addition, a series of clinical isolates (n=20) obtained from dental plaque

during this study (2.3.2-2.3.3) were analysed. An overnight culture was prepared in Mueller-Hinton Broth (MHB) and a 0.5 McFarland Standard ($1x10^8$ CFU/ml) prepared in MHB using a spectrophotometer at 625_{nm}. The McFarland standards were diluted 1:100 ($1x10^6$ CFU/ml) using MHB. Serial dilutions of antimicrobial mouthwashes (Listerine™ and CHX) were prepared in MHB, with Listerine™ starting at 50% (v/v) and CHX starting at 2% (v/v).

A 100-µl volume of diluted antimicrobial was added to the wells of 96 well plates, and 100 µl of the microbial suspension was subsequently added (200 µl total volume). Controls included sterile MHB (-VE control) and bacterial suspensions without antimicrobials (+VE control). The 96 well microtitre plates were incubated aerobically at 37°C for 24 h. After incubation the relative growth was estimated by a turbidity measurement of each well using spectrophotometric absorbance at 620_{nm}. Readings were standardised using wells with antimicrobial mouthwash dilutions without microbial species. The MIC value was recorded as the lowest concentration of antimicrobial agent that showed 80% reduction in absorbance reading, compared to the positive control (Klepser et al., 1998). MIC ranges were determined from 3 sets of triplicate experiments.

5.2.3.2 MICs for single and mixed species biofilms

Artificial saliva (A/S) was prepared in dH_20: 2.5 g/L porcine stomach mucin (Oxoid), 0.35 g/L sodium chloride (Fisher), 0.2 g/L potassium chloride (Fisher), 0.2 g/L calcium chloride dehydrate ($CaCl_2.2H_20$) (Fisher), 2.0 g/L yeast extract (Oxoid), 1.0 g/L Lab Lemco (Oxoid) and 5.0 g/L proteose peptone (Oxoid). The A/S mixture was aliquoted in 100 ml bottles and sterilised by autoclaving at 121°C for 15 min. A 125-µl volume of 40% (w/v) urea filter sterilised using a 0.22 µm pore size filter (Fisher) was added to 100 ml of A/S prior to use.

A 200-µl volume of A/S was added to each well of a 96 well plate and incubated for 24 h at 37°C to precondition the wells. The A/S was removed and 200 µl of a 1:100 diluted 0.5 McFarland standard ($1x10^6$ CFU/ml) of microbial species added. For multi-species inocula, the diluted preparations

were mixed before addition to the well. After biofilm development for 24 h at 37°C, the medium was discarded and the biofilm washed by adding 100 µl of phosphate buffered saline (PBS). Two hundred µl volumes of two-fold series dilutions of antimicrobial mouthwashes were added to each well, and the biofilms incubated for a further 24 h period. Media was removed and the biofilm washed in 100 µl of PBS. A 200-µl volume of MHB was added to the wells, and the biofilm re-suspended by repeat pipetting. Absorbance readings were determined and the 96 well plates re-incubated for a further 6 h. The MIC for *in vitro* biofilms was determined by measuring the difference in absorbance over the 24 h incubation period. The MIC was the concentration of antimicrobial that caused an 80% absorbance reduction compared to positive controls (Klepser et al., 1998). For both planktonic and biofilm mixed species combinations, 100 µl of the sub-MIC concentration was removed for species-specific microbial counts, using a spiral-plater (Don Whitley).

5.2.4 Dental plaque formation within the CDFF

5.2.4.1 Ethical approval

The collection of dental plaque required ethical approval from the Human Tissue Act (HTA) Governance Team at Cardiff University, and further approval from the School of Dentistry Research Committee (Appendix IV). The inclusion criteria was as follows: >18 years old, > 8 original teeth, no prescription medication and willing to avoid tooth brushing for 12-24 h before sample collection.

5.2.4.2 Dental plaque collection

Supragingival dental plaque was collected from recruited volunteers using dental instruments from 5 individuals. A dentist at the School of Dentistry (Paola Marino) performed the dental plaque collection once participant consent was obtained. Dental plaque was collected using paper points and initially stored in transport medium (section 2.2.3.1). Dental plaque from each volunteer was pooled to anonymise samples. Samples were gently inverted

and divided into 10x1 ml aliquots and stored in 15% (v/v) glycerol at -80°C until needed.

5.2.4.3 CDFF medium preparation

Biofilm medium (proteose peptone, 10 g/L; trypticase peptone, 5 g/L; yeast extract, 5 g/L, KCl, 2.5 g/L, haemin, 0.005 g/L, vitamin K_1, 0.001 g/L, L-cysteine HCl, 0.5 g/L and glucose, 10 g/L) was used to supplement the development of oral biofilms (McKee et al., 1985; Hill et al., 2010).

5.2.4.4 CDFF mediated development of 5 d oral biofilms

Oral biofilms were generated in a CDFF maintained at 37°C, on 4.75 mm diameter polytetrafluoroethylene (PTFE) plug inserts within a rotating turntable at a constant depth of 400 µm. To resemble the enamel pellicle formation, culture medium was initially re-circulated through the CDFF for 30 min with a turntable speed of 20 ml/h. A 100-µl volume of dental plaque was added to BM medium (1 L) and re-circulated through the CDFF for 24 h to 'seed' the system. After this time, the inoculum was disconnected and fresh un-inoculated medium fed into the CDFF, and the waste medium collected in a separate effluent bottle. The growth medium was delivered to the CDFF using a peristaltic pump (Watson–Marlow, 101U MK2) at a rate of 20 ml/h. The growth medium was continuously supplied to the CDFF for a period of 5 d. A PTFE scraper blade washed incoming media over the 15 pans, each containing 5 PTFE plugs (Wilson 1996).

5.2.4.5 Recovery and quantification of bacteria within the oral biofilm

A series of viable cell counts (CFU/ml) was performed on biofilm plugs removed from the CDFF (before and after subsequent incubation with different respiratory pathogens). Plugs were aseptically removed from the CDFF using sterile forceps and individually immersed in 5 ml of PBS and vortex-mixed for 2 min to remove the biofilm. Serial dilutions were prepared and re-suspensions were spirally plated (Don Whitley) on to BA, FAA and SAB agar media. Agars were incubated aerobically for 24 h at 37°C, or in the case of FAA, anaerobically for 48 h at 37°C. A total of 5 PTFE pans were

prepared for serial dilutions for each experimental factor to allow for ANOVA testing (IBM SPSS v20).

5.2.4.6 Biofilm imaging using live dead/PNA-FISH coupled with CLSM

PTFE plugs containing biofilms for imaging were fixed in 2 ml of 10% formalin. Species-specific PNA probes for respiratory pathogens (*S. aureus* and *P. aeruginosa*) and a bacterial universal PNA probe, were added to a series of 20 µm sections of dental plaque biofilms after exposure to different experimental conditions (2.2.7.1-2.2.7.3). Biofilms were imaged on a Zeiss LSM 880 microscope and images were processed using Zen Lite software.

5.2.5 Dental plaque challenge experiments: exposure to CHX and altered 'salivary' pH

Dental plaque biofilms formed upon CDFF PTFE pegs were subjected to challenge experiments including exposure to altered 'salivary' pH and CHX as outlined (Figure 5.2).

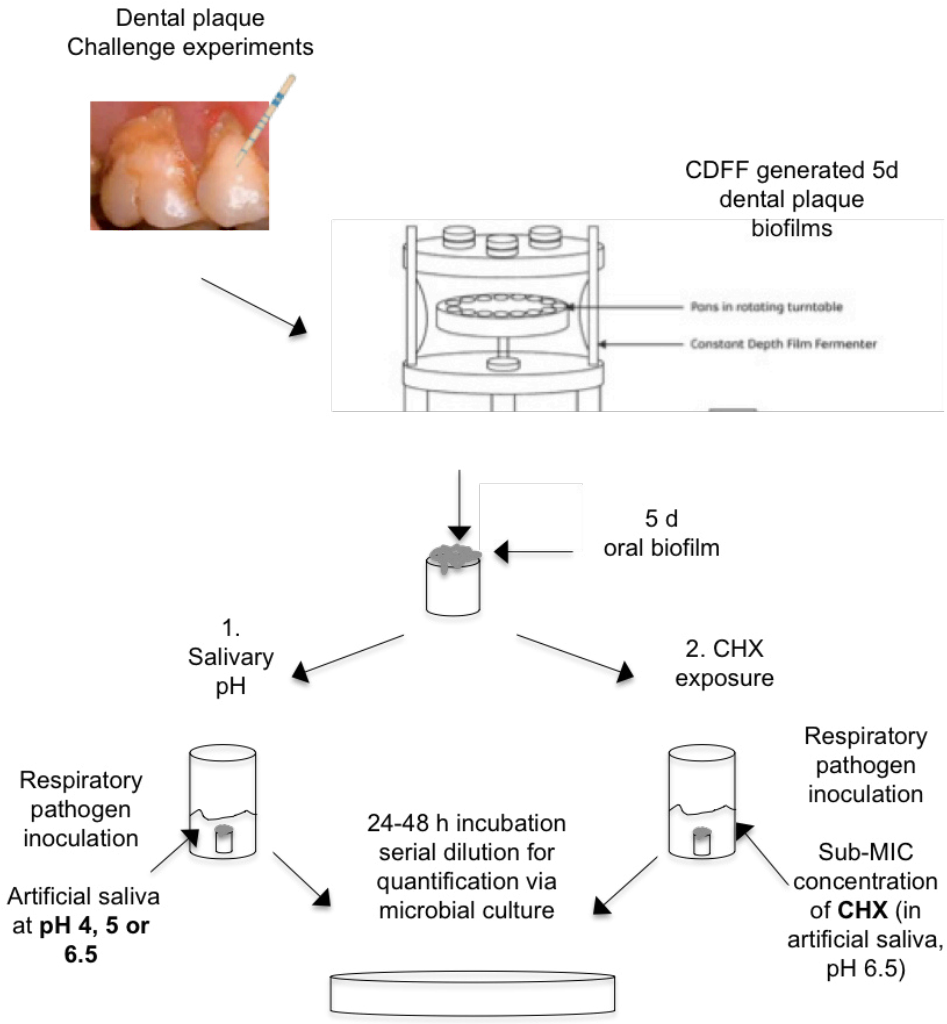

Figure 5.2 - Schematic of the challenge experiments performed on biofilms generated within the CDFF and further inoculated with respiratory pathogens and exposed to CHX/ variations in salivary pH.

5.2.5.1 Effects of CHX on colonisation of dental plaque by respiratory pathogens

CDFF plugs were removed after 5 d and aseptically transferred to universal containers. CHX gluconate solution was added at sub MIC concentrations (previously established for *P. aeruginosa* and *S. aureus*). The plugs were immersed in CHX (containing respiratory pathogens $1x10^2$ CFU/ml) and incubated at 37°C for 30 min, 1 h and 12 h. After incubation, biofilm bacteria were enumerated by culture or image analysis directly performed.

5.2.5.2 Effect of 'salivary' pH on respiratory pathogen colonisation of biofilms

The CDFF was established as outlined previously (section 5.2.3.4) to generate a 5 d oral biofilm using pooled dental plaque collected from 5 healthy volunteers. Plugs were removed from the CDFF containing 5 d biofilms and placed in 2 ml of AS for 1 h. The pans were removed aseptically and transferred to a series of universals containing different AS at different pHs and a different composition of respiratory pathogens. The following conditions were used: artificial saliva at pH of 4, 5 or 6.5, inoculated with either *S. aureus* or *P. aeruginosa* at an inoculum level of $1x10^2$ CFU/ ml. Once the plugs were immersed in AS (containing respiratory pathogens) the universals were incubated at 37°C for 24 h. Quantification of biofilm bacteria was performed as previously described (section 5.2.5.1). The pH of A/S was measured using a calibrated pH electrode (Fisher) following incubation with dental plaque biofilms.

5.2.6 Statistical analysis

All statistical analyses were performed using IBM SPSS v20. T-tests were used to analyse differences between MICs of CHX and Listerine™ for planktonic and biofilm growth of *S. mutans, C. albicans, S. aureus* and *P. aeruginosa*. In addition, t-tests were used to assess species viability post exposure of planktonic cells and biofilms to CHX. One-way ANOVA was

used to assess cell viability of mixed species biofilms generated within the CDFF post exposure to both artificial saliva at pH 4, 5 and 6.5 and post exposure to CHX at 30 min, 1 h and 12 h, with a Turkey HSD Post Hoc test for parametric analysis. To analyse differences in pH, recorded pH values were un-transformed to the original hydrogen ion concentration [H+], using the following equation *H+= -log^ pH value* (Boutilier & Shelton, 1979; Murphy, 1982). Finally, t-tests were undertaken to analyse the differences in enumeration between dual-species combinations of ETT biofilms.

5.3 Results

5.3.1 Sequencing of microorganisms used for *in vitro* analyses

Reference strain microorganisms (Table 5.2) were sequenced to confirm identification. DNA sequences were matched for organism identity using Basic Local Alignment Search Tool (BLAST; Table 5.2). BLAST uses an algorithmic approach to calculate a score of similar alignments. All sequence matches exceeded 85% coverage, confirming confident identification of reference organisms.

Table 5.2 - DNA sequencing results analysed via BLAST for species-specific identification. The sequencing results, in addition to *C. albicans* were analysed via BLAST to give species specific identification.

Reference Organism	Strain Details	BLAST Identification best match	Percentage (%) match
P. aeruginosa	**ATCC 25668**	*P. aeruginosa*	96
S. aureus	**NCIB 9518**	*S. aureus*	91
S. mutans	**GSM 2053^{t}**	*S. mutans*	97
C. albicans	**ATCC 90028**	*C. albicans*	99

5.3.2 Effect of oral microorganisms on promotion of pathogenic ETT biofilms

5.3.2.1 Single species ETT biofilm development

Biofilms were developed on sections of ETT surfaces for 10 d. Figure 5.3 illustrates a series of these biofilms, including monolayer of microbial cells (Images A, and B) and 3-dimensional structures (Images C, and D). The surface in image A (as identified by an arrow) is the inner lumen of the ETT. In this case, the biofilm has detached from the surface. The formation of complex biofilm structures is clearly evident (Figure 5.3, image B) with microbial cells aggregated together and encased in EPS. In these studies the lowest number of bacterial cells recovered in biofilms was for *S. aureus* ($6.80x10^6 \pm 6.89x10^6$ CFU/cm^2), and *P. aeruginosa* had the highest cell number in ETT biofilms ($1.81x10^7 \pm 1.52x10^7$ CFU/cm^2; Figure 5.4)[12]. To evaluate colonisation and relationships between microorganisms, dual species biofilms were also formed on sections of ETTs.

5.3.2.2 Effect of oral microorganisms in *in vitro* ETT biofilm development by *S. aureus* and *P. aeruginosa*

Dual species biofilms of *S. mutans* and *S. aureus* were developed and compared with single species controls. In single species biofilms, higher numbers of *S. mutans* ($1.15x10^7 \pm 1.02x10^7$ CFU/cm^2) were detected compared with *S. aureus* ($6.80x10^6 \pm 6.89x10^6$ CFU/cm^2). When the species were combined, a significantly ($p=0.037$) higher number of *S. aureus* ($3.17x10^7 \pm 3.29x10^7$ CFU/cm^2) were present compared with *S. mutans* ($9.09x10^6 \pm 7.94x10^6$ CFU/cm^2), with the latter showing decreased colonisation compared with its single species control (Figure 5.5 A). Biofilms were developed using *Streptococcus mutans* and *P. aeruginosa* in a similar way (Figure 5.5, B). In the case of single species control biofilms, *P. aeruginosa* ($1.81x10^7 \pm 1.52x10^7$ CFU/cm^2) was present in higher numbers than *S. mutans* ($1.15x10^7 \pm 1.02x10^7$ CFU/cm^2). When the two species were combined, a reduction in *S. mutans* occurred ($9.43x10^6 \pm 7.67x10^6$ CFU/cm^2)

[12] Tabulated raw data in Appendix IV

and there was an increase in *P. aeruginosa* ($2.02x10^7 \pm 1.44x10^7$ CFU/cm^2), although these changes were not deemed significant (p=0.050).

Biofilms involving *C. albicans* and *S. aureus* were developed (Figure 5.5, C). Single species biofilms had higher numbers of *C. albicans* ($1.04x10^7 \pm 1.21x10^7$ CFU/cm^2) compared to *S. aureus* ($6.80x10^6 \pm 6.89x10^6$ CFU/cm^2). When *C. albicans* was combined in biofilm with *S. aureus,* a significant (p=0.031) reduction in *C. albicans* numbers occurred ($3.12x10^6 \pm 1.55x10^6$ CFU/cm^2). The numbers of *S. aureus* were also lowered ($3.74x10^6 \pm 2.88x10^6$ CFU/cm^2), but not significantly.

Candida albicans was cultured in biofilms with *P. aeruginosa (*Figure 5.5, D), and these experiments showed that colonisation of *P. aeruginosa* was higher ($2.37x10^7 \pm 2.28x10^7$ CFU/cm^2), but not statistically significant (p=0.984) than *C. albicans.* When combined with *P. aeruginosa* there was a reduction in the numbers of *C. albicans* ($5.23x10^6 \pm 1.94x10^6$ CFU/cm^2), although this was not deemed significant (p=0.499)[13].

[13] Tabulated raw data in Appendix IV

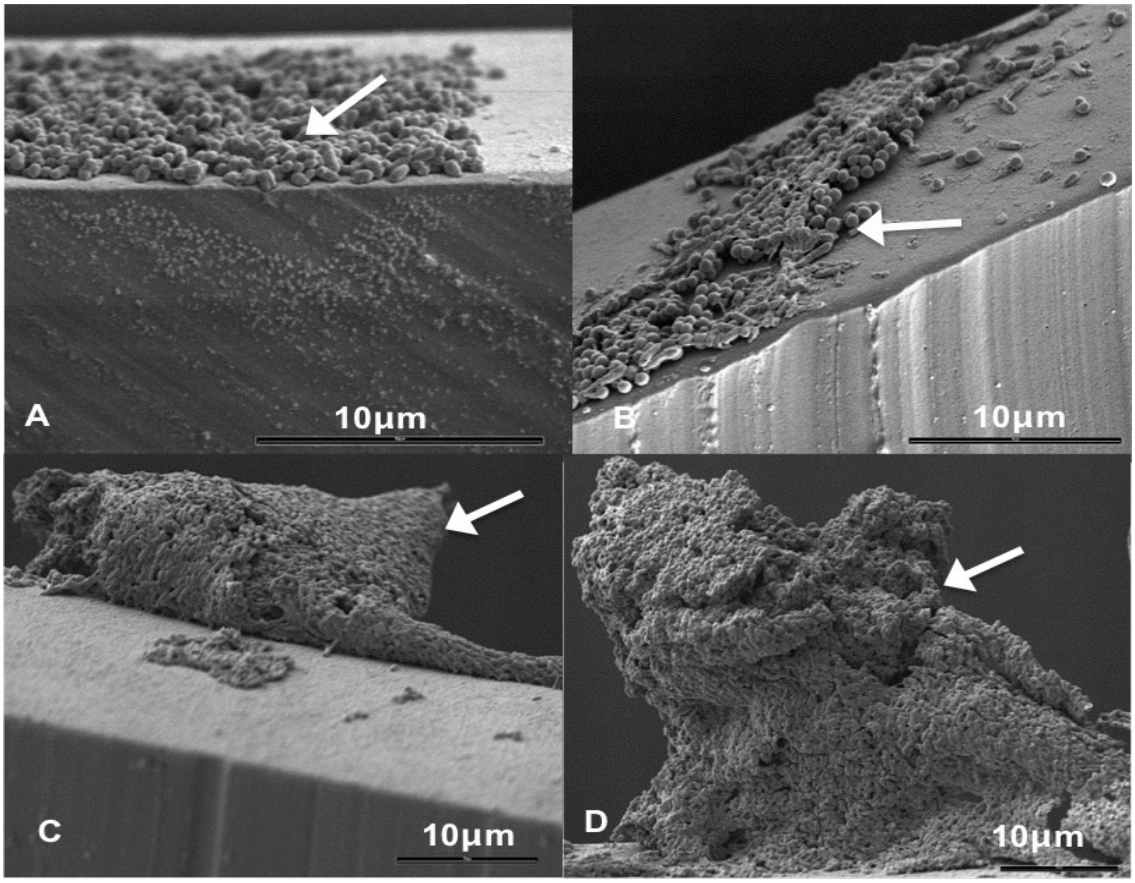

Figure 5.3 - SEM micrographs of; A and B illustrate initial stages of biofilm development and a monolayer of attached cells; C and D illustrate mixed bacterial species and *C. albicans* biofilms on ETT surfaces. Arrows indicate bacterial cells.

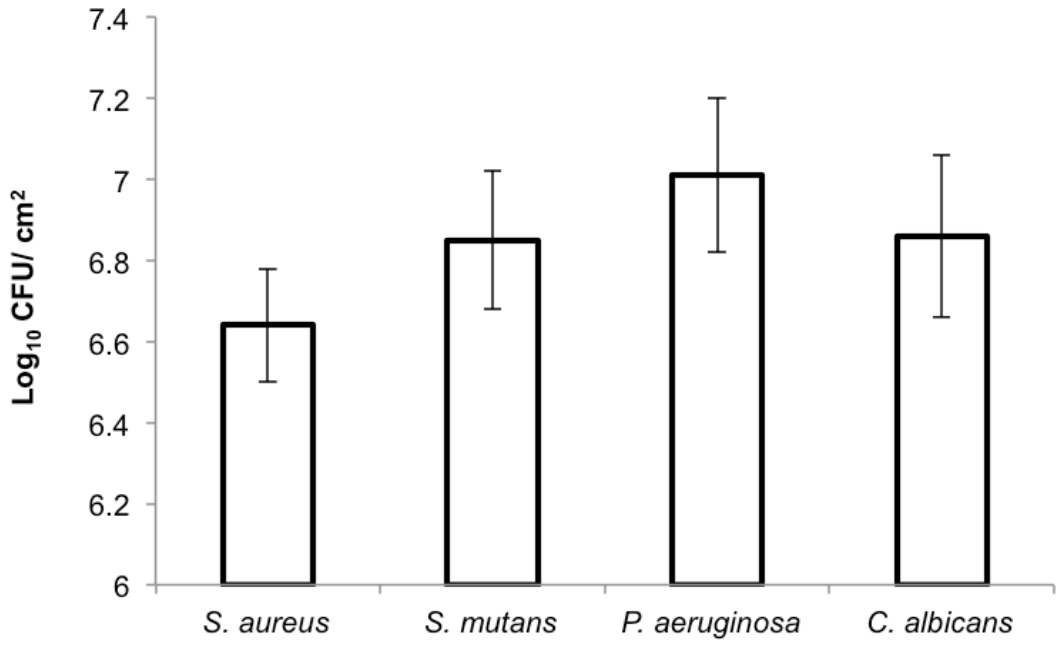

Figure 5.4 - Colony counts for single species biofilm developed on ETT over 10 d.

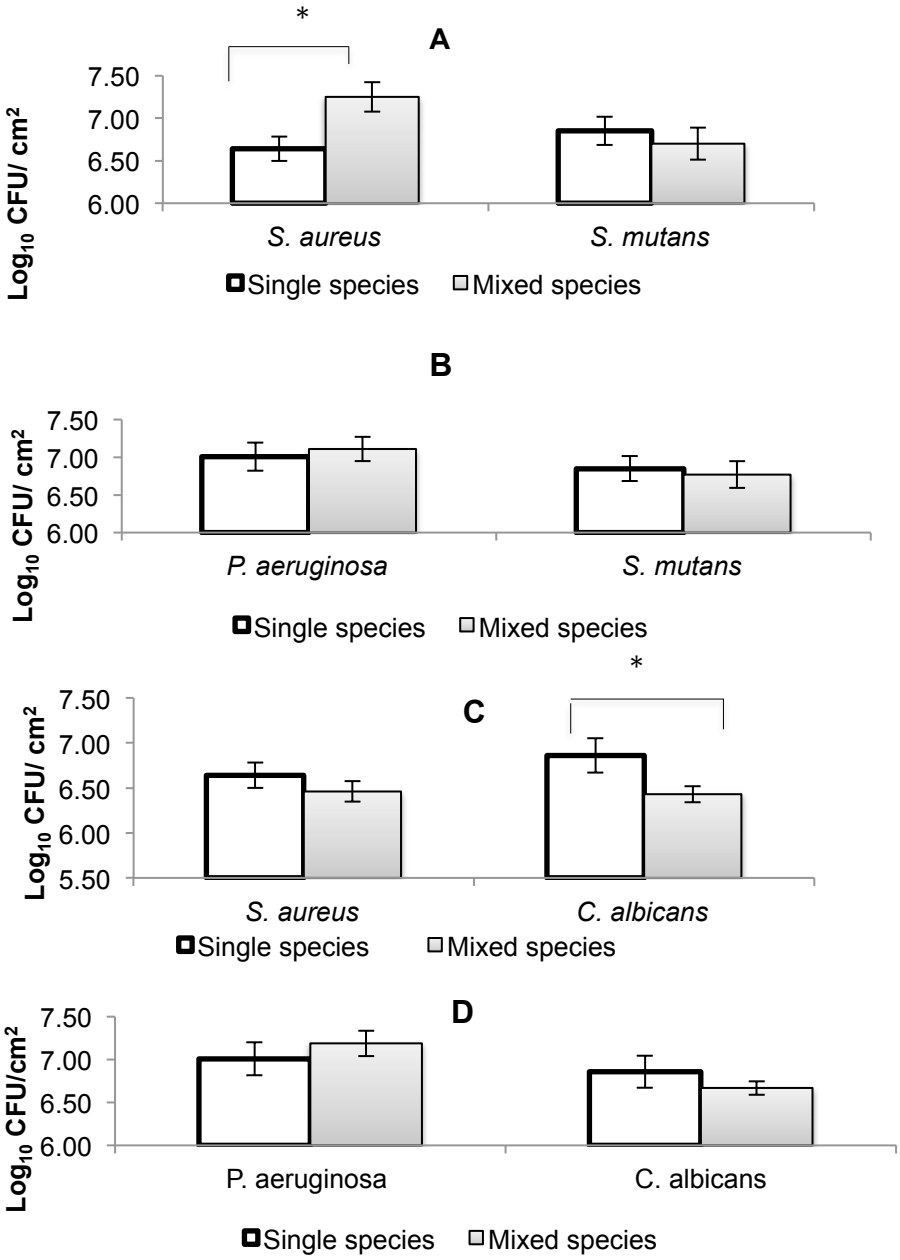

Figure 5.5 - Colony counts from in vitro ETT biofilms. A, *S. aureus* alone and with *S. mutans;* B, *P. aeruginosa* in mixed species biofilms with *S. mutans* compared to control data; C, single and mixed species ETT biofilm colonisation by *S. aureus* and *C. albicans*; D, *P. aeruginosa* was mixed with *C. albicans.*

5.3.3 Minimum inhibitory concentrations (MICs) of antimicrobial mouthwashes against test microorganisms

5.3.3.1 MICs against single species planktonic cells and biofilms

MICs for CHX against planktonic oral organisms (*S. mutans* and *C. albicans)* and respiratory pathogen *S. aureus* were <0.0000586% (<the lowest concentration tested; Table 5.3). For planktonic *P. aeruginosa* the MIC was noticeably higher at 0.0008%. Against biofilms, the minimum biofilm inhibitory concentration (MBIC) values increased significantly for *S. mutans* (0.0049%, p=0.01), *C. albicans* (0.0039%, p=0.025) and *S. aureus* (0.0025%, p=0.039). The MIC was 0.0049% for *S. mutans,* 0.0039% for *C. albicans* and 0.0025% for *S. aureus*. The MBIC value for *P. aeruginosa* was elevated to 0.0076%, from a planktonic value of 0.0008%, although not statistically significant (p=0.248).

A similar pattern was observed for the MICs of Listerine™ Gum Defence for planktonic and biofilm growth. The MIC values ranged between 2-4% for planktonic growth to 10-37.5% for biofilm cells. The MBICs of all 4 species were significantly higher than equivalent planktonic growth (*S. mutans* p=0.004*, C. albicans* p=<0.001*, S. aureus* p=0.036 and *P. aeruginosa* p=0.006) (Table 5.3). The MICs against *P. aeruginosa* biofilms (37.5% ± 2.78) were also noticeably higher compared with *S. aureus* (16.17% ± 0.29) and the oral microbial species *(S. mutans* (10% ± 0) and *C. albicans* (15.67% ± 4.93)) (Table 5.3).

Table 5.3 - Planktonic and biofilm MICs of CHX and Listerine™ Gum Defence[14] for test microorganisms.

		CHX (% v/v)		Listerine™ Gum Defence (% v/v)	
	Data set	**Planktonic**	**Biofilm**	**Planktonic**	**Biofilm**
	1	<0.0000586	0.0015	1.65	16.00
S. aureus	**2**	<0.0000586	0.0030	1.50	16.00
NCIB 9518	**3**	<0.0000586	0.0030	5.10	16.50
	Average	<0.0000586	0.0025	2.75	16.17
	SD	0	0.0009	2.04	0.29
	SEM	0	0.0005	1.18	0.17
	1	0.0007	0.0030	3.00	34.50
P. aeruginosa	**2**	0.0009	0.0038	3.13	38.00
ATCC 15682	**3**	0.0007	0.0160	6.25	40.00
	Average	0.0008	0.0076	4.13	37.50
	SD	0.0001	0.0073	1.84	2.78
	SEM	0.0001	0.0042	1.06	1.61
	1	<0.0000586	0.0018	2.40	10.00
S. mutans	**2**	<0.0000586	0.0060	2.80	10.00
GSM 2053^{t}	**3**	<0.0000586	0.0070	2.40	10.00
	Average	<0.0000586	0.0049	2.53	10.00
	SD	0	0.0028	0.23	0.00
	SEM	0	0.0016	0.13	0.00
	1	<0.0000586	0.0040	2.40	10.00
C. albicans	**2**	<0.0000586	0.0016	2.25	18.00
ATCC 90028	**3**	<0.0000586	0.0060	2.40	19.00
	Average	<0.0000586	0.0039	2.35	15.67
	SD	0	0.0022	0.09	4.93
	SEM	0	0.0013	0.05	2.85

[14] Each MIC value was obtained from a triplicate data set

5.3.3.2 MIC of CHX against mixed species planktonic and biofilms

Dual-species combinations of *S. aureus* with *S. mutans/C. albicans* and *P. aeruginosa* with *S. mutans/C. albicans* were co-cultured to compare the planktonic MICs and mixed species biofilm MBICs. For all dual-species biofilms, the MBICs were significantly increased (*P. aeruginosa/ S. mutans* p=0.017, MBIC 0.008% ± 0.002; *S. aureus/ C. albicans* p-0.001, MBIC 0.012% ± 0.0005; *P. aeruginosa/ C. albicans* p=0.007, MBIC 0.01% ± 0.002) when compared to planktonic MICs, with the exception of *S. aureus* and *S. mutans* (MBIC 0.02% ± 0.003, p=0.168) (Figure 5.6). In addition, for biofilm growth there was a greater variability between technical replicates as indicated by the larger error bars in figure 5.6. Mixed species MBICs were of similar values to the MBICs obtained from single species. Tabulated data in appendix IV.

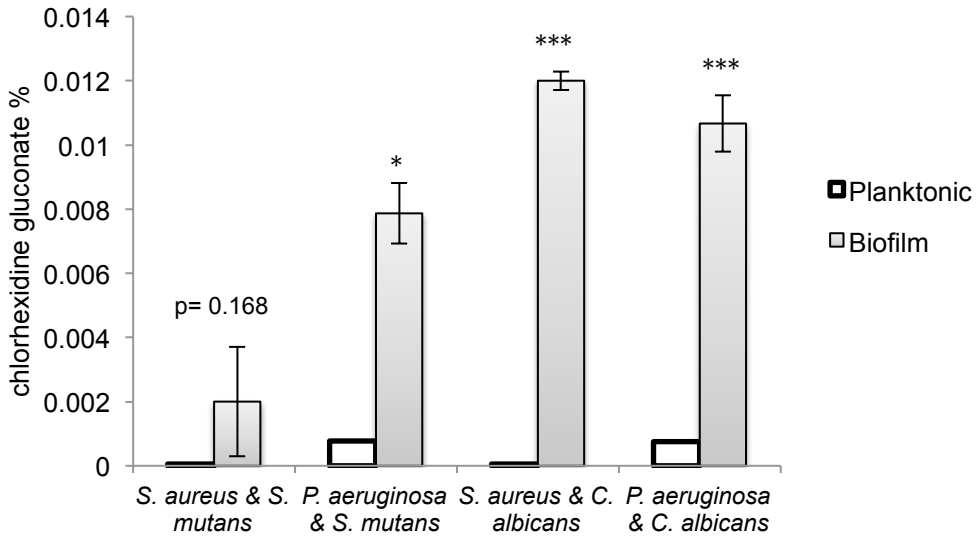

Figure 5.6 – A bar graph comparing the MIC values for dual-species species. A total of four combinations of oral organisms and respiratory pathogens isolated from dental plaque are used.

5.3.3.3 Species-specific recovery post-antimicrobial mouthwash exposure

When compared to planktonic cultures, co-culture of *S. aureus* and *S. mutans* in biofilms resulted in increased CFU/ml of *S. aureus* ($1.36x10^4$ ± $1.21x10^4$ CFU/ml to $4x10^4$ ± $1.73x10^3$ CFU/ml) and a significant decrease in *S. mutans* CFU/ml (p=0.028, CFU/ml decrease from $2.47x10^3$ ± $6.66x10^2$ to $1.46x10^2$ ± $4.71x10^1$ CFU/ ml) (log data table 5.4). Numbers of *P. aeruginosa* significantly increased (p=0.044) $8.8x10^4$ ± 0 CFU/ml to $4.27x10^5$ ± $1.27x10^5$ CFU/ml) when cultured with *S. mutans* in a biofilm compared to planktonic cultures. Neither *S. mutans* nor *C. albicans* were detected by microbial culture when co-cultured with *P. aeruginosa.* Finally, there were no significant differences in CFU/ml of *S. aureus* and *C. albicans* between planktonic and biofilm growth. Raw tabulated data in appendix IV.

Table 5.4 - Total counts of cultured microorganisms after CHX exposure for 24 h. T-tests were performed to compare enumeration difference between planktonic and biofilm growth post-CHX exposure.

	Dual – microbial combination species	sub-MIC[15] Log_{10} CFU/ml	sub-MBIC Log_{10} CFU/ml	Significance
1	*S. aureus*	4.13	4.60	ns*
	S. mutans	3.39	2.15	p=0.03
2	*P. aeruginosa*	4.94	5.63	p=0.04
	S. mutans	0.00	0.00	ns
3	*S. aureus*	4.71	4.52	ns
	C. albicans	4.37	3.93	ns
4	*P. aeruginosa*	4.58	5.71	ns
	C. albicans	0.00	0.00	ns

counts in triplicate, *ns – not significant

[15] Sub-MIC/MBIC concentration of CHX as determined for individual species in 5.2.3.1

5.3.3.4 MICs of antimicrobials against clinical isolates

The MICs of CHX and Listerine™ Gum Defence against 20 clinical isolates of 5x *S. aureus* and 5x *P. aeruginosa* and the oral microorganisms 5x *S. mutans* and 5x *C. albicans* (recovered from dental plaque of mechanically ventilated patients; section 2.2.3) are presented in table 5.5 and figure 5.7. The MBIC for CHX against clinical *S. aureus* isolates (MBIC 0.006% ± 0.004) were significantly higher compared to planktonic (MIC 0.001% ± 0.001, p=0.021). In addition, the mean MIC for clinical *S. aureus* biofilms was higher than for the reference strain *S. aureus* biofilm and this was statistically significant (p=0.018; Figure 5.7).

The highest CHX concentration required to inhibit growth of species identified from dental plaque was 0.05% (v/v) for *P. aeruginosa*. However, there was a large degree of variability in MBICs of clinical isolates, 0.048% ±0.024 (also shown by the error bars in figure 5.7). For *P. aeruginosa,* the MBIC of clinical isolates was (0.048%) significantly higher compared to the *P. aeruginosa* reference biofilm (MBIC 0.0076%), and all planktonic *P. aeruginosa* isolates (reference, and clinical) (p=<0.0001). In addition, the MICs for CHX against biofilm and planktonic oral microorganisms *S. mutans* and *C. albicans* were determined. The MBICs for *S. mutans* and *C. albicans* were significantly higher, p=<0.005 (0.009% and 0.004% respectively) than against planktonic equivalents (0.001% and 001% respectively).

The Listerine™ Gum Defence MICs against clinical isolates are shown in figure 5.8. For *S. aureus,* there was a significant difference (p<0.001) between planktonic MICs (3.017% ± 1.581) and biofilm MBICs (22.033% ±6.050). This was evident using both clinical and reference isolates. Similarly the MBICs of clinical *P. aeruginosa* isolates (15.235% ±40.100) were significantly higher (p=<0.001) compared to planktonic isolates 2.383%± 1.014), even considering the large variation in MBICs obtained for *P. aeruginosa* isolates. Biofilms of *S mutans* clinical isolates had significantly

higher MICs than planktonic counterparts ($p=<0.001$) and those of reference strains grown planktonically ($p=<0.001$) or as biofilms ($p=0.004$).

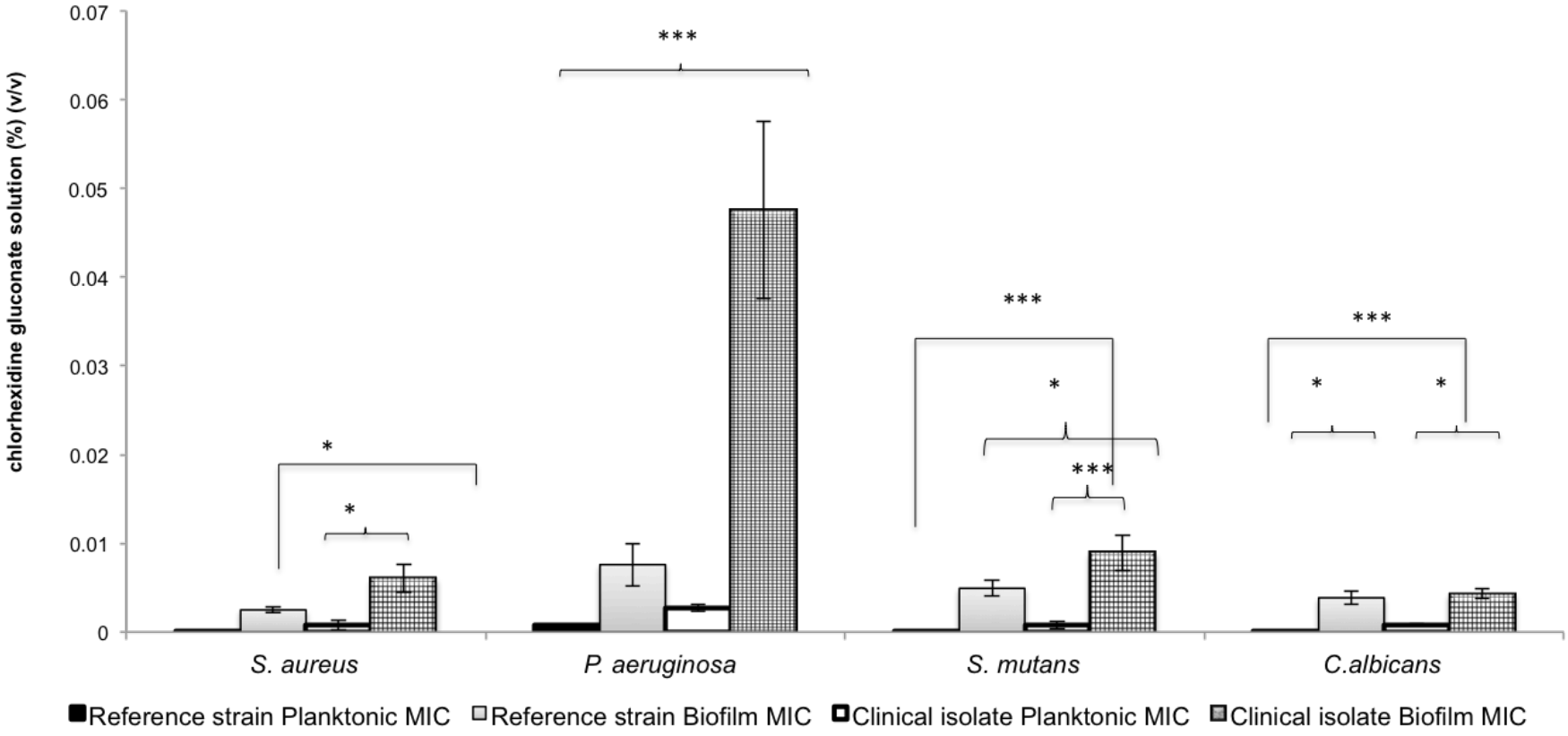

Figure 5.7 - MICs of CHX gluconate against reference and clinical microbial strains (obtained during the clinical study), Analysed via ANOVA with a Turkey HSD post-hoc test, whereby ***p ≤0. 001, **p ≤0.01 and *p≤ 0.05.

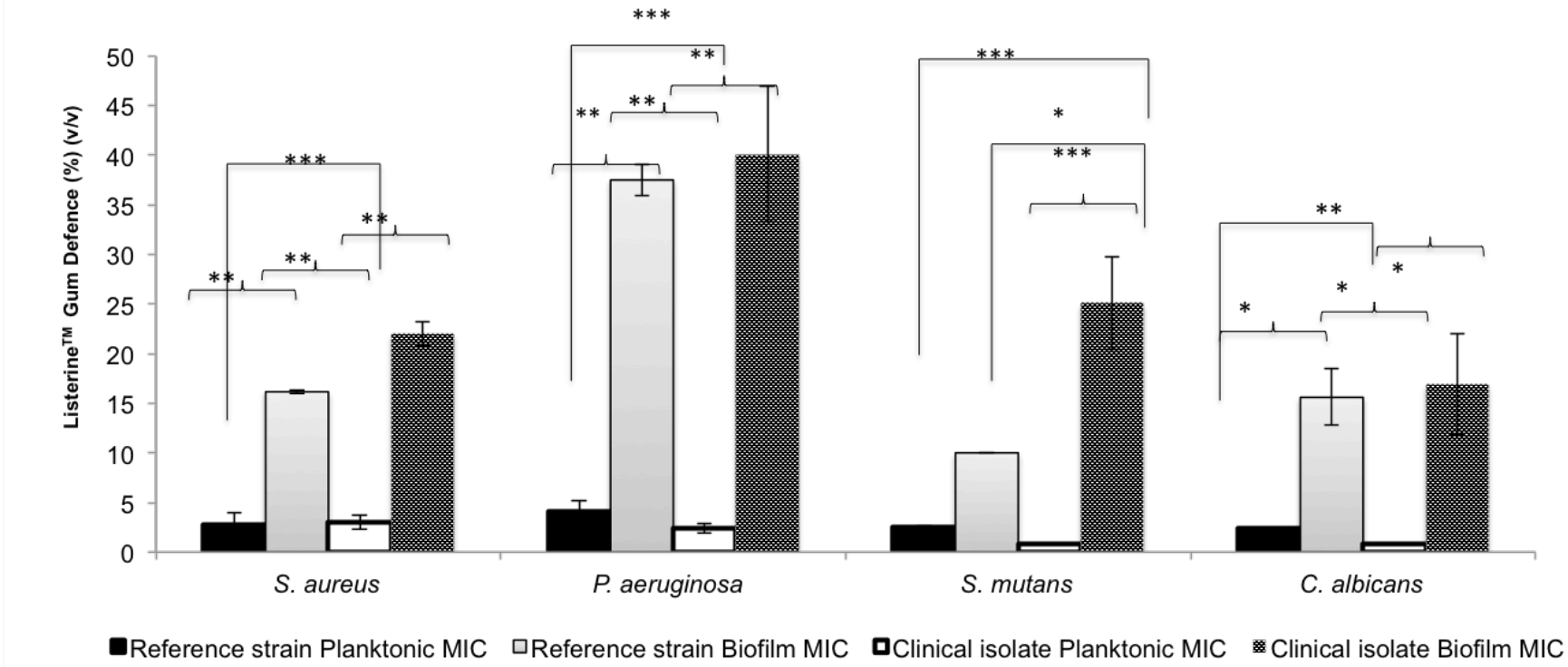

Figure 5.8 - MICs of Listerine™ Gum Defence against reference strains and clinical isolates Analysed via ANOVA with a Turkey HSD post-hoc test, whereby ***p ≤0. 001, **p ≤0.01 and *p≤ 0.05.

Table 5.5 - MICs of CHX against planktonic and biofilms formed by clinical isolates of *C albicans, P. aeruginosa, S. aureus* and *S. mutans.*

Chlorhexidine															
Planktonic MIC								Biofilm MIC							
Clinical Isolate	Isolate ref	dataset1	dataset2	dataset3	Mean			Clinical Isolates	Isolate no	dataset1	dataset2	dataset3	Mean		
C. albicans	CA01	0.0008	0.0008	0.0008	0.00078			*C. albicans*	CA01	0.0020	0.0035	0.0050	0.00350		
C. albicans	CA02	0.0008	0.0008	0.0007	0.00078	**Mean**	**0.0008**	*C. albicans*	CA02	0.0040	0.0045	0.0050	0.00450	**Mean**	**0.0044**
C. albicans	CA03	0.0012	0.0012	0.0010	0.00113	**SD**	0.0002	*C. albicans*	CA03	0.0005	0.0080	0.0025	0.00367	**SD**	**0.0014**
C. albicans	CA04	0.0007	0.0007	0.0007	0.00072	**SEM**	**0.0001**	*C. albicans*	CA04	0.0064	0.0070	0.0070	0.00667	**SEM**	**0.0010**
C. albicans	CA05	0.0008	0.0007	0.0009	0.00083			*C. albicans*	CA05	0.0004	0.0058	0.0038	0.00333		
P. aeruginosa	PA42	0.0032	0.0034	0.0034	0.0033			*P. aeruginosa*	PA42	0.0512	0.0501	0.0502	0.0503		
P. aeruginosa	PA48	0.0034	0.0038	0.0035	0.0036	**Mean**	**0.0027**	*P. aeruginosa*	PA48	0.0620	0.0484	0.0501	0.0533	**Mean**	**0.0480**
P. aeruginosa	PA09	0.0019	0.0018	0.0018	0.0018	**SD**	0.0085	*P. aeruginosa*	PA09	0.0042	0.0133	0.0040	0.0071	**SD**	**0.0240**
P. aeruginosa	PA192	0.0019	0.0100	0.0018	0.0018	**SEM**	**0.0004**	*P. aeruginosa*	PA192	0.0810	0.0550	0.0750	0.0700	**SEM**	**0.0110**
P. aeruginosa	PA150	0.0030	0.0030	0.0030	0.0030			*P. aeruginosa*	PA150	0.0555	0.0580	0.0580	0.0570		
S. aureus	SA12	0.0030	0.0031	0.0030	0.0030			*S. aureus*	SA12	0.0015	0.0061	0.0055	0.0043		
S. aureus	SA49	0.0004	0.0004	0.0004	0.0004	**Mean**	**0.0007**	*S. aureus*	SA49	0.0032	0.0063	0.0058	0.0051	**Mean**	**0.0061**
S. aureus	SA56	0.0001	0.0001	0.0001	0.0001	**SD**	**0.0012**	*S. aureus*	SA56	0.0032	0.0060	0.0060	0.0051	**SD**	**0.0040**
S. aureus	SA173	0.0001	0.0001	0.0001	0.0001	**SEM**	**0.0005**	*S. aureus*	SA173	0.0025	0.0030	0.0055	0.0036	**SEM**	**0.0020**
S. aureus	SA175	0.00018	0.0006	0.0002	0.0002			*S. aureus*	SA175	0.0150	0.0090	0.0125	0.0122		
S. mutans	SM01	0.0001	0.0002	0.0006	0.0003			*S. mutans*	SM01	0.0035	0.0210	0.0230	0.0158		
S. mutans	SM02	0.0016	0.0017	0.0016	0.0017	**Mean**	**0.0007**	*S. mutans*	SM02	0.0100	0.0090	0.0095	0.0095	**Mean**	**0.0090**
S. mutans	SM03	0.0003	0.0004	0.0004	0.0003	**SD**	**0.0005**	*S. mutans*	SM03	0.0100	0.0060	0.0095	0.0085	**SD**	**0.0050**
S. mutans	SM04	0.0004	0.0005	0.0005	0.0005	**SEM**	**0.0003**	*S. mutans*	SM04	0.0150	0.0020	0.0095	0.0088	**SEM**	**0.0035**
S. mutans	SM05	0.0002	0.0010	0.0015	0.0009			*S. mutans*	SM05	0.0020	0.0015	0.0025	0.0020		

Table 5.6 – MICs of Listerine™ Gum Defence against planktonic and biofilms formed by clinical isolates c
albicans, P. aeruginosa, S. aureus and *S. mutans.*

Listerine™ Gum Defence															
Planktonic MIC								Biofilm MIC							
Clinical Isolate	Isolate no	dataset1	dataset2	dataset3	Mean			Clinical Isolates	Isolate no	dataset1	dataset2	dataset3	Mean		
C. albicans	CA01	<0.78	<0.78	<0.78	<0.78			*C. albicans*	CA01	10.00	10.00	11.00	10.33		
C. albicans	CA02	<0.78	<0.78	<0.78	<0.78	**Average**	**0.78**	*C. albicans*	CA02	12.00	12.50	10.00	11.50	**Average**	**16.900**
C. albicans	CA03	<0.78	<0.78	<0.78	<0.78	**SD**	**0.00**	*C. albicans*	CA03	10.00	10.00	10.50	10.17	**SD**	**11.441**
C. albicans	CA04	<0.78	<0.78	<0.78	<0.78	**SEM**	**0.00**	*C. albicans*	CA04	38.00	38.00	35.00	37.00	**SEM**	**5.117**
C. albicans	CA05	<0.78	<0.78	<0.78	<0.78			*C. albicans*	CA05	13.00	18.00	15.50	15.50		
P. aeruginosa	PA42	2.50	2.50	2.60	2.53			*P. aeruginosa*	PA42	41.00	41.00	41.00	41.00		
P. aeruginosa	PA48	4.50	4.20	4.70	4.47	**Average**	**2.38**	*P. aeruginosa*	PA48	44.00	50.00	43.50	45.83	**Average**	**40.100**
P. aeruginosa	PA09	1.80	1.70	1.10	1.53	**SD**	**1.01**	*P. aeruginosa*	PA09	12.00	12.00	17.00	13.67	**SD**	**15.235**
P. aeruginosa	PA192	2.65	2.35	2.75	2.58	**SEM**	**0.45**	*P. aeruginosa*	PA192	50.00	50.00	50.00	50.00	**SEM**	**6.813**
P. aeruginosa	PA150	2.50	2.50	2.20	2.40			*P. aeruginosa*	PA150	50.00	50.00	50.00	50.00		
S. aureus	SA12	2.80	2.85	3.40	3.02			*S. aureus*	SA12	17.50	17.00	17.00	17.17		
S. aureus	SA49	<0.78	<0.78	<0.78	<0.78	**Average**	**3.02**	*S. aureus*	SA49	20.50	20.00	20.00	20.17	**Average**	**22.033**
S. aureus	SA56	<0.78	<0.78	<0.78	<0.78	**SD**	**1.58**	*S. aureus*	SA56	19.50	18.00	19.50	19.00	**SD**	**6.050**
S. aureus	SA173	<0.78	<0.78	<0.78	<0.78	**SEM**	**0.71**	*S. aureus*	SA173	40.00	19.50	38.00	32.50	**SEM**	**1.210**
S. aureus	SA175	<0.78	<0.78	<0.78	<0.78			*S. aureus*	SA175	10.00	36.00	18.00	21.33		
S. mutans	SM01	<0.78	<0.78	<0.78	<0.78			*S. mutans*	SM01	15.50	20.50	18.50	18.17		
S. mutans	SM02	<0.78	<0.78	<0.78	<0.78	**Average**	**0.78**	*S. mutans*	SM02	30.00	41.00	40.00	37.00	**Average**	**25.100**
S. mutans	SM03	<0.78	<0.78	<0.78	<0.78	**SD**	**0.00**	*S. mutans*	SM03	43.00	27.00	38.50	36.17	**SD**	**10.516**
S. mutans	SM04	<0.78	<0.78	<0.78	<0.78	**SEM**	**0.00**	*S. mutans*	SM04	17.00	21.00	16.00	18.00	**SEM**	**4.703**
S. mutans	SM05	<0.78	<0.78	<0.78	<0.78			*S. mutans*	SM05	19.00	15.00	14.50	16.17		

5.3.4 Dental plaque biofilms generated in the CDFF

5.3.4.1 CDFF biofilm development

The CDFF was used to develop complex and reproducible biofilms derived from supragingival and subgingival dental plaque from healthy volunteers. A 5 d biofilm was developed on PTFE plugs as described in section 5.2.4 using constantly supplied BM at a pH between pH 6.77-6.89. Microbial enumeration of the dental plaque inocula and from the 5 d CDFF biofilms was undertaken (Table 5.7).

Facultative anaerobes, aerobic bacterial species and *C. albicans* (budding cells and hyphae production identified by CLSM) were recovered from the CDFF generated biofilm (Figures 5.9 and 5.10). There were no significant differences in the recovery of aerobic and anaerobic species. There was a significant increase ($p=<0.001$) in the recovery of *C. albicans* following re-culture of dental plaque biofilm formation in the CDFF (Table 5.7). Furthermore, potential respiratory pathogens (*S. aureus* and *P. aeruginosa*) were not isolated on selective media from either the dental plaque or the 5 d CDFF biofilms (Table 5.7) (2.2.3).

5.3.4.2 Composition of dental plaque CDFF biofilms inoculated with respiratory pathogens

Dental plaque derived CDFF biofilms (cultured for 5 d) were exposed to A/S (pH 6.5) and inoculated with *S. aureus* or *P. aeruginosa* for 24 h. Subsequent viable counts (CFU/ml) of aerobic and anaerobic bacteria and *C. albicans* were reduced by ~1,000 fold after culture with *S. aureus* and *P. aeruginosa* (Figure 5.11).

The following sections report the effects of CHX treatment and exposure to artificial saliva at different pH upon dental plaque inoculated with respiratory pathogens (to mirror colonisation of dental plaque during MV). These investigations were undertaken to ascertain whether such environmental changes could promote respiratory pathogen colonisation.

Table 5.7 – Comparison of $\log_{10}$ CFU/ ml microbial enumeration of species recovered from pooled dental plaque, and species recovered from a CDFF generated biofilm of pooled dental plaque.

	S. aureus		*P. aeruginosa*		*Candida albicans*		Aerobic spp.		Anaerobic spp.	
Biofilm set	Dental plaque	CDFF regrowth	Dental plaque	CDFF regrowth	Dental plaque	CDFF regrowth	Dental plaque	CDFF regrowth	Dental plaque	CDFF regrowth
1	0.00	0.00	0.00	0.00	0.00	7.51	8.28	7.90	8.36	7.48
2	0.00	0.00	0.00	0.00	0.00	7.49	8.15	7.92	8.49	7.49
3	0.00	0.00	0.00	0.00	0.00	7.45	8.20	7.93	8.45	7.54
4	0.00	0.00	0.00	0.00	0.00	7.42	8.28	8.08	8.00	8.30
5	0.00	0.00	0.00	0.00	0.00	7.43	8.32	8.04	7.97	8.32
6	0.00	0.00	0.00	0.00	0.00	7.49	8.16	8.00	8.04	8.37
7	0.00	0.00	0.00	0.00	0.00	8.04	8.11	8.04	7.98	7.97
8	0.00	0.00	0.00	0.00	0.00	8.04	8.15	8.11	8.00	8.04
9	0.00	0.00	0.00	0.00	0.00	7.99	7.89	8.04	8.04	7.95
Average	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**7.74**	**8.17**	**8.01**	**8.15**	**8.06**
SD	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.27**	**0.13**	**0.07**	**0.22**	**0.34**
SEM	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.09**	**0.04**	**0.02**	**0.07**	**0.11**

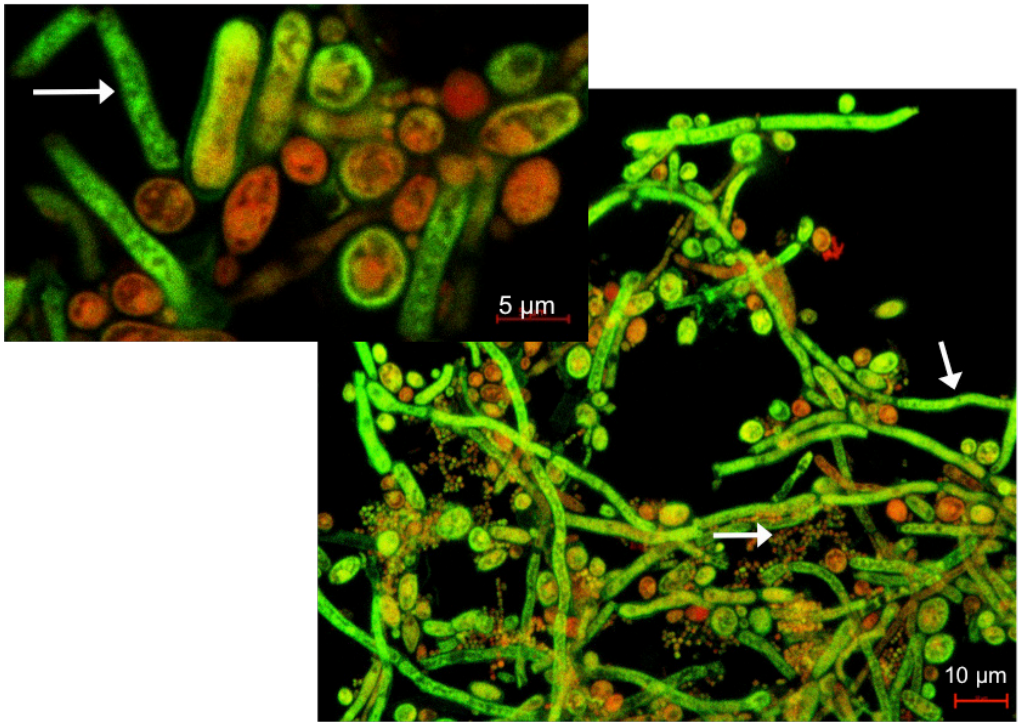

Figure 5.9 - Confocal laser scanning micrograph of microorganisms from a 5 d dental plaque CDFF biofilm treated with A/S at pH 6.5 with a live (green) dead (red) stain. Cellular morphologies include yeast hyphae (as shown by the arrows), yeast budding cells, and smaller clusters of bacterial cells.

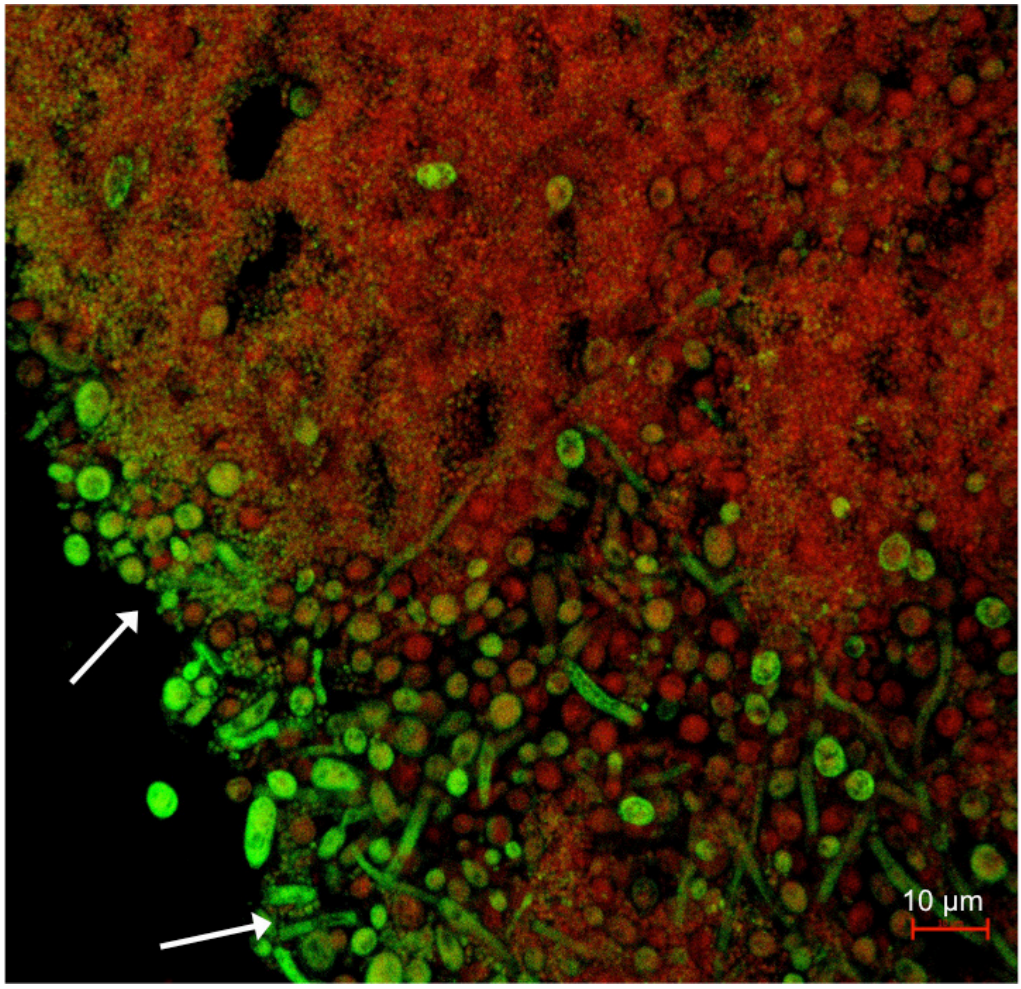

Figure 5.10 - Confocal laser scanning micrograph of microorganisms from a 5 d dental plaque CDFF biofilm treated with A/S at pH 6.5 with a live (green) dead (red) stain. Live cells (predominantly Candida) are on the outer of the biofilm (as shown by the arrows), with bacterial aggregates within the biofilm (stained red).

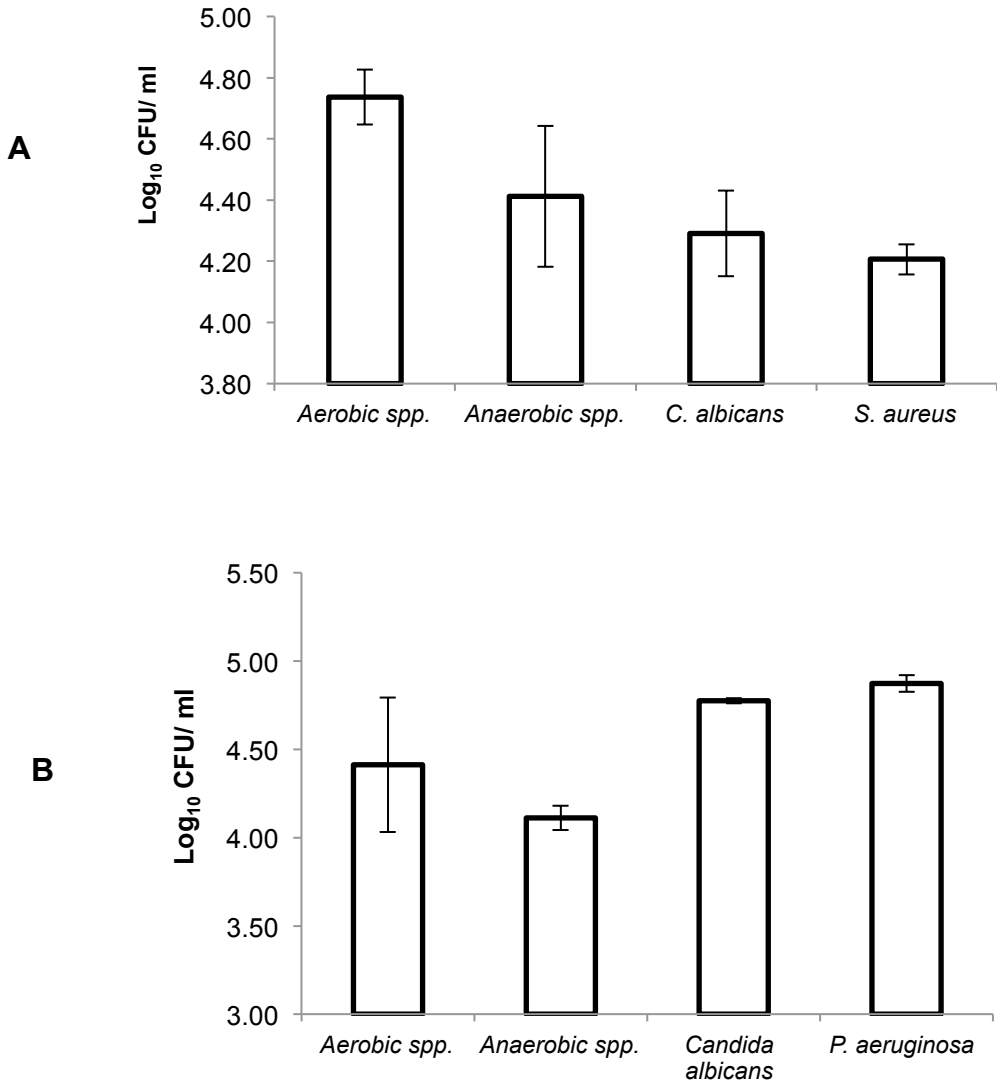

Figure 5.11 - Colony counts from 5 d CDFF biofilm inoculated with A, *S. aureus* and B, *P. aeruginosa* for 24 h.

5.3.5 Effect of CHX upon dental plaque biofilms inoculated with respiratory pathogens

5.3.5.1 Effect of CHX treatment on composition of CDFF dental plaque biofilms

CDFF dental plaque biofilms were treated with 0.0125% (v/v) CHX in A/S (for *P. aeruginosa*) or 0.00125% (v/v) CHX (for *S. aureus*) for 30 min, 1 h and 12 h. Total aerobic species, facultative and strict anaerobic species and *C. albicans* were all quantified before and after treatment (Figure 5.12). Whilst the numbers of aerobic species after 30 min and 1 h CHX treatments were similar, there was a significant ($p=<0.001$) reduction in numbers after 12 h CHX exposure. Significant reduction in anaerobic bacteria levels occurred following 1 h CHX treatment ($p=0.017$) and further reductions were seen after 12 h ($p=0.002$). No differences in numbers of *C. albicans* were detected for all durations of CHX treatment at both concentrations. Tabulated data in appendix IV.

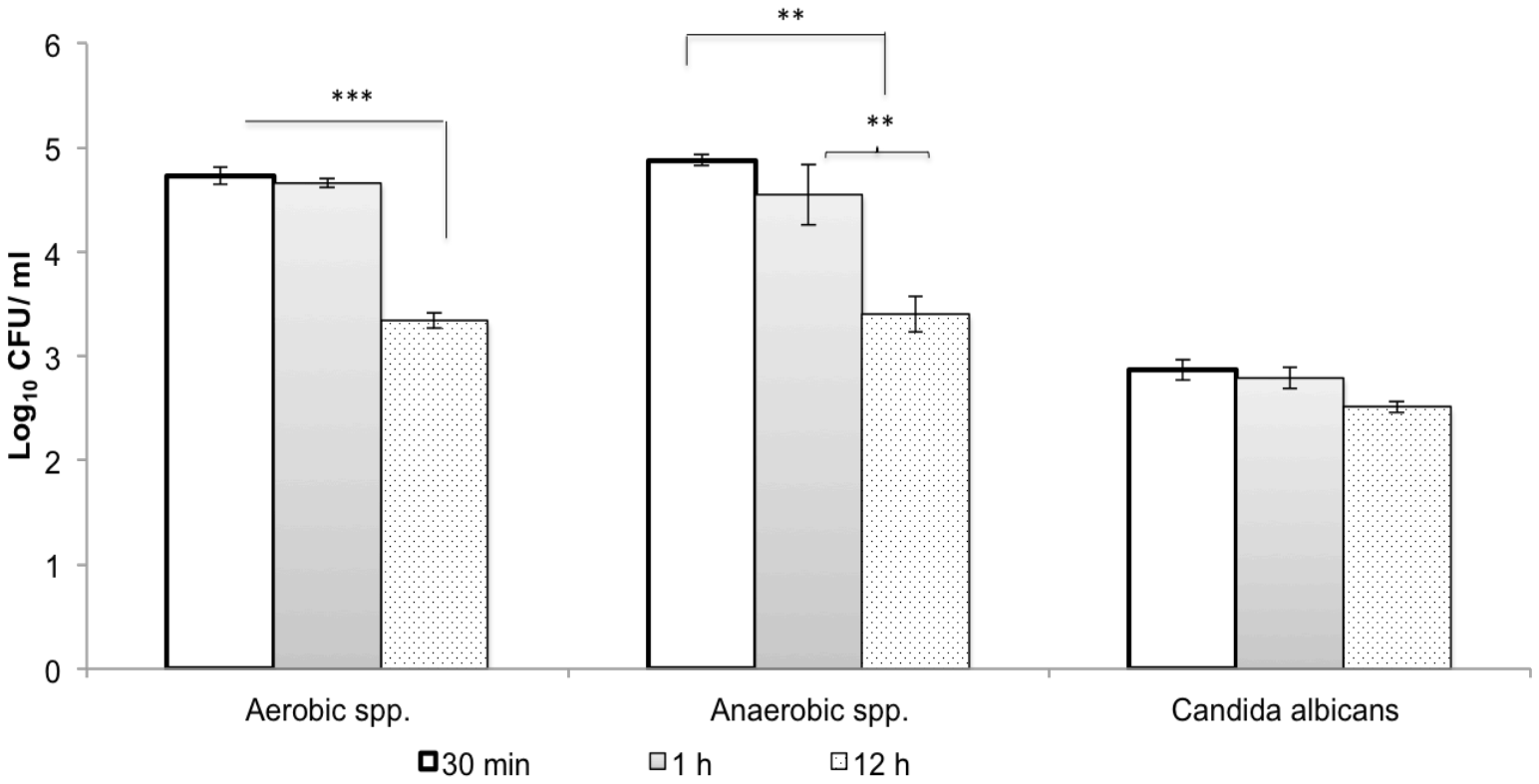

Figure 5.12 – Colony counts from dental plaque 5 d CDFF biofilm exposed to CHX for 30 min, 1 h and 12 h whereby ***p ≤0. 001, **p ≤0.01 and *p≤ 0.05.

5.3.5.2 Effect of 0.00125% (v/v) CHX on *S. aureus* colonisation of CDFF dental plaque biofilms

Dental plaque derived CDFF biofilms that had subsequently been seeded with *S. aureus* were analysed after 0.00125% CHX treatment. Compared with 30 min CHX treatment, noticeable increases in *S. aureus* and *C. albicans* numbers were detected following 1 h treatment. However, no significant differences in levels of mixed aerobic or anaerobic species were evident for 30 min and 1 h CHX exposures (Figure 5.13). After 12 h CHX treatment, *S. aureus* was not detected by culture, although *C. albicans* levels were similar to those after 1 h exposure ($p=0.190$). In addition, significant increases in total aerobic ($p=<0.001$) and anaerobic counts ($p=<0.001$) occurred following 12 h CHX treatment.

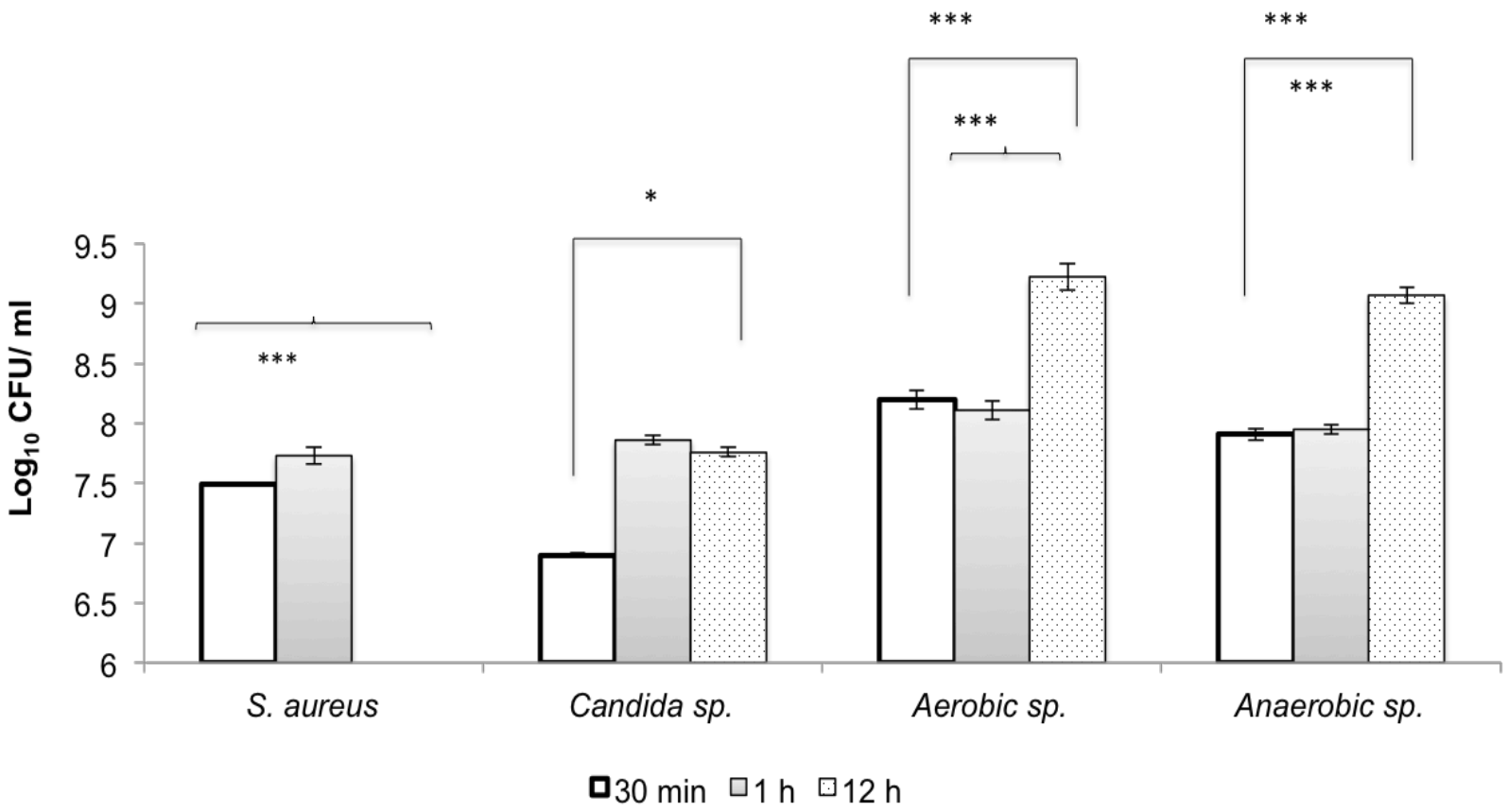

Figure 5.13 - Colony counts from dental plaque 5 d CDFF biofilms inoculated with artificial saliva (A/S) containing *S. aureus* and exposed to CHX for 30 min, 1 h and 12 h, whereby ***p ≤0. 001, **p ≤0.01 and *p≤ 0.05.

5.3.5.3 Effect of 0.0125% (v/v) CHX treatment on *P. aeruginosa* colonisation of CDFF dental plaque biofilms

Figure 5.14 presents the results of CHX treatment of *P. aeruginosa* inoculated CDFF dental plaque biofilms. After 30 min CHX treatment, *P. aeruginosa* was not detected by culture, whilst $\log_{10}$ 7.5-8.5 CFU/ml of *C. albicans,* non-*P. aeruginosa* aerobic bacteria and anaerobic bacteria were present. After 1 h CHX exposure, a significant increase in numbers of *C. albicans* and aerobic bacteria was evident ($p=<0.001$ and $p=<0.001$ respectively), although no differences were observed for anaerobic bacteria. Similar to 30 min CHX exposure, no *P. aeruginosa* were detected after 1 h CHX exposure. Following CHX treatment or 12 h, numbers of *P. aeruginosa* significantly increased ($p=<0.001$) to $\log_{10}$ 8.5 CFU/ml. In addition, *C. albicans* or other (non-*P. aeruginosa* species) aerobic bacteria were not detected by culture after 12 h CHX exposure. There was however, a significant increase in the levels of anaerobic bacteria after 12 h CHX exposure compared with 30 min ($p=0.011$) and 1 h treatments ($p=0.001$).

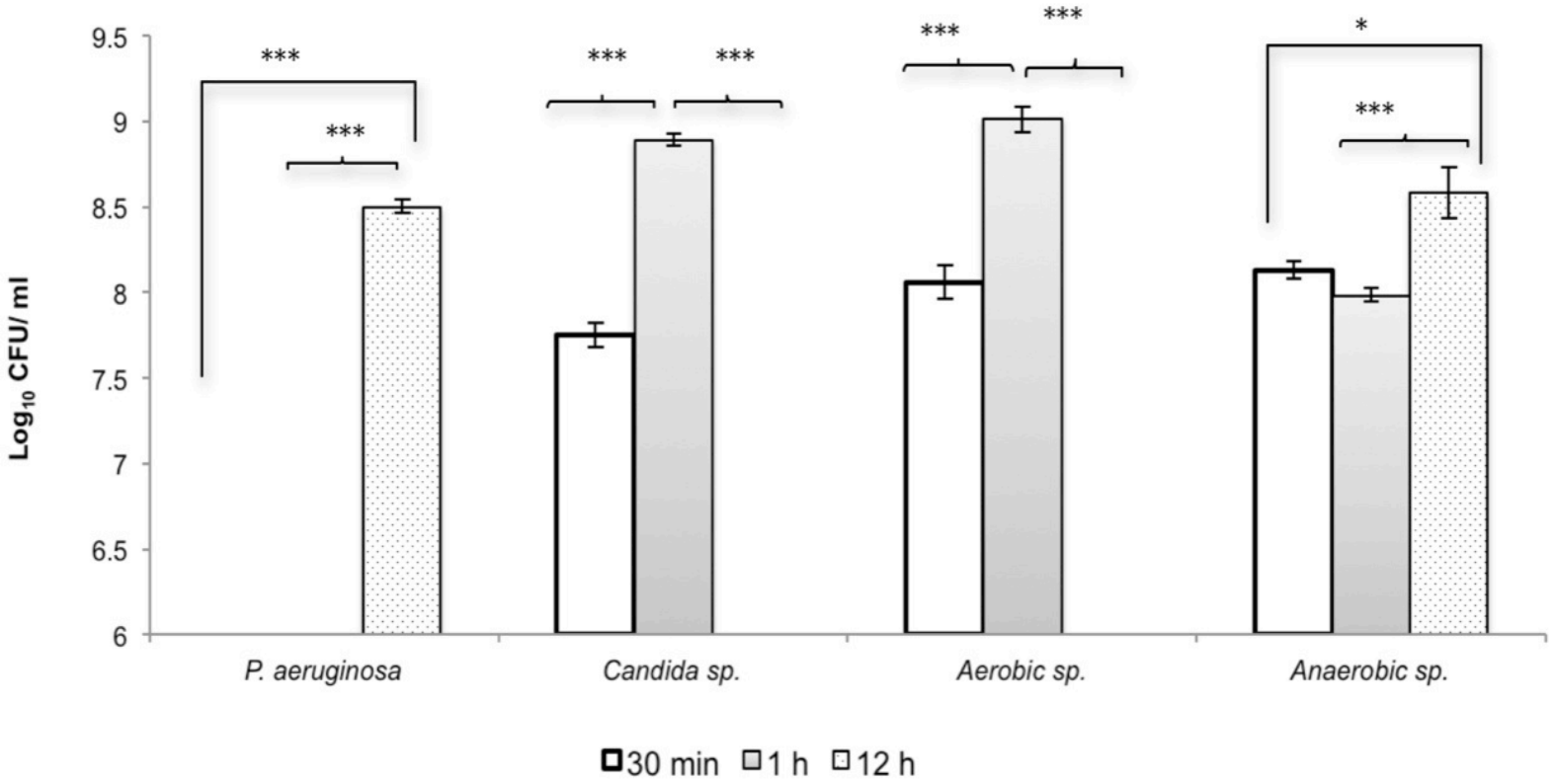

Figure 5.14 - Colony counts from dental plaque 5 d CDFF biofilm with inoculated with *P. aeruginosa* and exposed to CHX for 30 min, 1 h and 12 h, whereby ***p ≤0. 001, **p ≤0.01 and *p≤ 0.05.

5.3.6 Effect of artificial saliva pH on respiratory pathogen colonisation of dental plaque CDFF biofilms

5.3.6.1 Composition of CDFF dental plaque biofilms after exposure to artificial saliva (A/S) at different pH

Dental plaque derived CDFF biofilms were exposed to artifical saliva at pH 4, pH 5 and pH 6.5 in the absence of respiratory pathogens and incubated for 24 h at 37°C. For aerobic and anerobic bacteria there was no significant differences in numbers cultured from biofilms exposed to A/S at pH 4.5 or 6.5 (Figure 5.15). At all A/S pHs, the numbers of *C. albicans* was lower compared with aerobic and anaerobic bacteria as shown in figure 5.14, and *C. albicans* levels were significantly lower (p=0.006) at pH 6.5 ($4.07x10^2$ ± $1.15x10^1$ CFU/ ml), compared with pH 5 ($9.37x10^2$ ± $2.29x10^2$ CFU/ ml). All tabulated data in appendix IV.

The pH of A/S was measured prior to addition to a 5 d CDFF dental plaque biofilm and 24 h after exposure (Table 5.8) to determine whether dental plaque changes the A/S pH following exposure. A significant increase (p=<0.001) in A/S pH occurred for the pH 4 A/S following dental plaque biofilm exposure (pH 4.58 ± 0.02). Similarly A/S at pH 5 showed a significant increase (p=0.001) to pH 5.68 ± 0.07 when added to a dental plaque biofilm. In addition, the pH of A/S (initially at pH 6.5) decreased significantly (p=0.007) pH 6.21 ± 0.03 following exposure to dental plaque biofilm for 24 h.

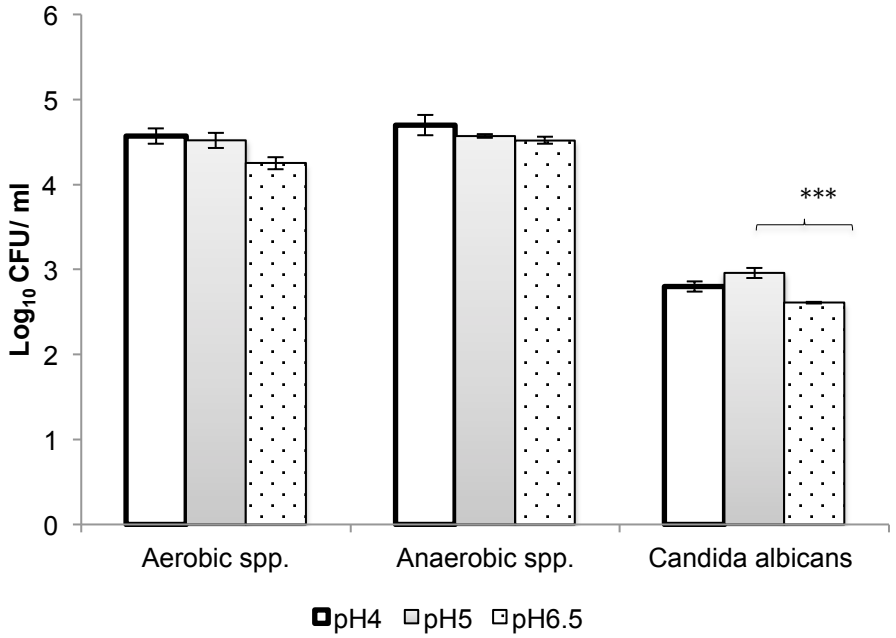

Figure 5.15 – Colony counts of aerobic and anaerobic bacteria and *C. albicans* from dental plaque 5 d CDFF biofilm after 24 h exposure with artificial saliva (A/S) at differing pHs, whereby ***p ≤0. 001, **p ≤0.01 and *p≤ 0.05.

Table 5.8 – pH measurements of artificial saliva (A/S) before and after exposure to a 5d mixed species dental plaque biofilm.

	A/S pH					
Biofilm	Pre-5d CDFF	Post-5d CDFF	Pre-5d CDFF	Post-5d CDFF	Pre-5d CDFF	Post-5d CDFF
1	4.00	4.59	5.00	5.63	6.50	6.19
2	4.00	4.56	5.00	5.64	6.50	6.25
3	4.00	4.59	5.00	5.76	6.50	6.20
Mean	**4.00**	**4.58**	**5.00**	**5.68**	**6.50**	**6.21**
SD	**0.00**	**0.02**	**0.00**	**0.07**	**0.00**	**0.03**
SEM	**0.00**	**0.01**	**0.00**	**0.04**	**0.00**	**0.02**

5.3.6.2 Effect of artificial pH on dental plaque biofilms inoculated with *S. aureus*

Dental plaque biofilms seeded with *S. aureus* were exposed to A/S at pH 4, pH 5 and pH 6.5. CLSM imaging and culture enumeration were undertaken to assess *S. aureus* colonisation of dental plaque CDFF biofilms after 24 h. *Staphylococcus aureus* were detected by FISH staining on the outer most layer and within the biofilm (Figure 5.16).

As shown in figure 5.17, no growth of *S. aureus* occurred after incubation with aritfiical saliva at pH 4, whilst significant increases were observed at pH 5 to $3.35x10^{7} \pm 5.82x10^{6}$ CFU/ ml (p=<0.001) and at pH 6.5 to $3.22x10^{9} \pm 2.64x10^{8}$ CFU/ ml (p=<0.001). No significant differences in *C. albicans* levels were seen following treatment with A/S pH 4, pH 5 or pH 6.5 (p=0.140). A similar pattern was observed for both aerobic and anaerobic bacteria at the different A/S pHs whereby similar enueration occurred between pH 4 and pH5, with a significant increase in aerobic species (p=<0.001) and anaerobic species (p=<0.001) respectively (Figure 5.17) following exposure to pH 6.5. All tabulated data is in appendix IV.

In addition, the pH was measured post-exposure to a 5 d dental plaque biofilm inouclated with *S. aureus* (Table 5.9). For A/S commencing at pH 4, a significant increase occurred after 24 h inoculation with *S. aureus* to pH 5.34 ± 0.20 (p=<0.001). For A/S at pH 5, a significantly higher pH of 5.86 ± 0.16 (p=0.001) was detected 24 h after inoculation with *S. aureus*. Similar pH values (pH 6.45 ± 0.32) were obtained for for A/S initially at pH 6.5 after exposure to dental plaque biofilms inoculated with *S. aureus* (p=0.728).

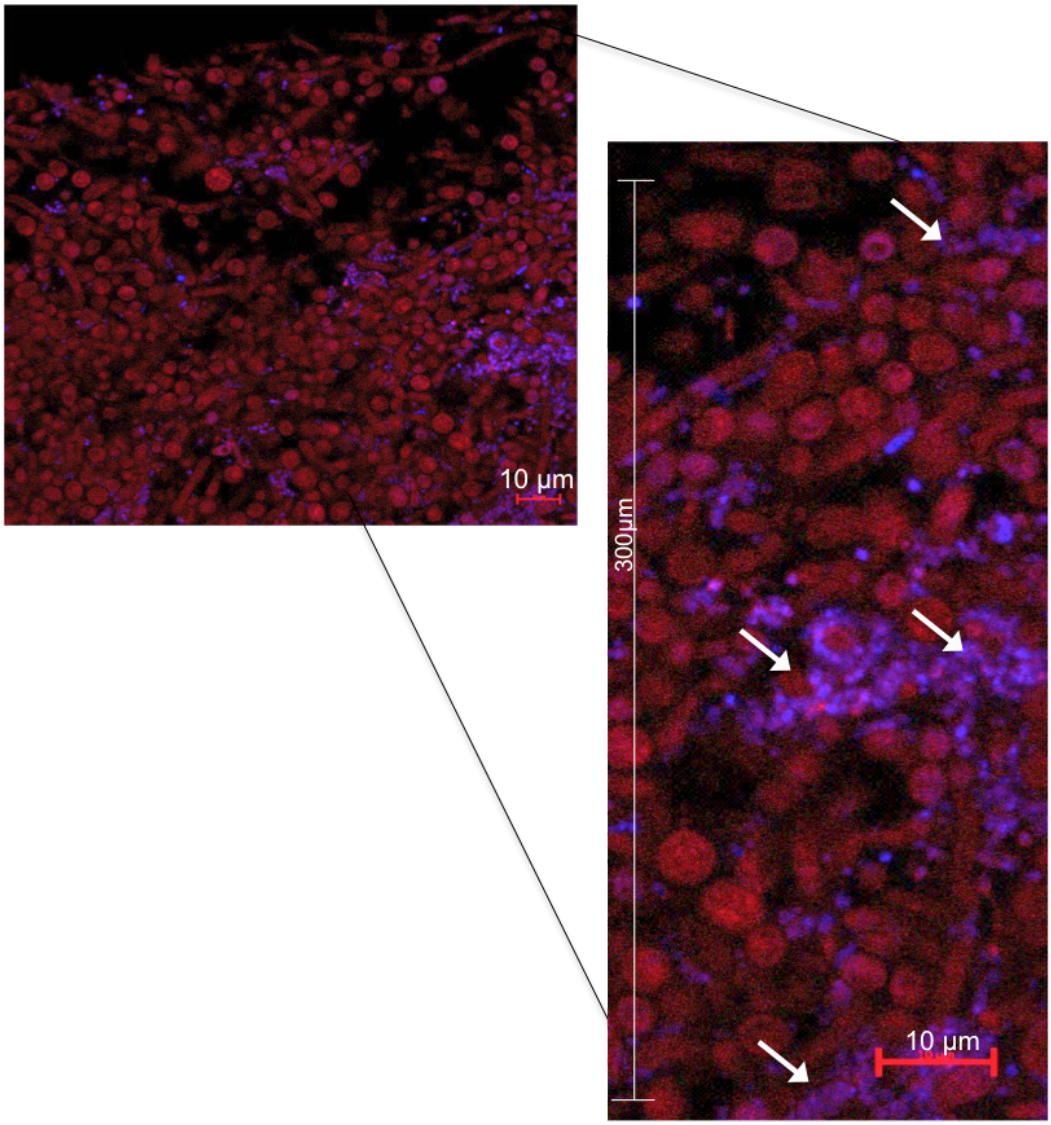

Figure 5.16 – Confocal laser scanning micrograph of microorganisms from a 5 d dental plaque CDFF stained with a bacterial universal probe (Cy3, red staining) and coupled with a species-specific probe for *S. aureus* (Cy5) as depicted by the arrows.

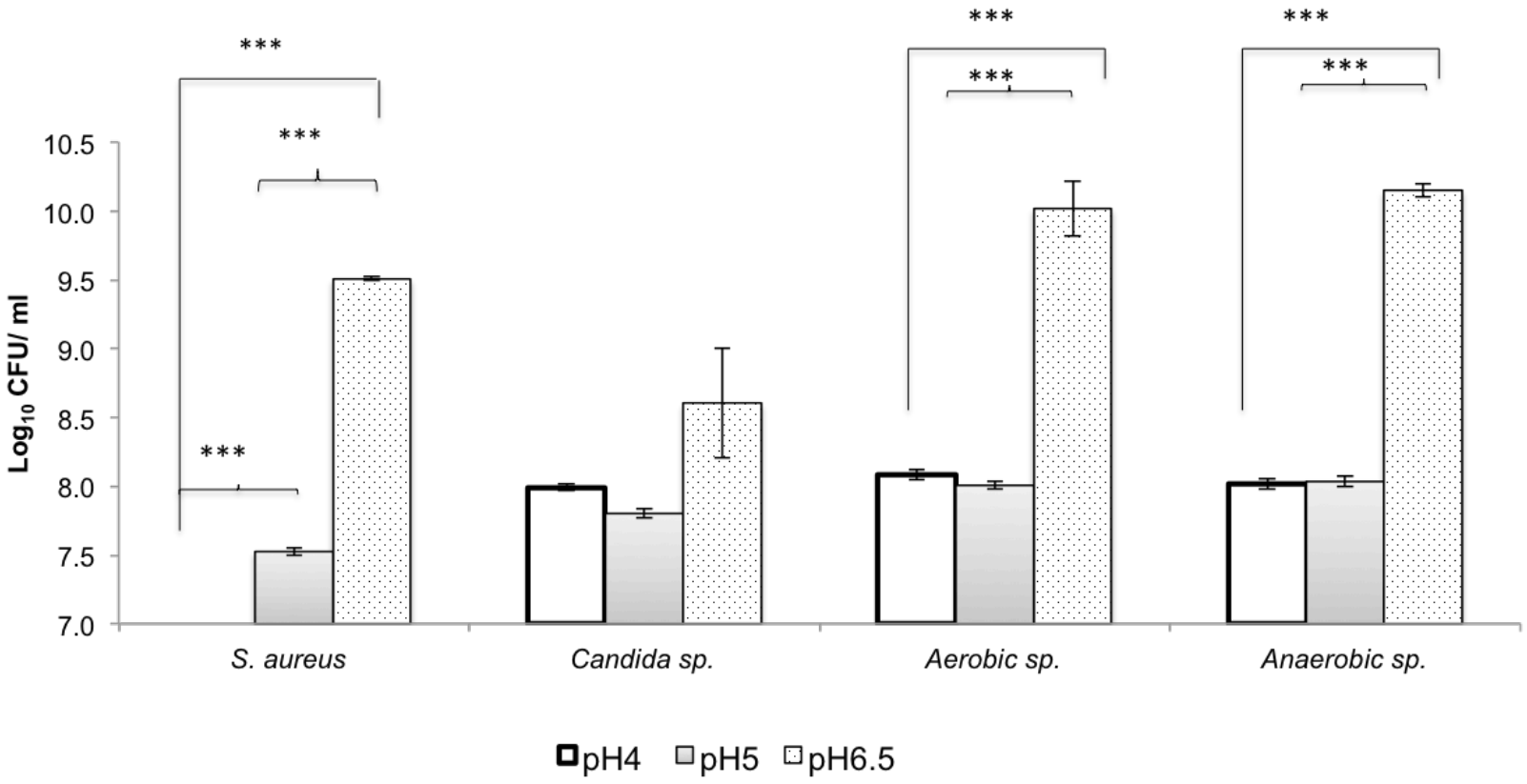

Figure 5.17 – Colony counts from dental plaque 5 d CDFF biofilm inoculated with *S. aureus* in artificial saliva (A/S) of different pHs, whereby ***p ≤0. 001, **p ≤0.01 and *p≤ 0.05.

Table 5.9 - pH of artificial saliva (A/S) pre- and post-exposure with a 5d dental plaque biofilm inoculated with *S. aureus.*

	A/S pH					
S. aureus	Pre-exposure	Post-exposure	Pre-exposure	Post-exposure	Pre-exposure	Post-exposure
1	4.00	5.54	5.00	5.76	6.50	6.14
2	4.00	5.32	5.00	6.05	6.50	6.78
3	4.00	5.15	5.00	5.78	6.50	6.43
Mean	**4.00**	**5.34**	**5.00**	**5.86**	**6.50**	**6.45**
SD	**0.00**	**0.20**	**0.00**	**0.16**	**0.00**	**0.32**
SEM	**0.00**	**0.11**	**0.00**	**0.09**	**0.00**	**0.19**

5.3.6.3 Effect of A/S pH on CDFF dental plaque biofilms inoculated with *P. aeruginosa*

No *P. aeruginosa* were detected by culture from dental plaque CDFF biofilms after A/S at pH 4 exposure for 24 h. Similar counts of between 7.5-8.0 log_{10} CFU/ml for *C. albicans,* aerobic and anaerobic bacteria were evident following exposure to A/S at pH 4 and pH 5 (Figure 5.18). There was a significant (p=<0.001) increase in *P. aeruginosa* numbers following incubation in A/S pH 6.5, compared to both pH 4 and pH 5. There was a reduction in numbers of *C. albicans* after exposure to A/S pH 6.5, however this was not significant (p=0.065). The presence of *P. aeruginosa* and *C. albicans* was evident in biofilms using CLSM imaging (Figure 5.19). *Candida albicans* hyphae were clearly detected at outermost biofilm layers. The highest numbers of aerobic ($1.45x10^9 \pm 3.61x10^8$ CFU/ ml and anaerobic bacteria ($1.04x10^{10} \pm 1.97x10^9$ CFU/ ml) were evident after exposure to A/S at pH 6.5. There was a significant (p=<0.001) increase in the numbers of aerobic and anaerobic bacteria at pH 6.5. All tabulated data is in appendix IV.

The pH of A/S was measured after incubation with dental plaque biofilms inoculated with *P. aeruginosa* (Table 5.10). A significant increase in the pH of A/S 4, to pH 5.26 ± 0.18 was evident (p=0.007). In addition, there was a significant increase in the pH of A/S after a starting pH of 5 to pH 6.35 ± 0.30 when exposed to dental plaque biofilms inoculated with *P. aeruginosa* (p=0.016). Finally, the pH of A/S initially at 6.5 remained similar following incubation with dental plaque biofilms inoculated with *P. aeruignosa* for 24 h with a resulting pH of 6.58 ± 0.16 (p=0.486).

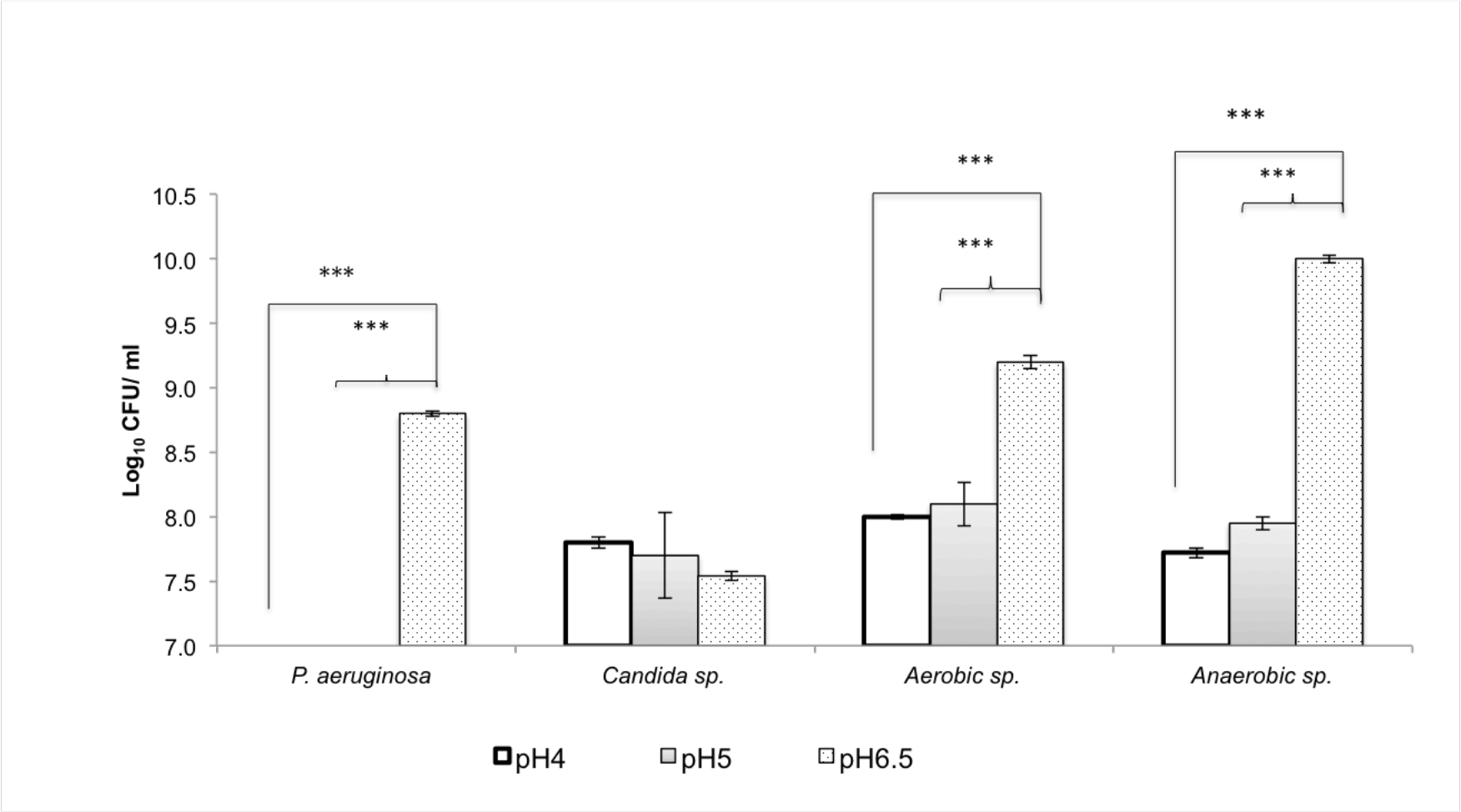

Figure 5.18 – Colony counts from dental plaque 5 d CDFF biofilm inoculated with *P. aeruginosa* in artificial saliva of different pHs, whereby ***p ≤0. 001, **p ≤0.01 and *p≤ 0.05.

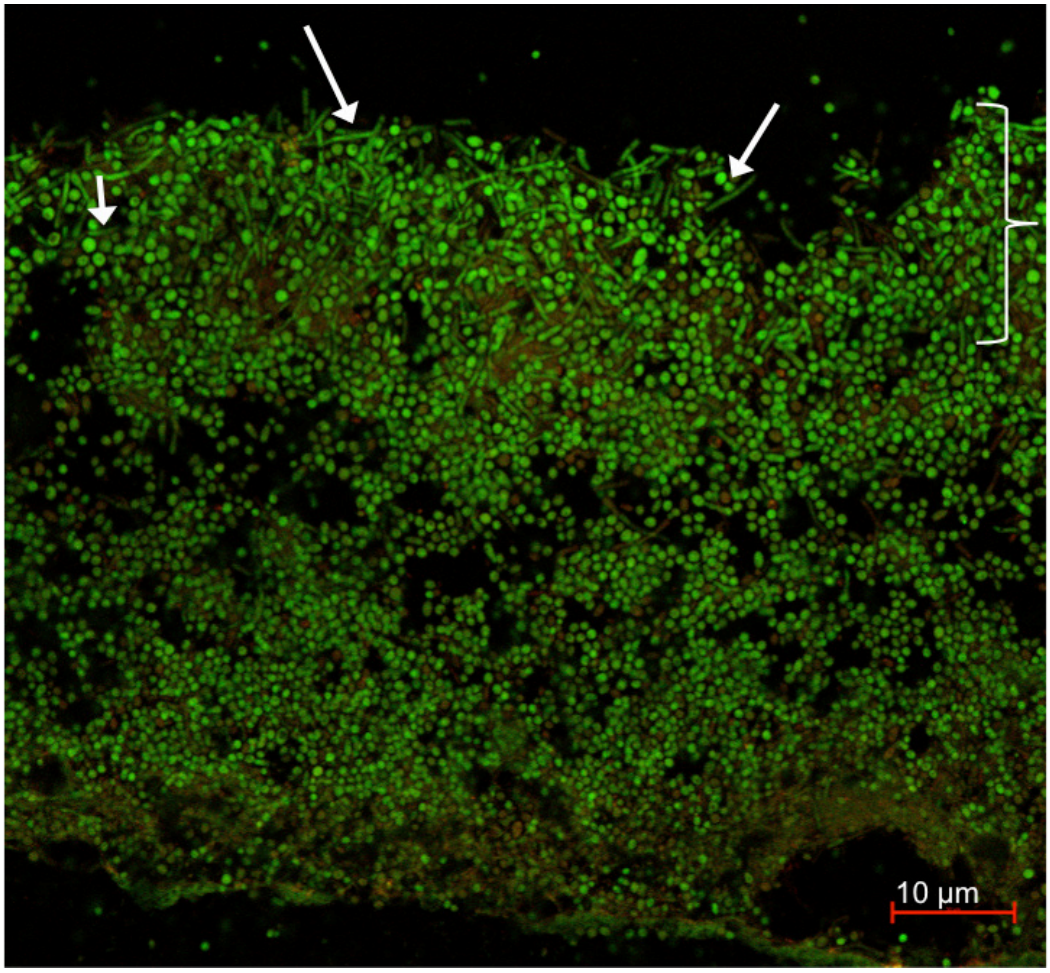

Figure 5.19 – Confocal laser scanning micrograph of microorganisms from a 5 d dental plaque CDFF inoculated with *P. aeruginosa* stained with a FITC-labelled probe. In addition, *Candida albicans* was stained with a FITC-labelled probe. The upper layers of the biofilm (bracketed in the image) contain extensive numbers of *C. albicans* hyphae (as shown by the arrows).

Table 5.10 – pH of artificial saliva (A/S) pre- and post-exposure with a 5d dental plaque biofilm inoculated with *P. aeruginosa*.

	A/S pH					
P. aeruginosa	Pre-exposure	Post-exposure	Pre-exposure	Post-exposure	Pre-exposure	Post-exposure
1	4.00	5.06	5.00	6.65	6.50	6.76
2	4.00	5.32	5.00	6.34	6.50	6.54
3	4.00	5.40	5.00	6.05	6.50	6.44
Mean	**4.00**	**5.26**	**5.00**	**6.35**	**6.50**	**6.58**
SD	**0.00**	**0.18**	**0.00**	**0.30**	**0.00**	**0.16**
SEM	**0.00**	**0.10**	**0.00**	**0.17**	**0.00**	**0.09**

5.4 Discussion

The overarching aim of the work presented in this chapter was to determine the role of selected variables in the oral environment (based on results from chapters 2, 3 and 4) that could promote or prevent colonisation of pathogenic microorganisms within dental plaque and ETT biofilms. Although frequently overlooked in clinical practice, over 60% of bacterial infections have a biofilm origin (Coenye & Nelis, 2010; Römling & Balsalobre, 2012) and these biofilm communities are often complex and polymicrobial. The transition of microorganisms from free-floating (planktonic) cells to biofilm growth and subsequent biofilm maturation is, in part, influenced by environmental factors (de la Fuente-Núñez et al., 2013). Biofilm growth will be affected by a variety of factors including nutrient availability, dynamics of fluid flow rates, pH of surrounding fluids, temperature, antimicrobial presence and oxygen levels (Peyyala & Ebersole, 2013).

Biofilms were developed on ETT surfaces over 10 d, after which the biofilm structure including EPS could be observed by SEM imaging (Figure 5.3). The majority of ETTs are composed of polyvinylchloride (PVC) material (Watson, 1980; Gorman et al., 2001). Triandafillu et al., 2003 investigated the extent of *P. aeruginosa* adhesion to PVC using both reference strains (including PAO1 AK44) and clinical isolates. There was a wide variation in the level of adhesion rates, however all strains of *P. aeruginosa* adhered upon contact with the PVC, with the greatest efficacy in adhesion observed for two clinical strains. Furthermore, Gorman et al., 2001 investigated biofilm formation upon PVC discs of re-cultured clinical *P. aeruginosa* isolates (originally obtained from ETT biofilms of mechanically ventilated patients). Atomic force microscopy revealed adhesion of *P. aeruginosa* to the PVC discs within 4h, and in a 5d period there was an increased formation of EPS and glycocalyx. During the current study, *P. aeruginosa* was the best biofilm former (greatest recovered cell number) on the PVC ETT surface (Figure 5.4), and it was not

significantly affected by the presence of either *S. mutans* of *C. albicans* presence in mixed species biofilms (Figure 5.5, B and D).

In addition to single species biofilms, a series of mixed species biofilms were developed to assess the role of oral organisms (*S. mutans* and *C. albicans*) in promoting respiratory pathogen colonisation and biofilm formation within the ETT, as previously evident in ETTs from extubated patients (chapter 2, figures 2.11-2.15). *Pseudomonas aeruginosa* was cultured in combination with *C. albicans* (Figure 5.5). Previous literature indicates an synergistic relationship between *P. aeruginosa* and *C. albicans* in *in vitro* biofilms, whereby the presence of *C. albicans* hyphae results in the release of phenazine by *P. aeruginosa,* allowing the bacteria to exploit *C. albicans* hyphae as a growth substrate (Harriott & Noverr, 2011). Phenazines are important in interactions between microorganisms and are extremely toxic at an acidic pH, which could include the microenvironment of dental plaque (microbial metabolism and acidic end products) (Gibson et al., 2009). Phenazines will inhibit *C. albicans* respiration pathways via carbon growth impairment and lead to increased levels of fermentation products (Morales et al. 2013). In such situations, *P. aeruginosa* induces extensive hyphae degradation reducing overall *C. albicans* colony formation (Douglas, 2003). Whilst limiting candidal growth by accumulating within the hyphae, this can promote the colonisation of *P. aeruginosa,* and this was observed during *in vitro* investigations within this study (Figure 5.5, D). In addition, When *P. aeruginosa* was combined with *C. albicans* for biofilm formation upon the ETT surface, although a slight reduction in the numbers of *C. albicans* occurred, there was no significant decrease, and this is perhaps due to a lack of hyphae. Although disruption of the biofilm composition may occur, *P. aeruginosa* does not degrade yeast forms of *C. albicans* (McAlester et al. 2008), which contributes to Candida's pathogenesis (Naglik et al. 2011; Méar et al. 2013)

In vivo interactions are likely to be more complex, largely due to the presence of the host immune response. Dectin-1, a C-type lectin receptor expressed on epithelial cells and macrophages, is an important pathogen recognition molecule in host defence against fungal pathogens, including *C. albicans* (Gantner et al., 2005). The ligand for Dectin-1 is β-glucan, which is highly abundant within yeast cells. The magnitude of the immune response is dependent on the balance of yeast to hyphal transition, as filamentous *C. albicans* can avoid immune activation via recognition of the Dectin-1 receptor on macrophages (Naglik et al., 2011). Nevertheless, this relationship between *P. aeruginosa* and *C. albicans* may be important when considering the possiblity of this pathogen exploiting the presence of oral commensal microorganisms in colonisation processes (Avila et al., 2009; Wade, 2013b).

A further key finding of mixed-species biofilm formation upon the ETT surface was the ability of *S. mutans* to enhance attachment and biofilm development of *S. aureus* compared to when cultured alone. *Streptococcus mutans* forms complex glucan-binding proteins, ultimately enabling *S. mutans* to adhere to surfaces, initiating biofilm formation (Lynch et al., 2013). Invertase catalyzes the hydrolysis of sucrose into fructose and glucose. At this stage, the glycolytic pathway can convert glucose into lactic acid. Additionally, glucose can be converted into a polymer known as a glucan, by glucosyltransferase (Gtf) enzymes (Loesche, 1996). *In vivo*, after medical devices (catheters and ETTs for example) are inserted, they quickly become coated with protein rich fluids, enhancing the attachment and rapid biofilm formation of opportunistic pathogens including *S. aureus* (Otto, 2008). During *in vitro* investigations within this project, *S. aureus* was cultured in nutrient and protein-rich culture media encouraging biofilm formation upon the ETT. The presence of *S. mutans* end products from metabolism may further promote and enhance the competitive growth of *S. aureus*. Lactic acid for example, ultimately produced by *S. mutans,* may stimulate the growth of *S. aureus* (Kao & Frazier, 1966). Recent work by Nair et al, 2014 explains that when exposed to lactic acid, *S. aureus* can deploy defense strategies towards the competing bacteria to

thrive in the same ecological niche (in this case, the surface of the ETT). Oral organisms including *S. mutans* promote biofilm formation, and enhance pathogenic attachments, and may as a consequence, contribute in the translocation of microorganisms towards the lower airways and the development of VAP (Perkins et al., 2010; Vandecandelaere & Coenye, 2015).

Antimicrobial mouthwashes are an important tool in promoting oral hygiene, and the effectiveness that these have against microbial species and oral biofilms has been extensively studied (Zanatta et al., 2007; Wise et al., 2008; Marsh, 2010; Hooper et al., 2011; Malic et al., 2013; Sands et al., 2014). Chlorhexidine and Listerine™ are frequently used mouthwashes and are effective against most oral microbiota. Chlorhexidine is key in many current oral intervention programs in an attempt to control the oral biofilm during MV (Jones 1997; Bellissimo-Rodrigues et al. 2009; Scannapieco et al. 2009; Klompas et al. 2014; Niazi et al. 2015). Listerine™ however is ineffective at both reducing dental plaque (potentially colonised by respiratory pathogens) and the incidence of VAP in mechanically ventilated patients (Berry 2013; Kollef 2015). Berry et al., 2013 revealed that Listerine™ was no more effective than the control group of sterile water in reducing the colonisation of dental plaque (n=398).

If bacteria that are not normally able to colonise dental plaque have reduced susceptibility to such antimicrobials compared with the existing oral bacteria then this selective pressure could facilitate subsequent colonisation of the oral biofilm following displacement of susceptible species. To test the hypothesis that those species with reduced susceptibility to administered antimicrobials would subsequently have an enhanced ability to colonise dental plaque biofilms, it was first necessary to establish the MICs of the antimicrobials against test organisms and then assess whether exposure of biofilms to sub-MIC environments promoted colonisation.

Importantly, the MIC of CHX against *P. aeruginosa* (both planktonic and in biofilm formation) was notably higher compared with that for *S. aureus, S. mutans* and *C. albicans* (Table 5.3). Additionally MICs of CHX against single species biofilms were all significantly higher compared to planktonic values (Table 5.3). Although an interesting observation, it is worth noting that direct comparison of the MIC/MBIC values between planktonic and biofilm culture is not strictly fair. While the numbers of organisms for each test was measured initially, it is likely that biofilm growth resulted in fewer cell replications. The antimicrobial activity of CHX was also determined against dual-species cultures. Microbial counts (CFU/ml) at sub-MIC CHX (0.0025-0.0076% CHX) revealed significant ($p=0.03$) reduction in oral microorganisms (*S. mutans* and *C. albicans*) when combined with *S. aureus*, and this was most evident in biofilms (Table 5.4). Once cultured into a biofilm and exposed to CHX at the sub-MIC concentration, *P. aeruginosa* was significantly increased when combined with *S. mutans* compared to levels exhibited during mixed species planktonic analysis.

It was also apparent that when cultured with *S. mutans, P. aeruginosa* numbers were enhanced (Table 5.4). In addition, after exposure of *S. mutans* and *P. aeruginosa* to sub-MIC CHX, *S. mutans* was not subsequently detected by culture. *In vitro P. aeruginosa* growth is rapid and this could have been a key factor in reduced levels of *S. mutans* (LaBauve & Wargo, 2012). Baldan et al. 2014 revealed that within mixed species biofilms, *P. aeruginosa* was able to outcompete other opportunistic pathogens including *S. aureus,* (pathogens associated in cystic fibrosis and respiratory infection). Both laboratory reference strains of *P. aeruginosa* (PA14 and PAO1) and isolates from early stage respiratory infection during the study by Baldan et al., 2014, successfully outcompeted *S. aureus* growth. Mucoidal *P. aeruginosa* from late stage infection did not however express the same ability to out compete growth of *S. aureus.* Within this current study, the MIC of CHX against *S. mutans* was lower than that of *P. aeruginosa* and as a consequence the

selective pressure of CHX would have been of greater detriment to *S. mutans*.

Clinical isolates generally exhibited similar MIC ranges compared to the reference strains used. Notably, the MICs of CHX were greater at 0.0480% ± 0.024 for clinical *P. aeruginosa* biofilms (Table 5.5) (compared to *P. aeruginosa* reference biofilms with an MIC 0.0076%). This relatively higher tolerance of *P. aeruginosa* biofilms to CHX could represent a limitation of such oral hygiene interventions in mechanically ventilated patients. Although studies have reported outcomes in the reduction in VAP (Munro et al., 2009) mortality and bacterial counts using 0.12% CHX (a concentration >2x MIC obtained against biofilms of clinical isolates of *P. aeruginosa* within this study), respiratory pathogens are colonising the dental plaque during MV (Balamurugan et al., 2012; Kusahara et a., 2012). Scannapieco et al., 2009 specifically reported a decrease in the numbers of *S. aureus* following decontamination with CHX, but not an overall reduction in the number of respiratory pathogens including Gram-negative species such as *P. aeruginosa* and *Klebsiella* species.

Antibacterial agents at sub-MIC levels may influence and promote biofilm formation. An *in vitro* investigation using *S. mutans* strain UA159 by Dong et al., 2012 found that exposure to sub-MIC levels of chlorhexidine (MIC was detected at 2.5mg/L) (amongst other antibacterial agents) resulted in the up regulation of biofilm formation genes including *gtfB*, *comD*, and *comE.* Future work investigating the expression of biofilm related genes of respiratory pathogens isolated from dental plaque of mechanically ventilated patients, (and following exposure to sub-MIC concentrations of CHX *in* vitro), could elucidate the extent at which CHX promotes biofilm formation of respiratory pathogens.

To further assess the influence of healthy dental plaque microbiota on respiratory pathogen integration into biofilms, the CDFF was used to

construct dental plaque biofilms >300μm employing pooled dental plaque from healthy volunteers (maturity of dental plaque between 12-24 h) (Figures 5.9 and 5.10). Using the CDFF, mixed species dental plaque biofilms were formed over 5 d. Live dead staining revealed a large quantity of cells stained with propidium iodide. Although this can be an indication of staining dead cellular material via intercalating with DNA, in a biofilm setting this may reflect the presence of eDNA within the EPS. Figure 5.10 reveals the extent of propidium iodide staining within the dental plaque biofilm. The presence of non-specific staining (staining outside the intact cells) may further indicate staining of the eDNA within the biofilm formed EPS. Confocal imaging of 5 d biofilms revealed a large proportion of *C. albicans* were in hyphal formation. Interestingly, *C. albicans* was not viable from dental plaque culture before supplementation into BM medium containing 1% glucose (Hill et al., 2010). Glucose is a source of energy for *C. albicans* proliferation and may further promote exaggerated colonisation triggering such morphological changes to hyphal form, usually observed as a virulence factor in invasive candidiasis (Brown et al. 2006; Vylkova et al., 2011). *Candida albicans* may be influenced to undergo morphogenesis by variation in extracellular pH although morphogenesis tends to occur at a greater rate with higher alkaline pH as opposed to acidic, *C. albicans* is tolerant to pH variation (Nadeem et al., 2013).

Established CDFF biofilms were inoculated with *S. aureus* and/or *P. aeruginosa* and treated with CHX (routinely administered as part of oral hygiene protocols during MV, to mirror clinical administration). The main aim of this work was to assess whether chlorhexidine, currently applied during MV as part of an oral intervention strategy, can promote further respiratory colonisation within the mixed species oral biofilm. Using the CDFF to generate biofilms was performed to confirm the results generated through the 96 well plate biofilm model and the MIC investigations (Tables 5.3 and 5.4). Chlorhexidine absorbs to the mucosal surface and the enamel pellicle via salivary proteins, with limited absorption upon formed dental plaque (Jones

1997). The concentration of active CHX may vary according to the amount of CHX being adsorbed upon the various surfaces within the oral cavity and this may have differential lasting antimicrobial activity, especially upon microbial biofilms. After a 12 h exposure period to CHX in suspension (as opposed to coated onto a surface) to mirror the clinical application in the oral cavity, *S. aureus* was not detected within the oral biofilm via microbial culture (Figure 5.13), however *P. aeruginosa* was detected amongst oral microorganisms after the same exposure periods to CHX (Figure 5.14). Gram-positive bacteria, such as *S. aureus* are perhaps exhibiting increased sensitivity to chlorhexidine due to the structure of the cell wall (Toté et al., 2010).

Dental plaque inoculated with respiratory pathogens was also subjected to variations of artificial saliva pH, to mirror the altered salivary pH evident in mechanically ventilated patients (whereby >25% of mechanically ventilated patients exhibited a decrease in salivary pH, and were also colonised with respiratory pathogens within their dental plaque during MV, Chapter 4, 4.3.3). The pH of saliva will directly modulate dental plaque pH (Marsh, 2006). An increase in the number and activity of acidophilic/acidogenic bacteria such as *S. mutans* in dental plaque may conversely lead to a reduction in the pH of saliva (Cvitkovitch, 2010). Changes in salivary flow and/or salivary pH will inevitably add ecological pressure to the dental plaque community resulting in changes in the overall composition. This phenomenon is referred to as the ecological plaque hypothesis, a term coined by Philip Marsh (Marsh, 1994). A relevant example is dental caries whereby an increased exposure to dietary sugars resulted in a caries-low pH, modulating the plaque over time eventually leading to aciduric organisms such as *S mutans* and lactobacilli species dominating the plaque community (Takahashi, 2005; Marsh, 2006).

In CDFF dental plaque biofilms, the pH of the A/S medium changed significantly after exposure to respiratory pathogens. From a starting salivary pH of 4 or 5, noticeable increases were observed post exposure to the CDFF dental plaque biofilm (Table 5.9). Biofilms, of mixed species, may both adapt

to the pH of saliva by entering a stationary growth phase if necessary, as fluctuations in the pH can have biocidal effects (Garrett et al., 2008). *Pseudomonas aeruginosa* was detected from an artificial saliva (A/S) pH of 6.5, however absent from an A/S of pH 4 and 5 respectively. If *P. aeruginosa* can exploit the dental plaque in the early stages of MV, and integrate in the biofilm, *P. aeruginosa* cells may be protected within the EPS against changes in internal and external pH levels.

At an A/S pH of 5, *S. aureus* was detected at a relatively lower abundance compared to normal oral microbiota, and *P. aeruginosa* was absent by culture. This is not surprising as *S. aureus* is known to be more tolerant than *P. aeruginosa* at pH 5 environments (Korting et al., 1992). Under these acidic conditions *S. aureus* would theoretically be better suited to colonising dental plaque biofilms, whilst *P. aeruginosa* would prefer a higher pH of 6-7.5 (Charyulu & Gnanamani, 2010).

In situations where saliva is not able to perform its buffering functions optimally, for example when saliva flow and pH decreases (as seen during MV, chapter 4; mirrored in challenge experiments 5.2.5) the pH of the dental plaque will decrease concurrently due to selective pressure from variations in the pH shifting the microbial community of dental plaque. Acid tolerant organisms including *S. mutans, Lactobacillus* species and late anaerobic colonisers will predominate within dental plaque. Acid-sensitive organisms may be competitively outcompeted, enhancing the ability of opportunistic respiratory pathogens (for example *S. aureus*) to survive and thrive within dental plaque.

Conclusion

Single and mixed species biofilm models were developed to examine the interactions between selected organisms, chlorhexidine and salivary pH. The ETT biofilm model of mixed-species colonisation revealed that oral

organisms including *S. mutans* enhance the colonisation of *S. aureus,* an important pathogen in the aetiology of VAP.

The application of CHX may be effective against oral biofilms in critically ill patients, reducing the overall bio-load in the oral cavity however this may not reduce all respiratory pathogens to the same extent. *Pseudomonas aeruginosa* exhibited the largest MIC towards chlorhexidine, especially once in biofilm formation and a total of 25 mechanically ventilated patients became colonised with *P. aeruginosa* during MV (2.3.3). Chlorhexidine applications may therefore alter both the abundance and composition of dental plaque, potentially allowing more respiratory pathogens to thrive and out-compete residual oral biofilm members and free-floating microorganisms within the oral cavity.

In addition, decreases in salivary pH significantly influenced the ability of respiratory pathogen colonisation and integration within dental plaque. *Staphylococcus aureus* was detected at a pH of 5 and 6.5, whereas *P. aeruginosa* was only detected via microbial culture within a salivary pH of 6. 5.

Using *in vitro* investigations, this experimental chapter highlights the potential limitations faced by current oral care practices within this patient population, whilst also emphasising the possible role of commensal oral microorganisms in promoting and enhancing respiratory pathogen colonisation and pathogenic biofilm formation within the dental plaque and the ETT biofilms.

6. General Discussion

6. General Discussion

VAP, a pneumonia occurring more than 48h after the initiation of mechanical ventilation, is the most frequently occurring hospital-acquired infection of patients who are in receipt of critical care (Joseph et al., 2010). It has a global prevalence estimated at 15% (Kollef, 2015) and a mortality rate between 20 and 70% (Joseph et al., 2010), although Kollef has suggested attributable mortality is likely to be towards the lower estimate (Kollef, 2015). A meta-analysis analysing individual patient data from 6284 patients from 24 different trials further revealed the attributable mortality rate of VAP at ~13% (Melsen et al., 2013). The prognosis of VAP is adversely influenced when the there is involvement of multidrug resistant biofilms (Raad et al., 2011; Grap et al., 2012; Seguin et al., 2014; Branch-Elliman et al., 2015).

Since the oral cavity is anatomically linked directly to the lower airways, associations between the oral microbial community and respiratory infection seem plausible. In the case of VAP, it has been suggested that commensal oral microorganisms can facilitate the colonisation of dental plaque and the lumens of endotracheal biofilms by respiratory pathogens (Doré et al., 1996; Bahrani-Mougeot et al., 2007). A study by Zuanzazzi et al., 2010, examined the colonisation of dental plaque and saliva by respiratory pathogens, pre and post surgery of 30 hospitalised patients (not all patients were ventilated). Zuanzazzi et al., 2010 documented colonisation by *P. aeruginosa* and Acinetobacter species in 25% and 60 % of dental plaque samples, respectively.

One of the aims of this research was to establish the factors involved in dental plaque colonisation by respiratory pathogens in mechanically ventilated critically ill patients. Initial experiments sought to analyse the extent of colonisation in mechanically ventilated patients using culture independent methods. Additionally, the persistence of respiratory pathogens in dental plaque for up to 3-months after cessation of mechanical ventilation was examined and this had never been investigated before. Persistence of

respiratory pathogens into the period of patient recovery may also be indicative of higher risk of future respiratory infection by acting as a future reservoir of infection.

Most previous studies have studied mechanically ventilated patients for only a short period following liberation from the ventilator. Scannapieco et al., 2009 measured the effect of chlorhexidine (CHX) on oral bacterial pathogens in mechanically ventilated patients. Using selective microbial culture, this comprehensive study simultaneously assessed the colonisation of the dental plaque by certain respiratory pathogens. Dental plaque was sampled every 48 h up to a period of two weeks, however there was no additional analysis following extubation. Although Scannapieco et al., 2009 did not publish dental plaque colonisation of respiratory pathogens at multiple time points during MV, they found dental plaque colonisation by *S. aureus, P. aeruginosa, Acinetobcater* species and Enterobacteriaceae.

Should dental plaque provide a reservoir of potential VAP pathogens, then it is desirable to understand the dynamics of the oral microbiome during MV and how this relates to contamination of both the endotracheal tube and lung parenchyma. Based on the results of microbial culture, the composition of dental plaque altered in one third of mechanically ventilated patients with inclusion of at least one respiratory pathogen (*S. aureus* and/or *P. aeruginosa).* Importantly, these bacterial species are considered to be the causative bacteria in up to 50% of VAP cases and frequently exhibit multidrug resistant genes, potentially limiting the efficacy of antibiotic treatment (Hunter, 2006; Parker et al., 2008; Bouza et al., 2012).

For the first time, this research has comprehensively analysed dental plaque microbiota of mechanically ventilated patients using high throughput sequencing. High-throughput sequencing generates a large amount of high quality and accessible data. Using this approach, over 100 microbial species, representing 40 bacterial genera were identified in dental plaque microbial communities during MV, highlighting high species richness and diversity.

Wang et al., 2013 performed metagenomic sequencing on dental plaque of 16 individuals (7 healthy and 9 patients with chronic periodontitis), identifying over 30 different genera. Wang et al., 2013 revealed large differences occurring in the composition of dental plaque between healthy control subjects and patients with chronic periodontitis. Bacteriodes was the most abundant phylum in all samples of periodontitis, whereas Actinobacteria and Proteobacteria were significantly increased in the healthy control plaque. At the genera level, *Prevotella* was most dominant in periodontitis plaque, (up to 45% of total bacterial communities), whereas, interestingly, not one genera was deemed most dominant in healthy dental plaque. Several different genera, including streptococci were present at similar levels. Comparably, during the current study, a substantial microbial change in the composition of dental plaque was evident during the course of MV and was noted by the detection of several potential respiratory pathogens, including *Staphylococcus aureus*, *Streptococcus pseudopneumoniae* and *Escherichia coli*.

Opportunistic pathogens in dental plaque contribute to the pathogenesis of both oral and systemic infections. Although not generally considered a permanent member of healthy dental plaque, *S. aureus* has been isolated from many areas of the oral cavity and nasal regions. Ohara-Nemoto et al., 2008 analysed the saliva, dental plaque and nasal cavity of 56 healthy adults; *Staphylococcus aureus* was the species most frequently isolated from saliva (50%, n=26). Interestingly during their study, *S. aureus* was isolated in over one-third of individuals' dental plaque (=n19). Whilst studies have shown the prevalence of *S. aureus* colonisation in the oral cavity of healthy individuals, there is little evidence in the literature to suggest *Streptococcus pseudopneumoniae* colonisation. *Streptococcus pseudopneumoniae,* a relatively new species genetically and morphologically different from *Streptococcus pneumoniae* was originally isolated from a lower respiratory tract infection. Although implicated in respiratory infections, the clinical importance of *S. pseudopneumoniae* is still to be fully determined (Keith et

al., 2006). *Escherichia coli,* another pathogen isolated within dental plaque during MV, is a commensal coloniser of the human large intestine and a frequent pathogen of urinary tract infections (Katouli, 2010). Previous studies have however reported the isolation of *E. coli* from saliva and oral tissues in hospitalised (non-ventilated) patients (Ewan et al., 2015), but currently there is little evidence for colonisation within dental plaque.

Interestingly, after ETT-extubation, both the prevalence and abundance of respiratory pathogens decreased. For the majority of patients, a loss of the colonising respiratory pathogen occurred and this was most apparent with *S. aureus*. However, for some patients, persistence of the targeted respiratory pathogens in dental plaque occurred highlighting that plaque remained a reservoir for potential recurrent infection. *Pseudomonas aeruginosa* was more persistent than *S. aureus* in dental plaque post ETT extubation. Nevertheless, whilst dental plaque provides a source of infectious bacteria during intubation, it would generally appear that its microbial composition quickly reverts back to one usually associated with health. These findings are important as they strongly indicate that dynamic changes to dental plaque composition occur in response to critical illness and treatment As such, identifying the factors involved in driving these microbial changes could facilitate the development of preventative and management strategies or serve as prognostic and diagnostic markers.

The ETT provides an essential interface between the lower airways of the patient and the ventilator. However, it also provides a conduit for microbial movement from the oral cavity to the lower airways (Cairns et al., 2011). This process typically would involve microbial aspiration from the oral cavity to the inflated retention cuff of the ETT. In the majority of ETTs, the thin polyurethane cuff material will contain folds, which form during inflation as the fully inflated cuff is larger than the tracheal diameter. This is because tracheal diameters vary between individuals and ETT cuffs have to be of a size to accommodate the tracheal size ranges. The microchannels that develop in a cuff provide pathways for microbial leakage. The effect also arises when a loss of cuff pressure occurs. Once below the cuff, subglottic

secretions can either enter the lungs or be drawn into the ETT lumen, which leads to biofilm formation.

Microbial culture, characterisation of the ETT biofilm microbiome and use of species-specific PNA probes (*in situ* hybridisation) coupled with confocal microscopy revealed that ETT biofilms of 47 mechanically ventilated patients, were colonised with members of the oral microbiota including *S. mutans* and strictly anaerobic species including *P. gingivalis.* Members of the oral microbiota colonising within the ETT may therefore contribute to the pathogenesis of VAP by acting as a reservoir for respiratory pathogens or indirectly by increasing pathogenic biofilm development in the ETT (Gil-Perotin et al., 2012).

In this study, the ETT biofilm from 21 patients was culture positive for respiratory pathogens. Within these 21 patients, dental plaque was colonised by respiratory pathogens (either *S. aureus* and/or *P. aeruginosa*) during MV in 18. It has been suggested that as ETT biofilms accumulate, there may be an increase in resistance to gas flow through the ETT and the biofilm can become displaced to the lower airways, increasing the risk of respiratory infection (Coppadoro et al., 2013). Aggregates of respiratory pathogens including *S. aureus* and *P. aeruginosa* identified (2.3.12) were indeed evident in ETT biofilms from patients using fluorescence *in situ* hybridisation and species-specific PNA probes. The fact that biofilm aggregates would be transferred to the lower airway might mean that the bacteria present would immediately be in an already resistant state to host defences and administered antimicrobials. Use of *in vitro* ETT biofilm model (5.3.2) further revealed that *S. mutans* significantly increased colonisation and biofilm formation by *S. aureus.* By-products of *S. mutans* metabolism, including lactic acid, may be influencing *S. aureus* growth, to outcompete the biofilm formation of *S. mutans* on the surface of the ETT (5.4).

Having established that microbiological composition changes occurred in the dental plaque of patients receiving MV and also after extubation, establishing

the underlying reasons for this is important. If the mechanistic basis for the acquisition of respiratory pathogens in dental plaque could be determined, then preventative approaches could be developed. Many factors could potentially serve as drivers for changes in the oral microbiome in mechanically ventilated patients, including underlying illness and effects of treatment. However, the research undertaken was primarily based on the hypothesis that changes in saliva would instigate alterations in the dental plaque community. To this end, the salivary factors assessed were flow, pH and proteomic composition on a longitudinal basis during MV. This is the first time that such an investigation has been undertaken.

It has been shown that mechanically ventilated patients develop dryness in the mouth (xerostomia) (Munro & Grap, 2004; Berry & Davidson, 2006; Prendergast et al., 2013). Xerostomia may arise in mechanically ventilated patients simply by the mouth being partially held open by the presence of the ETT. Administered medications, including diuretics (often prescribed to critically ill patients), and an absence of mechanical and chemical oral stimulation may also reduce the quantity of saliva produced. A reduced salivary flow rate during intubation was evident, with 52 out of 107 mechanically ventilated patients showing a reduction in saliva volume during MV (4.3.2).

A reduced salivary volume will promote plaque accumulation since there would be an associated reduction in the physical removal of plaque organisms by the flushing action of saliva, and a concurrent lowering of host antimicrobial components (Devine, 2003; Gorr & Abdolhosseini, 2011). Neither salivary flow nor salivary composition is typically measured during hospital stay. Although salivary flow rates vary significantly with age, gender and diet and flow rates range between 0.1-2 ml/min (Fenoll-Palomares et al., 2004), salivary reduction will inevitably influence dental plaque composition. Patients suffering with xerostomia, common in elderly individuals, may therefore develop dental oral dysbiosis leading to oral diseases including caries. A reduction in saliva (including a reduction in salivary antimicrobial

proteins and pH buffering systems) will inevitably lead to gradual dental plaque changes, specifically to caries associated microorganisms, able to tolerate an acidic environment (Turner et al., 2007; Su et al., 2011). Additionally, individuals with xerostomia often see increases in *Candida* colonisation within the oral cavity (Shinozaki et al., 2012).

A reduced salivary pH was also apparent during mechanical ventilation and this was most evident when microbial changes in plaque were detected (Figure 4.3). The reasons for this change are unclear, however, it is possible that a more acidic oral environment promotes respiratory pathogen colonisation. Marsh (Marsh, 1994) has described a mechanistic basis with relevant examples of dental caries and periodontal disease as to how changes in dental plaque and saliva pH may affect the composition of dental plaque (4.4). By *in vitro* modeling studies, this current research has demonstrated that *S. aureus* is more acid-tolerant (pH 5) than *P. aeruginosa.* This was evident from dental plaque challenge experiments using the CDFF, in which the growth of *S. aureus* was not inhibited at a pH of 5 (5.3.6.2). Respiratory pathogens may be better suited to an acidic environment and able to exploit this environment out competing other members of the community. Alternatively, it is possible that a reduction in saliva secretion will lead to a reduction in bicarbonate (primary pH buffer of saliva) (Hicks et al., 2003) resulting in a decreased ability to maintain salivary pH. Prasanthi et al., 2014 reported a correlation between reductions in stimulated saliva secretion, and reductions in buffering capacity and pH as a result of diuretic medications. In turn, a reduction in both salivary volume and pH could also promote increased colonisation by respiratory pathogens.

Proteomic analysis of saliva revealed changes in salivary proteins both during MV and into the post-ETT extubation period (Table 4.8). Particularly evident was the increased concentration of a spectrum of antimicrobial peptides including lysozyme, lactoperoxidase and cystatin in the post-ETT extubation period, compared with levels found during MV (Table 4.8). Several bacterial groups, predominantly Gram negative bacilli, that enter the

oral cavity after the early plaque colonisers can exploit these bacteria to adhere to the tooth surface indirectly (Perkins et al., 2010). Dental plaque colonisation of respiratory pathogens during MV may provoke a localised immune response. Proteomic and cytokine analysis during MV appeared to support this, significant increases in salivary levels of pro-inflammatory cytokines, including IL-8 and IL-1β occurred compared to healthy volunteers. Interestingly, levels of these cytokines reduced post extubation to levels similar to those of the healthy volunteers.

In vitro models of dental plaque and ETT biofilms were developed and used to assess the effect of an antimicrobial on respiratory pathogen colonisation of biofilms. Oral antiseptics, such as chlorhexidine have been used in general dentistry and critical care since the 1970s (Jones, 1997). Over the years there have been several studies assessing the effects of chlorhexidine in reducing VAP incidence and mortality (O'Reilly, 2003; Grap et al., 2011; Balamurugan et al., 2012). The CDFF modeling system was used to create robust dental plaque biofilms. Biofilm analysis revealed that the minimum biofilm inhibitory concentration was considerably higher, up to 1000-fold, than planktonic cell susceptibility (Malic et al., 2013; Smith et al., 2013). Following *in vitro* dental plaque biofilm inoculation with *S. aureus* and exposure to chlorhexidine for 12 h, growth of *S. aureus* was successfully inhibited. In contrast, a significant increase in numbers of *P. aeruginosa* was evident following a similar challenge with chlorhexidine, and only the highest chlorhexidine concentration tested was shown to inhibit the growth of this species.

Further studies

Based on this research, a number of areas have been identified for future work. Given that a number of salivary changes were found to occur during MV and in patients with microbial alteration in their dental plaque, it would be beneficial to assess specific salivary proteins for use as biomarkers to identify patients at higher risk of VAP, VAP prognosis and to assess effectiveness of interventions. Diagnostic biomarkers within saliva are an

emerging area of proteomic research (Thomadaki et al., 2011). Studies have investigated fluid collected from the lower airways of mechanically ventilated patients for proteomic content and potential biomarkers to aid the diagnosis of VAP (Wu et al., 2003). Antibacterial proteins including neutrophil proteases were expressed at significantly higher concentrations in those patients diagnosed with VAP (Lu et al. 2008; Wilkinson et al. 2012). Investigating the proteomic composition of saliva during MV has highlighted certain protein profiles, with elevated levels of antibody peptides and highly expressed antimicrobial peptides into the recovery period, warranting further clinical investigations.

Reductions in both salivary flow and salivary pH were evident during MV. Investigating therapeutic interventions involving salivary supplementation with antimicrobial peptides or the use of artificial saliva could be beneficial in reducing respiratory pathogen colonisation of dental plaque.

Further areas for investigation are as follows:

- Biomarkers that can predict VAP. Table 4.5 has provided a short list of proteins to focus on in future clinical investigations.
- Clinical water testing to ascertain the aetiology and origins of *P. aeruginosa* colonisation.
- Routine nasal swabbing for the detection of *S. aureus* in mechanically ventilated patients. In addition, in those patients with nasal *S. aureus* colonisation bacterial strain typing could be performed to match isolates recovered from dental plaque.
- Further biofilm modeling with the CDFF could use human saliva, adding the benefit of the complex proteomic composition. Larger quantities would be collected to allow for repeat experiments and generation of data capable of statistical analysis.
- Future work to enhance the biofilm CDFF model to allow viability of anaerobes and therefore creating PNA probes allowing multiplex images could further track certain species within the biofilm.

- Bacterial strain typing and further genomic analysis of biofilm-related genes would elaborate on whether patients' who remain colonised in the post-ETT extubation period harbor the same strain.
- Developing a co-culture model to look at antibacterial and anti-inflammatory/proinflammatory response at same time. Can you mimic the recorded microbial shift that occurs during mechanical ventilation to detect biomarkers to predict the onset of VAP?

Research Summary

Dental plaque of mechanically ventilated patients was shown to become colonised by a range of potential respiratory pathogens. Also during ventilation changes were observed in saliva volume, pH and concentration. These changes were accompanied by an increase in salivary pro-inflammatory cytokines. Following extubation, dental plaque colonisation by respiratory pathogens was not detected (for most patients) and levels of salivary cytokines were seen to be reduced to levels similar to those of healthy volunteers. Whilst the patient is mechanically ventilated, dental plaque does serve as a reservoir for respiratory pathogens. There were several salivary parameters, including reduced salivary flow, pH and the salivary proteome, that changed, and were associated with the presence of these respiratory pathogens in dental plaque.

VAP remains an important hospital acquired infection in critically ill patients (Kollef, 2015) and is associated with increased mortality, duration of stay and cost (Bahrani-Mougeot et al., 2007; Sundar et al., 2012). Performing longitudinal microbial culture coupled with high-throughput sequencing of the dental plaque microbiome revealed extensive changes occurring during MV. In over one-third of mechanically ventilated patients reductions in salivary volume and/or pH were also evident. Approaches that maintain adequate salivary flow and at a suitable pH for example the administration of artificial saliva, could prove inhibitory to respiratory pathogens colonising dental plaque and consequently could limit the translocation of pathogenic

microorganisms to the lower airways. In addition, the observed changes in salivary cytokines explored through high-throughput proteomic analysis might be of future value as diagnostic markers that reflect either the inflammatory responses of the lower airway, or accumulation of respiratory pathogens in the plaque. Such an approach would be highly beneficial as it would be a non-invasive tool for VAP diagnosis, and this is a feature lacking with most other approaches.

Key findings include:

- This is the largest study of oral microbial changes during MV and recovery from critical illness. During MV, dental plaque was shown to become heavily colonised with respiratory pathogens, including Gram-positive and Gram-negative species such as *S. aureus, P. aeruginosa, E. coli* and *S. pseudopneumoniae*.
- Analyses revealed that 70% and 55% of mechanically ventilated patients that demonstrated colonization of dental plaque with *S. aureus* and *P. aeruginosa,* respectively, were shown to have "lost" these species post-extubation.
- For the first time, the dental plaque microbial community of mechanically ventilated patients has been comprehensively analysed using high throughput sequencing.
- The full diversity of dental plaque during MV was demonstrated with 40 genera, and over 100 identified species with 8 species of potential respiratory pathogens detected.
- Decreases (a trend) in salivary volume were evident in over a third of mechanically ventilated patients. Reduced salivary pH was also observed, and was especially significant in patients where respiratory pathogens occurred in dental plaque during MV.
- The salivary proteomic concentration and composition differed during MV compared with that at commencement of MV. Significant increases in pro-inflammatory cytokines IL-6, IL-1β and IL-8 were also evident during MV in comparison to healthy volunteers. Post ETT-

extubation these levels reduced to those observed in healthy volunteers.

- *In vitro* biofilm model systems were successfully developed to allow the modeling of the changes *in vivo* and study the effects of bacterial challenges and antimicrobial interventions.
- The presence of *Streptococcus mutans* increased the numbers of *S. aureus* within in vitro ETT biofilms models.
- *In vitro* dental plaque biofilms inoculated with respiratory pathogens (*S. aureus* and/or *P. aeruginosa*), exhibited significant changes when challenged at different pH. *Staphylococcus aureus* remained viable at a pH of 5, whilst *P. aeruginosa* was only viable at a higher salivary pH of 6.5.
- Chlorhexidine challenge of *in vitro* oral biofilms inoculated with respiratory pathogens had differential effects on respiratory pathogens. Whilst able to limit the numbers of *S. aureus* within the oral biofilm, chlorhexidine was not effective at inhibiting *P. aeruginosa.*

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Appendix I

Clinical study additional information and data

- NHS ethics letter
- Critical care oral assessment plan
- Individual DMFT scores
- Individual patient demographics
- Individual patient target/selective microbial culture results
- *Pseudomonas aeruginosa* isolate PCR amplicons
- *Staphylococcus aureus* isolate PCR amplicons
- *Pseudomonas aeruginosa* isolate sensitivities
- *Staphylococcus aureus* isolate sensitivities

NHS ethics letter:

Part of the research infrastructure for Wales funded by the National Institute for Social Care and Health Research, Welsh Government.
Yn rhan o seilwaith ymchwil Cymru a ariannir gan y Sefydliad Cenedlaethol ar gyfer Ymchwil Gofal Cymdeithasol ac Iechyd, Llywodraeth Cymru

NISCHR

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05 March 2013

Dr Matt Wise
Consultant Adult Critical Care
Cardiff and Vale University Health Board
University Hospital of Wales
Heath Park
Cardiff CF14 4XW

Dear Dr Wise

Study title: **Dynamics of dental plaque biofilms in critically ill patients.**
REC reference: **13/WA/0039**
IRAS project ID: **121288**

Thank you for your letter of 01 March 2013, responding to the Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chairman, Dr. Pete Wall.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to withhold permission to publish, please contact the Co-ordinator Dr. Corinne Scott, corinne.scott@wales.nhs.uk.

Confirmation of ethical opinion
On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Mental Capacity Act 2005
I confirm that the committee has approved this research project for the purposes of the Mental Capacity Act 2005. The committee is satisfied that the requirements of section 31 of the Act will be met in relation to research carried out as part of this project on, or in relation to, a person who lacks capacity to consent to taking part in the project.

Ethical review of research sites
NHS sites
The favourable opinion applies to all NHS sites taking part in the study, subject to management permission being obtained from the NHS/HSC R&D office prior to the start of the study (see "Conditions of the favourable opinion" below).

Critical Care Oral plan:

Critical Care Mouth Care Plan: A B & C:

Always ensure hand hygiene - wear an apron and gloves when performing Mouth Care

Soft / Medium Bristle toothbrush / low foaming toothpaste ☐

Aqueous lip balm ☐

Sterile water ☐

Ensure fluid intake / unsweetened liquid to moisten mouth ☐

Artificial saliva ☐

Chlorhexidine gluconate (CHX) 1% gel ☐ 0.2% rinse ☐

Benzamine hydrochloride (dilute 50:50 with water if not tolerated) ☐

Topical Acyclovir ☐

Antifungal medication ..... ☐

Denture cleaning Soap / Sodium hypochlorite ☐

Denture pot ☐

Additional Medication Prescribed ..... YES / NO

Care Plan A 12 hourly care: **LOW RISK (Indicate √ care undertaken based on need)**

Natural teeth

Twice daily ensure patient has the necessary resources indicated above. Ensure:

- Ensure access to oral care twice daily – **EM / LE**
- Provide access to fluids and monitor **EM / MD / EE / LE**
- Administer medications (prescription / assessment)
- Water based gel to moisten lips **EM / LE (Dry lips)**

Denture care required - Yes / No

EM: Ensure dentures fit and are in good condition, Clean with identified cleaning agent

LE: Remove as appropriate

Overnight Storage – in a named lidded pot

Soak overnight sterile water, Long term: clean dentures and replace sterile water daily

Infection suspected: soak in Sodium hypochlorite 1%

Acrylic denture (2 mins), Chrome Cobalt denture (1mins) and rinse well

Care Plan B 4 - 6 hourly care: **MODERATE RISK (Indicate √ care undertaken based on need)**

- Assist and or Clean teeth (Low foaming toothpaste) 2-3 mins **EM / LE**
- Administer medications(see prescription / assessment)
- Moisten lips mouth / Ensure access to unsweetened fluids / adequate fluid intake **EM / MD / EE / LE**
- CHX or equivalent twice daily as indicated for ulceration of known origin (min 2 hours before or after brushing with toothpaste **EM / LE**

Care Plan C 2 - 4 hourly care: **HIGH RISK (Indicate √ care undertaken based on need)**

Early Morning 08.00 (EM):	**Mid Day 12.00 (MD):**	**Early Evening 18.00 (EE):**	**Late evening 22.00 (LE):**	**Night 02.00 (NT):**	**Additional / PRN**
Brush – Toothpaste (2-3 mins) Moisten mouth Apply lip balm Medicate as prescribed Pharyngeal suction **Check ET / TT cuff pressure[1]**	Brush CHX (2-3 mins) Moisten mouth Apply lip balm Medicate as prescribed Pharyngeal suction **Change ET / TT tapes[1]**	Brush – Toothpaste (2-3 mins) Moisten mouth Apply lip balm Medicate as prescribed Pharyngeal suction **Check ET / TT cuff pressure[1]**	Brush CHX (2-3 mins) Moisten mouth Apply lip balm Medicate as prescribed Pharyngeal suction **Reposition ET tube[1]**	CHX / moisten mouth - rinse if indicated Apply lip balm Medicate as prescribed Pharyngeal suction **Check ET position[1]**	**Assess mouth / oral cavity** Moisten lips / mouth Pharyngeal suction and or Subglottic suction (if available) **Endotracheal tube and Tracheostomy (ET/TT)[1]** Assess mouth for pressure damage, reposition ET and secure as required, **cuff pressure 20 - 30 cm H_2O** NBM / NG / PEG / JEJ / TPN - Omit toothpaste Brush with CHX 1% gel **ADMINISTER ANALGESIA AS PRESCRIBED**

The **aim** of this care process is to ensure that:

- The patient's mouth is assessed regularly, and that any issue is identified, escalated if necessary, treated appropriately and accurately documented.
- Ventilator Acquired Pneumonia will be prevented.
- The patient's oral hygiene, comfort and communication needs will be effectively met.

	Initial care performed, expand in the patient evaluation
EM /	**Comments**.....
MD /	.....
EE /	.....
LE /	.....
NT /	.....

PRN	Time	Initial			

CCMCP /Version 1 (12/12)

Individual DMFT scores:

			DMFT score			
Age	**PN**	**DMFT complete**	**D**	**M**	**F**	**Total**
51	**1**	✓	0	3	5	8
33	**2**	✓	0	4	0	4
31	**3**	✓	1	1	7	9
55	**4**	✓	3	12	4	19
62	**5**	✓	1	6	14	21
34	**6**	✓	0	0	0	0
54	**7**	✓	1	6	4	11
63	**8**	✓	0	9	3	12
77	**9**	✓	9	13	1	23
30	**10**	✗				
73	**11**	✓	1	9	6	16
68	**12**	✓	2	5	6	13
50	**13**	✓	0	5	9	14
44	**14**	✓	0	0	11	11
49	**15**	✓	2	5	4	11
28	**16**	✓	0	4	0	4
51	**17**	✓	1	18	4	23
39	**18**	✗				
69	**19**	✓	1	11	10	22
29	**20**	✓	0	2	1	3
18	**21**	✓	0	0	0	0
72	**22**	✓	0	1	11	12
19	**23**	✓	1	0	4	5
40	**24**	✓	0	4	10	14
27	**25**	✓	0	2	2	4
63	**26**	✗				
41	**27**	✓	0	3	2	5
55	**28**	✓	1	9	5	15
76	**29**	✓	1	9	5	15
58	**30**	✓	0	14	5	19
31	**31**	✗				
67	**32**	✓	0	5	6	11
85	**33**	✓	2	14	1	17
57	**34**	✓	1	7	10	18
58	**35**	✓	9	8	2	19
60	**36**	✗				
37	**37**	✓	3	5	7	15
37	**38**	✓	0	0	0	0
86	**39**	✓	0	14	3	17
60	**40**	✓	0	18	3	21
58	**41**	✓	0	21	5	26

74	**42**	✓	0	8	7	15
59	**43**	✓	3	1	2	6
22	**45**	✗				
48	**46**	✓	0	2	10	12
53	**47**	✓	0	9	3	12
57	**48**	✓	3	1	3	7
75	**49**	✓	0	1	15	16
39	**50**	✓	3	2	6	11
73	**51**	✓	0	2	6	8
65	**52**	✓	2	10	4	16
66	**53**	✓	0	6	5	11
66	**54**	✓	0	3	10	13
60	**55**	✓	0	0	8	8
49	**56**	✓	2	8	7	17
53	**57**	✓	0	6	5	11
38	**58**	✓	0	0	6	6
86	**59**	✓	8	10	10	28
39	**60**	✓	0	1	8	9
36	**61**	✓	0	7	7	14
21	**62**	✓	0	0	1	1
58	**63**	✗				
44	**64**	✓	4	19	0	23
42	**65**	✓	3	2	8	13
73	**66**	✓	0	0	0	0
68	**67**	✓	1	14	4	19
52	**68**	✓	0	0	8	8
82	**69**	✓	0	9	11	20
18	**70**	✓	0	0	2	2
26	**71**	✓	0	5	2	7
67	**72**	✓	0	7	3	10
59	**73**	✓	0	14	5	19
51	**74**	✓	1	12	4	17
75	**75**	✓	0	12	8	20
49	**76**	✓	1	5	10	16
70	**77**	✓	3	13	2	18
75	**78**	✓	7	11	6	24
51	**79**	✗				
71	**80**	✓	2	2	2	6
74	**81**	✓	1	12	5	18
45	**82**	✓	0	1	0	1
47	**83**	✓	1	0	0	1
58	**84**	✓	0	5	12	17
75	**85**	✓	0	1	13	14
74	**86**	✓	1	3	1	5
80	**87**	✓	0	4	7	11
40	**88**	✓	19	0	3	22
38	**89**	✓	9	4	2	15

66	**90**	✓	4	9	4	17
55	**92**	✓	2	4	7	13
42	**93**	✓	1	2	4	7
60	**94**	✓	3	5	5	13
41	**95**	✓	1	0	8	9
27	**96**	✓	0	1	0	1
77	**97**	✓	0	5	17	22
58	**99**	✓	0	4	5	9
26	**100**	✓	2	0	4	6
29	**101**	✓	12	8	5	25
30	**102**	✓	0	3	5	8
80	**103**	✗				
56	**104**	✓	0	10	8	18
67	**105**	✓	0	8	6	14
60	**106**	✓	1	6	12	19
47	**107**	✓	0	2	10	12
74	**108**	✗				
38	**109**	✓	0	1	6	7
60	**110**	✗				

	Total DMFT	**D**	**M**	**F**	**DMFT Score**
Average	96	1.46	5.70	5.39	12.54

Key:

DMF	Group
0-5	1
6-10	2
11-15	3
16-20	4
21-25	5
25+	6

Recruited patients	Patient NO.	Gender	Age	Admission details (reason for MV)	DFMT
1	PN001	M	51	Respiratory failure	8
2	PN002	M	33	Stroke/brain injury/seizures	4
3	PN003	M	31	Overdose/suicide attempt	9
4	PN004	F	55	Stroke/brain injury/seizures	19
5	PN005	M	62	Stroke/brain injury/seizures	21
6	PN006	F	34	Respiratory failure	0
7	PN007	F	54	Stroke/brain injury/seizures	11
8	PN008	M	63	Respiratory failure	12
9	PN009	F	77	Other	23
10	PN010	F	30	Stroke/brain injury/seizures	-
11	PN011	F	73	Respiratory failure	16
12	PN012	M	68	Respiratory failure	13
13	PN013	F	50	Stroke/brain injury/seizures	14
14	PN014	M	44	Respiratory failure	11
15	PN015	F	49	Stroke/brain injury/seizures	11
16	PN016	M	28	Other	4
17	PN017	F	51	OOHCA	23
18	PN018	M	39	Overdose/suicide attempt	-
19	PN019	M	69	Poly-trauma	22
20	PN020	F	29	Poly-trauma	3
21	PN021	F	18	Stroke/brain injury/seizures	0
22	PN022	M	72	OOHCA	12
23	PN023	M	19	Stroke/brain injury/seizures	5
24	PN024	M	40	Stroke/brain injury/seizures	14
25	PN025	F	27	Stroke/brain injury/seizures	4
26	PN026	F	63	General surgery - Stomach	-
27	PN027	M	41	Stroke/brain injury/seizures	5
28	PN028	F	55	Respiratory failure	15
29	PN029	M	76	Respiratory failure	15
30	PN030	F	58	Stroke/brain injury/seizures	19
31	PN031	F	31	Other	-
32	PN032	M	67	Stroke/brain injury/seizures	11
33	PN033	F	85	Respiratory failure	17
34	PN034	M	57	Other	18
35	PN035	M	58	OOHCA	19
36	PN036	M	60	OOHCA	-
37	PN037	M	37	Stroke/brain injury/seizures	15
38	PN038	M	37	Stroke/brain injury/seizures	0
39	PN039	F	86	Respiratory failure	17
40	PN040	F	60	Respiratory failure	21
41	PN041	F	58	Respiratory failure	26
42	PN042	M	74	Stroke/brain injury/seizures	15
43	PN043	M	59	Stroke/brain injury/seizures	6

44	PN045	F	22	Stroke/brain injury/seizures	-
45	PN046	M	48	OOHCA	12
46	PN047	M	53	Stroke/brain injury/seizures	12
47	PN048	M	57	Stroke/brain injury/seizures	7
48	PN049	F	75	General surgery - Stomach	16
49	PN050	M	39	Stroke/brain injury/seizures	11
50	PN051	F	73	Stroke/brain injury/seizures	8
51	PN052	M	65	Other	16
52	PN053	F	66	Respiratory failure	11
53	PN054	M	66	Other	13
54	PN055	F	60	Other	8
55	PN056	F	49	Stroke/brain injury/seizures	17
56	PN057	M	53	Respiratory failure	11
57	PN058	F	38	Stroke/brain injury/seizures	6
58	PN059	M	86	General surgery - Stomach	28
59	PN060	M	39	Stroke/brain injury/seizures	9
60	PN061	M	36	Poly-trauma	14
61	PN062	M	21	Poly-trauma	1
62	PN063	M	58	Respiratory failure	-
63	PN064	M	44	OOHCA	23
64	PN065	F	42	General surgery - Stomach	13
65	PN066	F	73	Respiratory failure	0
66	PN067	M	68	Respiratory failure	19
67	PN068	M	52	OOHCA	8
68	PN069	F	82	General surgery - Stomach	20
69	PN070	M	18	Poly-trauma	2
70	PN071	M	26	Poly-trauma	7
71	PN072	M	67	Respiratory failure	10
72	PN073	M	59	Other	19
73	PN074	F	51	Stroke/brain injury/seizures	17
74	PN075	M	75	Stroke/brain injury/seizures	20
75	PN076	M	49	Respiratory failure	16
76	PN077	M	70	Stroke/brain injury/seizures	18
77	PN078	M	75	Respiratory failure	24
78	PN079	F	51	Respiratory failure	-
79	PN080	F	71	Respiratory failure	6
80	PN081	M	74	Other	18
81	PN082	F	45	Stroke/brain injury/seizures	1
82	PN083	M	47	Respiratory failure	1
83	PN084	M	58	Poly-trauma	17
84	PN085	F	75	Respiratory failure	14
85	PN086	M	74	Stroke/brain injury/seizures	5
86	PN087	M	80	OOHCA	11
87	PN088	F	40	Dental/Oral cavity	22

88	PN089	M	38	Poly-trauma	15
89	PN090	M	66	Respiratory failure	17
90	PN092	M	55	Stroke/brain injury/seizures	13
91	PN093	F	42	Stroke/brain injury/seizures	7
92	PN094	M	60	Respiratory failure	13
93	PN095	M	41	Stroke/brain injury/seizures	9
94	PN096	F	27	Other	1
95	PN097	F	77	Poly-trauma	22
96	PN099	M	58	Other	9
97	PN100	M	26	Overdose/suicide attempt	6
98	PN101	M	29	Respiratory failure	25
99	PN102	M	30	Respiratory failure	8
100	PN103	F	80	Respiratory failure	-
101	PN104	M	56	OOHCA	18
102	PN105	M	67	OOHCA	14
103	PN106	F	60	Respiratory failure	19
104	PN107	F	47	Stroke/brain injury/seizures	12
105	PN108	M	74	OOHCA	-
106	PN109	M		Respiratory failure	7
107	PN110	M	60	Other	

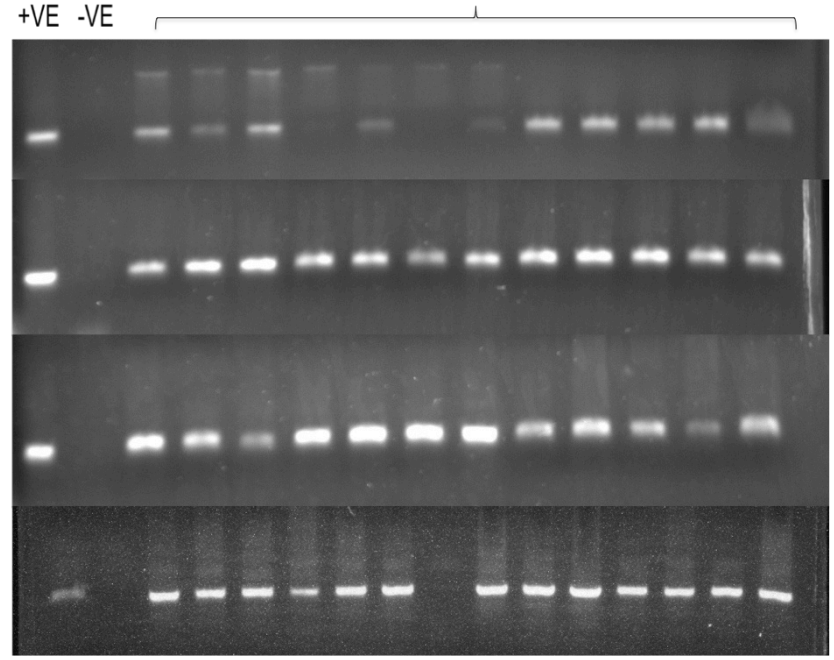
Individual patient dental plaque microbial culture results:

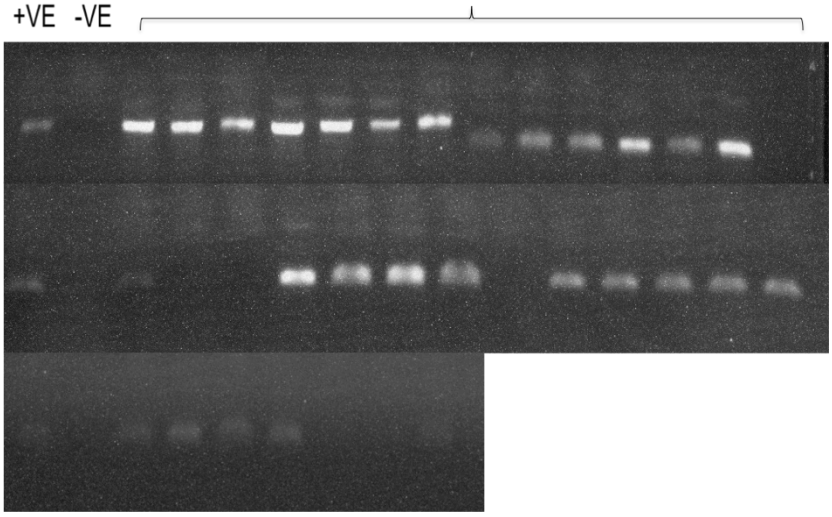
PN	Dental Plaque			
	S.aureus	*S. mutans*	*C.ablicans*	*P.aeruginosa*
1	No	Yes	Yes	No
2	No	No	Yes	No
3	No	Yes	Yes	No
4	No	Yes	Yes	No
5	Yes	Yes	Yes	No
6	No	Yes	Yes	Yes
7	Yes	Yes	Yes	Yes
8	Yes	No	Yes	Yes
9	Yes	Yes	Yes	No
10	No	Yes	No	No
11	No	Yes	Yes	No
12	Yes	Yes	Yes	No
13	~~Yes~~	Yes	No	No
14	No	Yes	Yes	No
15	Yes	Yes	Yes	No
16	No	No	No	No
17	No	Yes	Yes	No
18	~~Yes~~	No	Yes	Yes
19	No	Yes	Yes	No
20	No	Yes	Yes	No
21	Yes	Yes	No	No
22	No	Yes	No	No
23	No	No	No	No
24	Yes	Yes	Yes	No
25	Yes	Yes	No	No
26	No	No	Yes	No
27	Yes	Yes	Yes	No
28	Yes	Yes	Yes	No
29	No	Yes	No	No
30	No	Yes	Yes	Yes
31	No	Yes	Yes	No
32	No	No	Yes	No
33	No	No	Yes	No
34	No	Yes	Yes	No
35	No	Yes	Yes	No
36	No	No	No	No
37	Yes	Yes	Yes	No
38	No	No	Yes	Yes

39	No	No	Yes	No
40	No	No	Yes	No
41	No	No	Yes	No
42	Yes	Yes	Yes	No
43	Yes	No	No	No
44	EXEMPT			
45	Yes	Yes	Yes	No
46	Yes	No	Yes	No
47	No	No	Yes	Yes
48	Yes	No	Yes	No
49	Yes	Yes	Yes	Yes
50	Yes	No	Yes	Yes
51	Yes	Yes	Yes	Yes
52	Yes	No	Yes	No
53	No	No	Yes	No
54	Yes	Yes	No	No
55	No	Yes	Yes	No
56	Yes	No	Yes	No
57	No	Yes	Yes	No
58	No	No	Yes	No
59	No	No	Yes	No
60	No	Yes	Yes	Yes
61	No	No	No	No
62	No	No	No	Yes
63	No	No	No	No
64	Yes	Yes	Yes	No
65	No	No	Yes	No
66	No	No	No	No
67	Yes	No	Yes	No
68	Yes	No	Yes	No
69	No	No	No	No
70	No	No	Yes	No
71	No	Yes	Yes	No
72	No	No	Yes	No
73	No	Yes	Yes	No
74	No	No	Yes	Yes
75	No	No	Yes	No
76	No	No	Yes	No
77	Yes	No	Yes	No
78	No	No	Yes	No
79	No	No	Yes	Yes
80	Yes	No	Yes	No
81	Yes	Yes	Yes	No
82	No	No	Yes	No
83	No	Yes	Yes	Yes
84	No	Yes	Yes	No

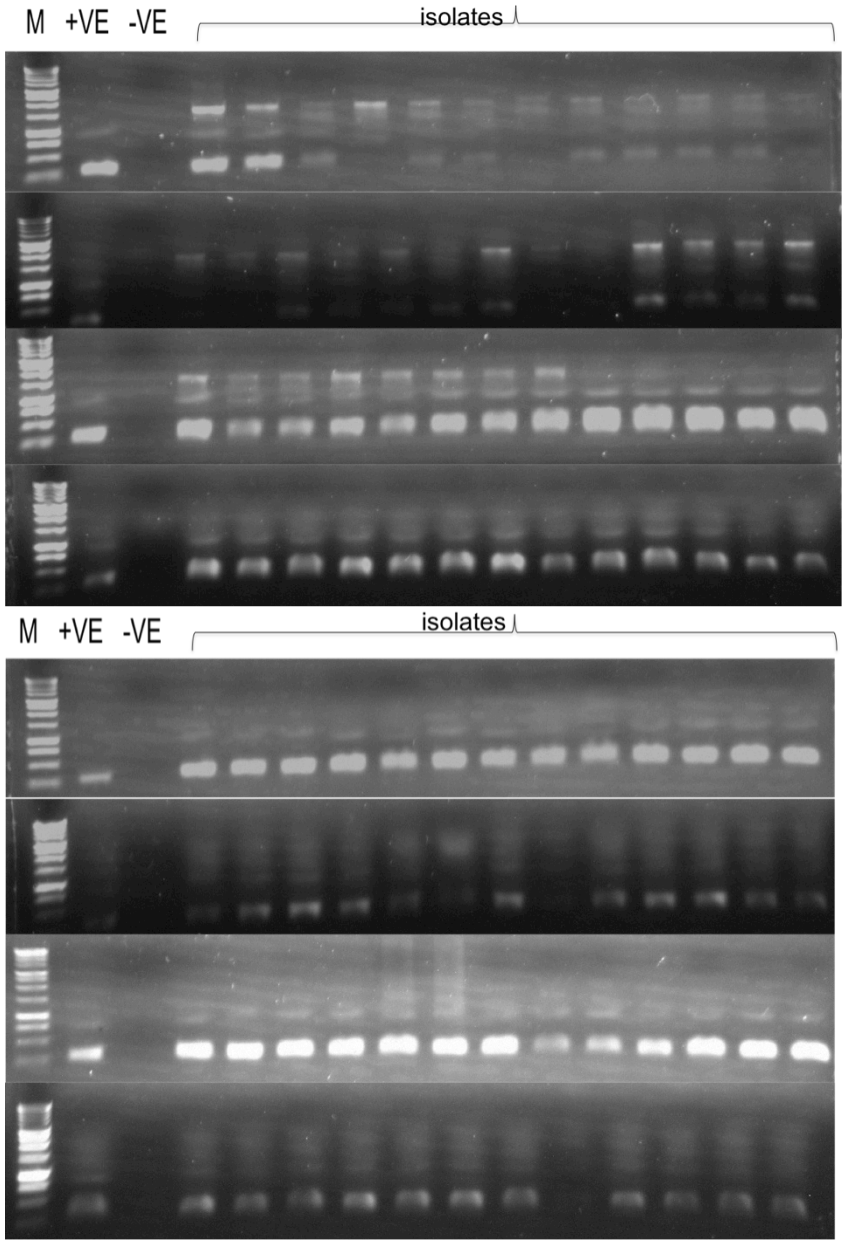
85	No	No	Yes	No
86	No	No	Yes	No
87	Yes	No	Yes	No
88	No	No	No	No
89	Yes	Yes	Yes	No
90	~~Yes~~	No	Yes	No
91				
92	Yes	No	Yes	No
93	No	No	Yes	No
94	Yes	Yes	Yes	No
95	Yes	No	Yes	Yes
96	No	No	No	No
97	No	Yes	No	No
98	WITHDRAW			
99	Yes	No	Yes	No
100	No	No	Yes	No
101	No	Yes	Yes	No
102	Yes	No	Yes	Yes
103	No	No	Yes	No
104	Yes	Yes	Yes	Yes
105	Yes	No	No	Yes
106	Yes	No	Yes	Yes
107	Yes	Yes	No	Yes
108	Yes	No	Yes	No
109	Yes	No	Yes	Yes
110	Yes	No	No	Yes

Pseudomonas aeruginosa isolates:





Staphylococcus aureus isolates:



Pseudomonas aeruginosa isolate sensitivities:

Patient		Antibiotics						
Patient	**Sample**	**Ceftazidime**	**Ciprofloxacin**	**Gentamicin**	**Meropenem**	**Piperacillin**	**Piperacillin-Tazobactam**	**Tobramycin**
PN006	DP	22	30	20	27	24	27	19
PN007	DP	22	30	20	28	24	27	22
PN008	DP	24	30	26	28	24	27	21
PN018	DP	22	30	19	27	24	28	21
PN018	NBL	23	30	18	32	25	27	20
PN030	DP	19	30	19	28	23	27	20
PN030	NBL	20	30	19	29	24	27	20
PN030	SUB	21	29	20	30	24	27	19
PN030	NBL	22	29	20	30	24	29	19
PN030	ETT	23	30	18	32	20	26	20
PN030	DP	19	31	19	31	18	26	20
PN038	DP	22	30	24	0	18	20	20
PN045	NBL	22	30	22	0	17	21	22
PN047	DP	22	29	19	20	21	26	24
PN047	DP	19	28	21	27	22	29	22
PN047	DP	22	30	19	27	23	26	19
PN047	DP	21	30	20	27	19	27	20
PN048	NBL	22	30	20	28	23	27	21
PN049	DP	21	30	19	28	17	18	19
PN049	DP	22	30	18	18	23	26	21
PN051	DP	21	30	20	27	23	28	21
PN060	DP	23	30	21	27	21	25	22
PN060	DP	22	30	21	18	14	19	21
PN060	DP	21	30	19	29	21	28	19
PN062	DP	22	31	21	29	22	27	19
PN062	NBL	22	28	21	28	22	27	19
PN062	DP	22	30	20	0	23	25	20
PN075	DP	20	30	20	29	21	26	19
PN078	NBL	21	30	20	28	20	28	20
PN079	SUB	22	30	18	30	21	26	20
PN079	DP	22	30	21	28	0	14	19
PN079	NBL	23	30	21	29	19	28	22
PN079	DP	22	27	19	6	11	15	21
PN079	DP	22	27	19	0	12	17	21
PN081	DP	23	30	20	0	21	26	20
PN083	NBL	23	30	21	10	19	27	21
PN095	SUB	22	29	20	29	20	29	19
PN095	DP	20	29	21	29	21	28	22
PN095	ETT	22	29	21	29	19	29	19
PN095	DP	22	30	21	0	18	29	18
PN095	NBL	21	30	18	10	22	29	19

PN095	SUB	21	30	23	28	23	27	20
PN102	DP	21	30	20	30	24	28	20
PN104	DP	20	30	19	30	21	28	20
PN105	ETT	24	30	19	28	24	27	21
PN105	NBL	25	30	19	29	24	29	21
PN105	DP	21	29	21	29	21	28	21
PN106	DP	19	29	21	27	23	27	19
PN107	DP	22	30	21	30	22	28	20
PN108	ETT	16	7	17	19	11	19	14
PN109	DP	20	30	21	31	23	28	20
PN109	DP	23	30	21	27	23	29	20
PN109	DP	22	29	21	29	25	28	19
PN110	DP	22	28	20	28	21	25	20
PN110	NBL	23	27	21	28	22	26	19
PN110	ETT	22	30	21	29	22	26	21
							Resistant	

Staphylococcus aureus isolate sensitivities:

Patient		Antibiotic					
Patient	**Sample**	**Cefepime**	**Cefoxitin**	**Ceftazidime**	**Ciprofloxacin**	**Clindamycin**	**Erythromycin**
PN004	SUB	25	0	20	35	0	0
PN005	DP	28	29	16	28	0	0
PN005	DP	27	29	19	28	0	0
PN007	DP	27	30	20	20	27	26
PN007	DP	28	29	18	21	36	25
PN007	DP	24	28	18	21	22	26
PN007	DP	27	0	28	35	0	0
PN009	DP	24	29	18	27	20	24
PN009	NBL	22	28	17	24	30	24
PN009	DP	23	29	16	22	30	18
PN015	DP	28	0	27	35	0	0
PN021	ETT	26	31	14	21	29	21
PN021	DP	27	29	17	20	30	24
PN021	NBL	28	30	18	20	30	25
PN021	DP	24	29	17	22	28	21
PN024	SUB	26	30	19	21	28	26
PN024	NBL	25	29	19	22	27	26
PN024	ETT	24	29	18	22	27	23
PN024	DP	23	30	19	26	30	23
PN024	NBL	27	28	16	28	29	22
PN025	DP	25	30	17	27	28	24
PN025	NBL	27	30	18	28	30	24
PN025	NBL	27	29	17	27	28	25
PN025	ETT	26	29	18	26	27	25
PN027	NBL	28	29	28	34	29	23
PN027	NBL	25	29	16	0	29	24
PN027	ETT	23	30	18	0	22	0
PN028	ETT	0	9	0	24	23	0
PN028	SUB	0	10	0	12	29	0
PN028	NBL	0	11	0	0	31	0
PN037	DP	24	30	18	26	28	18
PN037	NBL	25	30	19	27	24	27
PN037	NBL	31	30	20	25	28	26
PN037	NBL	24	30	17	24	28	22
PN042	DP	24	30	18	24	28	23
PN042	NBL	24	30	18	25	24	23
PN042	SUB	16	0	0	25	13	26

PN042	ETT	24	30	18	25	28	22
PN043	DP	24	30	18	24	28	22
PN045	DP	22	29	19	23	30	23
PN046	DP	24	30	18	25	28	22
PN046	NBL	24	30	18	25	28	22
PN046	DP	25	30	19	24	28	22
PN046	NBL	27	30	20	27	25	19
PN046	ETT	24	30	18	24	28	22
PN046	DP	22	30	20	23	30	22
PN048	NBL	25	30	18	24	24	18
PN049	DP	22	30	19	25	21	20
PN049	NBL	20	30	20	24	21	23
PN049	DP	25	0	18	11	10	22
PN050	DP	22	30	19	25	28	20
PN050	NBL	22	29	20	24	28	19
PN050	ETT	23	30	20	22	29	19
PN051	DP	23	30	19	0	28	0
PN051	DP	20	30	20	22	21	19
PN052	DP	24	31	18	24	0	0
PN052	NBL	23	30	19	24	30	23
PN052	ETT	21	30	19	22	21	22
PN054	NBL	23	30	20	24	22	23
PN056	DP	23	30	20	22	29	19
PN056	NBL	23	30	20	22	29	19
PN056	DP	19	29	21	22	22	23
PN056	NBL	20	30	19	22	29	23
PN056	SUB	19	30	21	22	21	23
PN056	SUB	19	30	21	23	21	22
PN057	NBL	19	29	20	23	22	22
PN057	ETT	20	30	18	23	22	19
PN058	SUB	19	29	20	21	22	22
PN063	NBL	20	29	18	22	22	19
PN068	DP	21	30	20	0	9	0
PN068	NBL	22	30	20	22	28	0
PN068	DP	22	30	19	21	28	28
PN077	NBL	22	29	19	21	27	22
PN077	DP	22	30	19	26	28	22
PN077	NBL	21	30	20	22	28	22
PN080	SUB	0	14	0	10	28	0
PN080	DP	0	13	0	0	32	0
PN080	DP	0	15	0	0	33	0
PN081	NBL	0	0	0	0	28	23
PN081	SUB	0	0	0	0	32	26
PN081	DP	0	9	0	0	33	29

PN081	DP	0	8	0	0	30	27
PN086	NBL	23	30	19	23	28	22
PN087	ETT	21	30	19	23	28	19
PN087	NBL	25	20	16	21	29	8
PN087	SUB	24	30	19	24	22	0
PN087	DP	23	30	20	22	28	0
PN089	NBL	23	30	20	24	28	22
PN089	SUB	23	29	21	22	21	22
PN089	NBL	25	30	22	23	28	24
PN089	DP	21	30	21	24	28	22
PN092	DP	23	30	22	0	21	23
PN092	NBL	21	30	20	23	21	24
PN094	NBL	24	29	21	22	28	23
PN095	DP	21	29	21	22	0	0
PN095	NBL	29	32	17	22	0	0
PN095	SUB	26	30	14	24	0	0
PN095	DP	22	30	18	24	0	0
PN097	NBL	23	30	21	24	24	23
PN099	DP	20	29	23	24	28	22
PN099	DP	23	30	19	22	27	19
PN102	DP	23	30	19	23	25	22
PN102	DP	20	29	22	21	0	0
PN104	DP	21	30	24	23	28	24
PN104	NBL	21	30	21	23	21	25
PN104	DP	20	30	22	24	21	23
PN105	NBL	20	30	21	20	28	23
PN105	ETT	24	30	22	21	21	24
PN106	ETT	22	30	23	21	28	23
PN108	DP	20	30	22	23	21	23
PN108	NBL	24	30	19	23	28	22
PN108	ETT	21	30	16	23	29	22
PN109	DP	20	30	17	24	24	22
PN110	NBL	0	0	0	10	32	0

Resistant	

Patient		Antibiotic					
Patient	**Sample**	**Fusidic Acid**	**Gentamicin**	**Meropenem**	**Penicillin**	**Tobramycin**	**Vancomycin**
PN004	SUB	0	26	26	0	22	0
PN005	DP	30	24	34	16	22	16
PN005	DP	36	24	38	17	23	16

PN007	DP	30	22	34	40	22	17
PN007	DP	36	22	36	16	22	16
PN007	DP	31	24	32	29	21	16
PN007	DP	24	23	35	0	22	0
PN009	DP	33	23	36	42	20	18
PN009	NBL	33	23	33	33	23	16
PN009	DP	32	22	31	28	22	16
PN015	DP	0	23	36	0	25	0
PN021	ETT	35	24	36	17	21	16
PN021	DP	36	23	39	16	19	16
PN021	NBL	35	22	36	15	23	16
PN021	DP	30	22	32	29	21	17
PN024	SUB	30	23	31	15	21	16
PN024	NBL	30	22	30	17	22	16
PN024	ETT	29	23	33	30	22	18
PN024	DP	31	22	30	39	20	18
PN024	NBL	32	23	33	36	21	19
PN025	DP	30	23	32	30	21	16
PN025	NBL	31	22	31	34	22	17
PN025	NBL	30	22	30	40	22	16
PN025	ETT	32	23	32	29	21	18
PN027	NBL	30	24	31	40	21	17
PN027	NBL	30	22	30	30	23	17
PN027	ETT	30	22	10	0	21	17
PN028	ETT	30	25	0	0	19	14
PN028	SUB	33	22	13	7	22	16
PN028	NBL	30	22	12	0	22	17
PN037	DP	31	25	30	17	20	18
PN037	NBL	30	23	30	17	21	17
PN037	NBL	30	24	30	19	19	18
PN037	NBL	30	24	30	20	20	20
PN042	DP	30	24	30	20	20	20
PN042	NBL	30	24	30	20	19	18
PN042	SUB	30	24	18	0	14	15
PN042	ETT	30	22	30	30	19	19
PN043	DP	30	24	30	18	18	18
PN045	DP	30	22	32	21	22	19
PN046	DP	30	22	30	28	19	19
PN046	NBL	30	22	30	28	19	19
PN046	DP	30	22	30	28	18	14
PN046	NBL	29	22	29	21	22	18
PN046	ETT	28	23	30	28	18	21
PN046	DP	29	0	31	18	20	18
PN048	NBL	30	22	30	28	20	20

PN049	DP	30	19	29	19	22	20
PN049	NBL	29	25	32	29	24	0
PN049	DP	19	20	30	20	14	0
PN050	DP	30	22	30	28	19	20
PN050	NBL	29	19	30	19	20	20
PN050	ETT	29	23	30	28	20	20
PN051	DP	29	19	30	28	24	20
PN051	DP	30	22	29	22	24	21
PN052	DP	29	22	30	28	22	19
PN052	NBL	29	22	30	29	21	4
PN052	ETT	29	22	30	20	20	11
PN054	NBL	30	22	30	21	19	19
PN056	DP	29	22	30	19	22	19
PN056	NBL	30	24	30	29	19	21
PN056	DP	30	18	30	29	21	19
PN056	NBL	30	24	29	28	21	19
PN056	SUB	30	18	30	28	22	20
PN056	SUB	30	18	28	21	21	21
PN057	NBL	30	22	30	19	21	22
PN057	ETT	29	23	30	21	21	21
PN058	SUB	29	18	30	30	22	0
PN063	NBL	29	18	30	30	22	0
PN068	DP	30	20	30	30	20	0
PN068	NBL	26	21	29	28	0	28
PN068	DP	11	21	30	29	21	19
PN077	NBL	29	20	21	22	19	19
PN077	DP	30	20	32	23	20	0
PN077	NBL	30	20	30	29	21	19
PN080	SUB	30	10	20	0	10	21
PN080	DP	30	10	21	0	10	21
PN080	DP	33	11	21	0	0	21
PN081	NBL	10	27	30	0	20	0
PN081	SUB	29	23	7	0	20	9
PN081	DP	31	24	14	0	23	18
PN081	DP	21	24	9	0	22	17
PN086	NBL	29	22	29	21	22	20
PN087	ETT	30	24	29	21	24	20
PN087	NBL	29	22	29	17	23	20
PN087	SUB	30	22	30	28	22	20
PN087	DP	30	22	29	29	21	20
PN089	NBL	30	24	30	29	21	19
PN089	SUB	29	22	30	28	20	21
PN089	NBL	29	23	29	31	20	20
PN089	DP	29	23	29	28	21	19

PN092	DP	30	22	27	24	21	20
PN092	NBL	29	23	29	29	22	20
PN094	NBL	30	22	29	29	21	19
PN095	DP	29	22	29	32	21	20
PN095	NBL	17	25	35	29	22	19
PN095	SUB	19	24	33	29	18	18
PN095	DP	19	23	29	18	21	0
PN097	NBL	30	21	31	31	22	18
PN099	DP	30	23	30	28	21	21
PN099	DP	29	23	30	29	21	18
PN102	DP	29	22	33	29	19	19
PN102	DP	30	22	34	30	21	20
PN104	DP	30	24	30	21	21	17
PN104	NBL	30	24	30	29	22	18
PN104	DP	30	23	30	28	20	21
PN105	NBL	30	23	30	31	22	19
PN105	ETT	30	23	29	29	20	18
PN106	ETT	29	23	30	30	21	19
PN108	DP	30	23	30	28	19	20
PN108	NBL	29	22	29	28	21	20
PN108	ETT	31	24	30	28	20	19
PN109	DP	32	22	32	30	20	17
PN110	NBL	25	12	0	0	12	20

Resistant	

Appendix II

Community profiling data

- Mothur output
- Raw sequencing data

Mothur output:

#Mothur commands
make.contigs(file=Sands3242.files, processors=25)
summary.seqs(fasta=Sands3242.trim.contigs.fasta, processors=25)
screen.seqs(fasta=Sands3242.trim.contigs.fasta, group=Sands3242.contigs.groups,
summary=Sands3242.trim.contigs.summary, maxn=0, maxambig=0, maxhomop=6,
minlength=310, maxlength=339)
unique.seqs(fasta=Sands3242.trim.contigs.good.fasta)
count.seqs(name=Sands3242.trim.contigs.good.names,
group=Sands3242.contigs.good.groups)
align.seqs(fasta=Sands3242.trim.contigs.good.unique.fasta,
reference=../../silva.bacteria.fasta, flip=t, processors=25)
summary.seqs(fasta=Sands3242.trim.contigs.good.unique.align,
count=Sands3242.trim.contigs.good.count_table, processors=25)
screen.seqs(fasta=Sands3242.trim.contigs.good.unique.align,
count=Sands3242.trim.contigs.good.count_table,
summary=Sands3242.trim.contigs.good.unique.summary, start=1044, end=6333,
maxhomop=5)
filter.seqs(fasta=Sands3242.trim.contigs.good.unique.good.align, vertical=T, trump=.)
unique.seqs(fasta=Sands3242.trim.contigs.good.unique.good.filter.fasta,
count=Sands3242.trim.contigs.good.good.count_table)
pre.cluster(fasta=Sands3242.trim.contigs.good.unique.good.filter.unique.fasta,
count=Sands3242.trim.contigs.good.unique.good.filter.count_table, diffs=2, processors=25)
chimera.uchime(fasta=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.fast
a, count=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.count_table,
dereplicate=t)
remove.seqs(fasta=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.fasta,
accnos=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.uchime.accnos)
classify.seqs(fasta=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick.fa
sta,
count=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.count_t
able, reference=../../trainset9_032012.rdp.fasta, taxonomy=../../trainset9_032012.rdp.tax,
cutoff=80, processors=25)
remove.lineage(fasta=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick
.fasta,
count=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.count_t
able,
taxonomy=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick.rdp.wang.t
axonomy, taxon=Chloroplast-Mitochondria-unknown-Archaea-Eukaryota)
cluster.split(fasta=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick
.fasta,
count=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.pick.co
unt_table,
taxonomy=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick.rdp.wang.p
ick.taxonomy, splitmethod=classify, taxlevel=4, cutoff=0.15)
make.shared(list=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick
.an.unique_list.list,
count=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.pick.co
unt_table, label=0.03)
classify.otu(list=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.a
n.unique_list.list,
count=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.uchime.pick.pick.co
unt_table,
taxonomy=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick.rdp.wang.p
ick.taxonomy, label=0.03)

sub.sample(shared=Sands3242.trim.contigs.good.unique.good.filter.unique.precluster.pick.pick.an.unique_list.shared, size=1016)
dist.seqs(fasta=Sands3242.final.fasta, output=phylip, processors=25)
clearcut(phylip=Sands3242.final.phylip.dist)
collect.single(shared=Sands3242_final_subsample.shared, calc=chao-invsimpson-shannon, freq=5)
rarefaction.single(shared=Sands3242_final_subsample.shared, calc=sobs, freq=5)
summary.single(calc=nseqs-sobs-chao-ace-invsimpson-npshannon-coverage)
unifrac.weighted(tree=Sands3242.final.phylip.tre, name=Sands3242.final.names, group=Sands3242.final.groups, distance=square, processors=25, random=F, subsample=1016)
get.oturep(phylip=Sands3242.final.phylip.dist, list=Sands3242.final.list, fasta=Sands3242.final.fasta, label=0.03)

Raw sequencing data (Species level 97%):

Species 97%	1	2	3	4	6	7	8	9	10
Veillonella_parvula	107	9	300	158	537	377	963	44	8
Escherichia/Shigella_flexneri	0	0	0	0	0	0	3	0	0
Streptococcus_pseudopneumoniae	2	0	0	101	12	1	0	155	766
Enterococcus_villorum	623	983	135	0	0	0	0	0	0
Staphylococcus_aureus	11	2	35	0	0	0	0	1	241
Lactobacillus_gasseri	9	0	0	0	0	5	0	0	0
Prevotella_nanceiensis	0	0	0	0	24	0	0	0	0
Granulicatella_adiacens	0	0	26	17	55	3	9	44	0
Prevotella_melaninogenica	3	4	42	27	22	238	0	1	0
Prevotella_oris	0	3	9	72	0	0	0	82	0
Haemophilus_influenzae	3	0	0	0	0	0	0	0	0
Unclassified	4	0	138	59	1	6	4	102	1
Lactobacillus_salivarius	0	0	0	0	0	0	0	0	0
Lactobacillus_rhamnosus	17	0	0	0	106	285	0	1	0
Enterococcus_faecalis	0	1	20	0	0	0	0	0	0
Haemophilus_parainfluenzae	0	0	0	4	0	0	0	0	0
Prevotella_salivae	0	0	33	49	62	41	0	0	0
Parvimonas_micra	0	1	33	195	4	0	0	173	0
Streptococcus_parasanguinis	10	0	26	29	4	1	10	0	0
Neisseria_flavescens	0	0	0	0	0	0	0	0	0
Streptococcus_cristatus	0	0	1	37	26	13	16	159	0
Peptostreptococcus_stomatis	0	0	0	0	0	0	0	22	0
Gemella_haemolysans	0	0	0	3	0	0	0	1	0
Streptococcus_vestibularis	0	0	0	0	7	22	0	0	0
Lactobacillus_crispatus	0	0	0	0	0	0	0	0	0
Streptococcus_gordonii	0	0	0	9	142	12	0	7	0
Streptococcus_infantis	0	0	0	0	0	0	0	0	0
Streptococcus_constellatus	0	2	0	10	0	0	0	17	0
Prevotella_aurantiaca	0	0	0	0	0	0	0	0	0
Streptococcus_anginosus	0	0	15	4	0	0	1	7	0
Solobacterium_moorei	0	4	0	0	0	0	0	2	0
Prevotella_histicola	0	0	0	70	0	2	0	0	0
Megasphaera_micronuciformis	0	0	0	8	0	1	0	5	0
Streptococcus_peroris	0	0	0	6	0	0	0	46	0
Dialister_invisus	0	0	0	7	0	0	0	4	0
Selenomonas_sputigena	0	0	0	1	0	0	0	7	0
Prevotella_denticola	0	0	0	11	0	0	0	9	0
Eikenella_corrodens	0	0	0	1	0	0	0	1	0
Abiotrophia_defectiva	0	0	0	0	0	0	0	0	0
Streptococcus_agalactiae	0	0	0	0	0	0	0	0	0
Selenomonas_infelix	0	0	0	1	0	0	0	0	0
Aggregatibacter_segnis	0	0	0	0	0	0	0	4	0
Aggregatibacter_aphrophilus	0	0	0	0	0	0	0	0	0
Streptococcus_sanguinis	0	0	0	0	1	0	0	0	0
Prevotella_tannerae	0	0	0	0	0	0	0	25	0
Actinomyces_meyeri	6	0	0	6	0	0	2	3	0
Lactobacillus_paracasei	0	0	0	0	4	4	0	0	0
Granulicatella_elegans	0	0	0	0	0	0	0	0	0
Prevotella_loescheii	0	0	0	3	0	0	0	6	0
Oribacterium_sinus	0	0	0	3	0	0	0	2	0
Finegoldia_magna	0	0	0	0	0	0	0	0	0
Gemella_morbillorum	0	0	0	0	0	0	0	1	0
Dialister_pneumosintes	0	0	45	5	0	0	0	4	0
Prevotella_nigrescens	0	0	0	0	0	0	0	0	0
Serratia_nematodiphila	214	0	30	0	0	0	0	1	0
Selenomonas_artemidis	0	0	0	2	0	0	0	0	0
Clostridium_bolteae	0	0	0	0	0	0	0	0	0
Anaeroglobus_geminatus	0	0	0	1	0	0	0	0	0
Tannerella_forsythia	2	0	41	0	0	0	0	0	0
Streptococcus_lactarius	0	0	0	0	0	0	0	0	0

Streptococcus_oralis	0	0	0	0	0	0	0	0	0
Staphylococcus_devriesei	0	0	0	0	0	0	0	0	0
Neisseria_bacilliformis	0	0	0	2	0	0	0	0	0
Selenomonas_noxia	0	0	0	16	0	0	0	0	0
Actinomyces_viscosus	0	0	1	2	1	0	0	0	0
Streptococcus_mutans	0	0	0	6	0	2	0	0	0
Stenotrophomonas_pavanii	0	0	0	0	0	0	0	0	0
Prevotella_oulorum	0	0	0	2	0	0	0	0	0
Catonella_morbi	0	0	0	4	0	0	0	4	0
Kingella_oralis	0	0	0	0	0	0	0	1	0
Bacteroides_dorei	0	0	0	0	0	0	0	0	0
Filifactor_alocis	0	0	0	11	0	0	0	18	0
Prevotella_veroralis	0	0	0	1	0	0	0	2	0
Robinsoniella_peoriensis	0	0	0	0	0	0	0	0	0
Prevotella_maculosa	0	0	0	1	0	0	0	0	0
Prevotella_pleuritidis	0	0	45	4	0	0	0	11	0
Treponema_denticola	0	0	0	0	0	0	0	4	0
Eubacterium_yurii	0	0	0	0	0	0	0	0	0
Prevotella_intermedia	0	0	0	24	0	0	0	0	0
Eubacterium_saphenum	0	0	0	0	0	0	0	1	0
Gemella_asaccharolytica	0	0	0	0	0	0	0	0	0
Gemella_sanguinis	0	0	0	0	0	0	0	0	0
Prevotella_zoogleoformans	0	0	0	0	0	0	0	0	0
Prevotella_oralis	0	0	7	0	0	0	0	1	0
Bacillus_niacini	0	0	9	0	0	0	0	0	0
Eubacterium_infirmum	0	0	0	0	0	0	0	11	0
Treponema_maltophilum	0	0	0	0	0	0	0	1	0
Phocaeicola_abscessus	0	0	0	0	0	0	0	0	0
Actinomyces_dentalis	0	0	0	0	0	0	0	0	0
Porphyromonas_gingivalis	0	0	0	0	0	0	0	0	0
Treponema_amylovorum	0	0	0	0	0	0	0	16	0

Appendix III

Additional information: Salivary analysis during mechanical ventilation

- Ethics approval documents for saliva collection from healthy volunteers
- Full breakdown of salivary parameters for all patients
- Protein identification from healthy saliva
- Protein identification from patient saliva
- Cytokine quantification from saliva, ETT fluid and plasma

Ethical approval documents for saliva collection from healthy volunteers:

(Version 1, 20th March 2014)

CONSENT FORM

COLLECTION OF SALIVA FOR PROTEOMIC RESEARCH PURPOSES

Name of Researchers: Kirsty M Sands, Professor David W Williams, Dr Melanie J Wilson, Dr Matthew P Wise and Professor Michael AO Lewis

Please initial

1. I confirm that I have read and understood the study information sheet and have had an opportunity to ask any questions and had sufficient time to come to my decision. _____

2. I give permission that the saliva be collected and stored within the Cardiff School of Dentistry only for use in salivary proteomics in this study and then discarded via autoclaving. _____

3. I understand that my participation is voluntary and that I am free to withdraw at any time prior to my samples being used in the study without giving any reason, without my medical care or legal rights being affected. _____

4. I understand that results from this study may be used in medical/scientific journals and/or publications, but donated saliva will not be identifiable. _____

5. I understand that the sample is given as a "gift" and that I will have no right to a share of any profits which might arise from research using it (e.g. as part of a new medical treatment or test). _____

_____	_____	_____
Name of Cardiff University volunteer	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

Ethical approval documents for saliva collection from healthy volunteers

Version 2, 14th April 2014

Information Sheet

Cardiff School of Dentistry

Collection of saliva for proteomic research purposes

Study title
Collection of saliva for proteomic research purposes

Invitation

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully. Please feel free to ask us any questions if anything is unclear, in order to help you decide whether you wish to take part.

1. What is the purpose of the salivary proteomic study?

Saliva contains hundreds of proteins, several of which contain antimicrobial peptides. In critically ill patients, it is accepted that salivary flow may be reduced as a direct consequence of mechanical ventilation (MV) *i.e.* a direct result from the apparatus leading to the patient's mouth remaining open. Understanding the salivary flow, pH, and composition of proteins in critically ill patients can provide an insight into the involvement salivary peptides can have in dental plaque biofilm formation. This may be important when dental plaque changes are associated with those bacteria that can cause pneumonia. If any changes in the salivary protein composition are established, these proteins can then be targeted for specific and quantitative analysis. Healthy volunteers will be used to provide comparable data to critically ill patients.

This research project has been considered and accepted by the Research Ethics Committee within the School of Dentistry.

2. Why have I been chosen

You have been chosen as you meet the required inclusion/exclusion criteria and are deemed able to readily donate a small volume of saliva. A total of 5 individuals will contribute to this study.

3. **Do I have to take part?**

There is no obligation for you to take part in this study

4. **How can I contribute and what will happen if I take part?**

If you decide to contribute you will be requested to chew gently on a cotton swab specifically designed to collect saliva, a total of 5 samples will be collected over a period of 14 days. Your saliva will be stored in the -80°C until processed for the extracellular proteins. Your name will not be written on the microcentrifuge tube, or used at any time throughout the analysis. A number will be generated and used from point of collection, analysis and until saliva discarded. The saliva sample will be subject to a protein assay and analysis via SDS-PAGE. Any remaining sample will be discarded via autoclaving at 121°C for 50 min. You will not be contacted after regarding the results of the sampling, and no results will affect the future health/knowledge of the individual. No genetic testing will be carried out on any samples.

If at any stage you choose to withdraw consent, no future contact will be necessary. Any samples already collected to that date would have been used in proteomic analysis (pooling of samples at time of collection).
Samples that have been processed and used will not be able to be removed from the study and will continue to remain part of the study results.

Any complaints can be made via contact with Dr Fiona Gagg – the Laboratory Manager.

5. **What about confidentiality?**

Your sample will be collected in a numbered receptacle and will be pseudoanonymised to the research team. Prior to use your saliva will be pooled with the saliva collected from other volunteering individuals. Your identity will not be disclosed in any results or publications arising from this study.

6. **What do I have to do?**

You will be requested to chew gently on a cotton swab specifically designed to collect saliva. This cotton swab will then be returned to the researcher for saliva collection.

7. **Are there any risks?**

There are no obvious risks to you in taking part in this study.

8. **What are the benefits of taking part and what will happen to the results?**

Whilst there is no personal benefit from taking part in this study, participation will provide healthy volunteer control data for extracellular human salivary proteins. The control data can then be referred to in comparison from data obtained from critically ill patients. The results of the study will be used to compare data findings from a patient study.

9. Who is organising and funding the research?

The research is being organised by Miss Kirsty Sands and Professor David Williams (School of Dentistry, Cardiff University). The funder of the study is GlaxoSmithKline (as a result of a small grant success).

10. Contact for Further Information

Further information on this study can be obtained from Miss Kirsty Sands, PhD Student Sandsk1@cardiff.ac.uk

[1] **Version 2, April 14th, 2014**

Full breakdown of salivary volume for all patients:

PN	Sample date	Saliva volume (ml)	Time collection (s)	protein conc (mg/ml)	Saliva flow (ml/min)	protein content per volume collected (mg)
PN002	30/07/13	0.3	45 s	28.5	0.40	8.55
PN002	05/08/13	0.02	45 s	16.8	0.03	0.34
PN003	02/08/13	0.5	45 s	7.95	0.67	3.98
PN003	06/08/13	0.1	45 s	4.40	0.13	0.44
PN003	08/08/13	0.1	45 s	3.60	0.13	0.36
PN004	04/08/13	1.7	45 s	7.60	2.27	12.92
PN004	09/08/13	0.04	45 s	5.25	0.05	0.21
PN004	10/08/13	0.01	45 s	5.10	0.01	0.05
PN004	17/08/13	0.45	45 s	4.50	0.60	2.03
PN004	23/08/13	0.7	45 s	2.40	0.93	1.68
PN004	26/08/13	0.72	45 s	2.50	0.96	1.80
PN004	31/08/13	0.4	45 s	1.05	0.53	0.42
PN004	16/05/14	0.35	45 s	10.95	0.47	3.83
PN004	20/05/14	0.05	45 s	3.15	0.07	0.16
PN005	17/08/13	0.1	45 s	11.55	0.13	1.16
PN005	19/08/13	0.5	45 s	6.3	0.67	3.15
PN006	17/08/13	0.01	45 s	18	0.01	0.18
PN006	04/10/13	0.06	45 s	4.05	0.08	0.24
PN007	20/08/13	0.4	45 s	7.65	0.53	3.06
PN007	22/09/13	0.2	45 s	0.9	0.27	0.18
PN008	20/08/13	0.05	45 s	6.45	0.07	0.32
PN008	29/08/13	0.4	45 s	4.4	0.53	1.76
PN010	25/08/13	1	45 s	3.1	1.33	3.10
PN010	11/09/13	0.2	45 s	2.2	0.27	0.44
PN012	30/08/13	0.35	45 s	17.25	0.47	6.04
PN012	02/09/13	0.05	45 s	6	0.07	0.30
PN012	09/09/13	0.3	45 s	16.5	0.40	4.95
PN013	26/08/13	0.01	45 s	29.25	0.01	0.29
PN013	02/09/13	0.3	45 s	37.50	0.40	11.25
PN014	01/09/13	0.2	45 s	45.00	0.27	9.00
PN015	28/08/13	0.065	45 s	16.5	0.09	1.07
PN015	30/08/13	0.15	45 s	18.6	0.20	2.79
PN016	03/09/13	0.44	45 s	9.90	0.59	4.36
PN016	11/09/13	0.1	45 s	2.00	0.13	0.20
PN017	04/10/13	0.15	45 s	4.65	0.20	0.70
PN018	03/10/13	0.7	45 s	14.19	0.93	9.93
PN019	16/10/13	0.18	45 s	19.95	0.24	3.59
PN020	08/10/13	0.15	45 s	18.75	0.20	2.81
PN021	07/10/13	0.40	45 s	9.3	0.53	3.72

PN021	09/10/13	0.05	45 s	8.4	0.07	0.42
PN022	09/10/13	0.50	45 s	11.4	0.67	5.70
PN022	12/10/13	1.10	45 s	9.6	1.47	10.56
PN023	13/10/13	0.03	45 s	5.25	0.04	0.16
PN024	12/10/13	0.9	45 s	10.05	1.20	9.05
PN025	19/10/13	0.05	45 s	15.9	0.07	0.80
PN028	31/10/13	0.9	45 s	15.23	1.20	13.71
PN028	03/11/13	0.1	45 s	10	0.13	1.00
PN028	10/11/13	0.9	45 s	5.9	1.20	5.31
PN033	12/11/13	0.05	45 s	15.90	0.07	0.80
PN033	17/11/13	0.25	45 s	6.60	0.33	1.65
PN035	15/11/13	0.05	45 s	9.30	0.07	0.47
PN036	21/11/13	1	45 s	5.55	1.33	5.55
PN037	05/01/14	0.02	45 s	20.7	0.03	0.41
PN037	25/01/14	0.4	45 s	5.7	0.53	2.28
PN038	13/01/14	0.1	45 s	5.7	0.13	0.57
PN039	29/01/14	0.12	45 s	2.70	0.16	0.32
PN040	14/01/14	0.26	45 s	18.00	0.35	4.68
PN040	17/01/14	0.3	45 s	7.35	0.40	2.21
PN041	17/01/14	1.3	45 s	3.00	1.73	3.90
PN042	28/01/14	0.08	45 s	6.8	0.11	0.54
PN042	04/02/14	0.12	45 s	3.3	0.16	0.40
PN043	17/01/14	0.04	45 s	10.2	0.05	0.41
PN043	20/01/14	0.15	45 s	5.4	0.20	0.81
PN045	03/02/14	0.045	45 s	4.8	0.06	0.22
PN045	15/02/14	0.28	45 s	4.625	0.37	1.30
PN046	06/02/14	0.35	45 s	30	0.47	10.50
PN046	08/02/14	0.15	45 s	11.55	0.20	1.73
PN046	18/02/14	0.035	45 s	37.125	0.05	1.30
PN046	03/03/14	0.35	45 s	3.75	0.47	1.31
PN046	18/03/14	0.05	45 s	33	0.07	1.65
PN046	25/03/14	0.075	45 s	28.5	0.10	2.14
PN047	11/02/14	0.25	45 s	20.55	0.33	5.14
PN048	11/02/14	1.4	45 s	5.7	1.87	7.98
PN050	18/02/14	0.1	45 s	20.25	0.13	2.03
PN051	19/02/14	0.02	45 s	30	0.03	0.60
PN052	23/02/14	0.01	45 s	16.5	0.01	0.17
PN052	27/02/14	1.2	45 s	12	1.60	14.40
PN054	26/02/14	0.35	45 s	5.1	0.47	1.79
PN054	28/02/14	0.035	45 s	24	0.05	0.84
PN054	11/04/14	0.065	45 s	1.65	0.09	0.11
PN055	01/03/14	0.05	45 s	16.5	0.07	0.83
PN057	05/03/14	0.1	45 s	9.3	0.13	0.93
PN058	10/03/14	0.33	45 s	4.2	0.44	1.39

PN060	12/03/14	0.08	45 s	18	0.11	1.44
PN060	20/03/14	0.075	45 s	18.3	0.10	1.37
PN060	27/03/14	1.08	45 s	25.05	1.44	27.05
PN061	14/03/14	0.12	45 s	17.7	0.16	2.12
PN062	17/03/14	0.04	45 s	18.45	0.05	0.74
PN064	28/03/14	0.04	45 s	3.9	0.05	0.16
PN066	10/04/14	0.05	45 s	18.15	0.07	0.91
PN068	14/04/14	0.13	45 s	11.55	0.17	1.50
PN070	24/04/14	0.05	45 s	4.5	0.07	0.23
PN075	07/05/14	0.2	45 s	4.8	0.27	0.96
PN075	09/05/14	1.5	45 s	4.8	2.00	7.20
PN077	06/05/14	0.3	45 s	3.9	0.40	1.17
PN079	21/05/14	0.08	45 s	20.25	0.11	1.62
PN082	29/05/14	0.2	45 s	5.55	0.27	1.11
PN082	31/05/14	0.45	45 s	9.9	0.60	4.46
PN083	26/05/14	0.25	45 s	12.45	0.33	3.11
PN083	29/05/14	0.1	45 s	4.2	0.13	0.42
PN086	05/06/14	0.05	45 s	8.7	0.07	0.44
PN089	09/06/14	0.03	45 s	10.5	0.04	0.32
PN093	25/06/14	0.25	45 s	20.25	0.33	5.06
PN094	05/07/14	0.1	45 s	14.85	0.13	1.49
PN094	30/07/14	0.1	45 s	9.75	0.13	0.98
PN095	13/07/14	0.1	45 s	36	0.13	3.60
PN097	10/07/14	0.03	45 s	24	0.04	0.72
PN097	13/07/14	0.02	45 s	18	0.03	0.36
PN099	10/07/14	1.85	45 s	19.65	2.47	36.35
PN099	12/07/14	0.4	45 s	2.85	0.53	1.14
PN099	14/07/14	0.7	45 s	4.5	0.93	3.15
PN099	21/07/14	1.6	45 s	9.75	2.13	15.60
PN099	04/08/14	0.05	45 s	6.6	0.07	0.33
PN102	25/07/14	0.1	45 s	2.85	0.13	0.29
PN102	03/08/14	0.2	45 s	2.1	0.27	0.42
PN104	18/07/14	0.4	45 s	25.05	0.53	10.02
PN105	21/07/14	0.08	45 s	25.5	0.11	2.04
PN106	19/07/14	0.1	45 s	25.35	0.13	2.54
PN106	15/08/14	0.1	45 s	2.7	0.13	0.27
PN107	27/07/14	0.2	45 s	17.25	0.27	3.45
PN107	30/07/14	0.1	45 s	16.5	0.13	1.65
PN107	08/08/14	0.1	45 s	24.9	0.13	2.49
PN108	31/07/14	0.4	45 s	1.8	0.53	0.72
PN109	02/08/14	0.5	45 s	1.8	0.67	0.90
PN109	05/08/14	0.5	45 s	1.7	0.67	0.85
PN109	07/08/14	0.1	45 s	1.6	0.13	0.16

Protein identification from healthy saliva:

Peptide no'	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count
1	Serum albumin	ALBU_HUMAN	70931.0625	5.92	23
2	Alpha-2-macroglobulin-like protein 1	A2ML1_HUMAN	162153.75	5.5	21
3	Serotransferrin	TRFE_HUMAN	78853.14063	6.81	15
4	Polymeric immunoglobulin receptor	PIGR_HUMAN	84197.39063	5.58	14
5	Heat shock 70 kDa protein 1A/1	HSP71_HUMAN	70238.96875	5.48	13
6	Complement C3	CO3_HUMAN	188271.5469	6.02	13
7	Alpha-enolase	ENOA_HUMAN	47415.23828	7.01	13
8	Alpha-amylase 2B	AMY2B_HUMAN	58178.87109	6.64	12
9	Actin, cytoplasmic 2	ACTG_HUMAN	42041.71875	5.31	12
10	Mucin-5B	MUC5B_HUMAN	608560.75	6.2	12
11	Ig alpha-1 chain C region	IGHA1_HUMAN	38320.44922	6.08	11
12	Keratin, type II cytoskeletal 1	K2C1_HUMAN	66136.96094	8.15	11
13	Ig alpha-2 chain C region	IGHA2_HUMAN	37146.82813	5.71	10
14	Fructose-bisphosphate aldolase A	ALDOA_HUMAN	39763.21094	8.3	10
15	14-3-3 protein sigma	1433S_HUMAN	27848.66992	4.68	10
16	Involucrin	INVO_HUMAN	68529.17969	4.62	9
17	Heat shock cognate 71 kDa protein	HSP7C_HUMAN	71038.17188	5.37	9
18	Ezrin	EZRI_HUMAN	69461.71094	5.94	9
19	Protein disulfide-isomerase	PDIA1_HUMAN	57402.58984	4.76	9
20	Pyruvate kinase PKM	KPYM_HUMAN	58359.89844	7.96	9
21	Phosphoglycerate kinase 1	PGK1_HUMAN	44908.03906	8.3	9
22	Lactotransferrin	TRFL_HUMAN	79649.50781	8.5	8
23	Zinc-alpha-2-glycoprotein	ZA2G_HUMAN	34421.05078	5.71	7
24	6-phosphogluconate dehydrogenase, decarboxylating	6PGD_HUMAN	53519.83984	6.8	7
25	14-3-3 protein zeta/delta	1433Z_HUMAN	27865.68945	4.73	7
26	Small proline-rich protein 3	SPRR3_HUMAN	18510.13086	8.86	7
27	Peroxiredoxin-1	PRDX1_HUMAN	22280.23047	8.27	6
28	Heat shock 70 kDa protein 6	HSP76_HUMAN	71352.13281	5.81	6
29	Cystatin-A	CYTA_HUMAN	10999.66016	5.38	6
30	Leukocyte elastase inhibitor	ILEU_HUMAN	42806.67969	5.9	6
31	Moesin	MOES_HUMAN	67869.75781	6.08	6
32	Gelsolin	GELS_HUMAN	85966.10156	5.9	6
33	Transaldolase	TALDO_HUMAN	37654.42188	6.36	6
34	Haptoglobin	HPT_HUMAN	45728.42188	6.13	6
35	Cystatin-S	CYTS_HUMAN	16433.90039	4.95	5
36	Thioredoxin	THIO_HUMAN	11959.67969	4.82	5
37	Profilin-1	PROF1_HUMAN	15182.51953	8.44	5
38	Ig gamma-3 chain C region	IGHG3_HUMAN	42088.14063	8.23	5
39	Immunoglobulin lambda-like polypeptide 5	IGLL5_HUMAN	23324.56055	9.08	5
40	Triosephosphate isomerase	TPIS_HUMAN	31001.63086	5.65	5

41	Immunoglobulin J chain	IGJ_HUMAN	18454.90039	5.12	5
42	Prolactin-inducible protein	PIP_HUMAN	16791.73047	8.26	5
43	Macrophage-capping protein	CAPG_HUMAN	38704.41016	5.82	5
44	Fatty acid-binding protein, epidermal	FABP5_HUMAN	15430.48047	6.6	5
45	78 kDa glucose-regulated protein	GRP78_HUMAN	72380.40625	5.07	5
46	Transketolase	TKT_HUMAN	68386.57813	7.58	5
47	Serpin B3	SPB3_HUMAN	44582.53125	6.35	5
48	Coronin-1A	COR1A_HUMAN	51545.64844	6.25	5
49	Cystatin-SN	CYTN_HUMAN	16561.28906	6.73	4
50	Heme-binding protein 2	HEBP2_HUMAN	22861.18945	4.58	4
51	Cystatin-SA	CYTT_HUMAN	16664.15039	4.85	4
52	Ig lambda-3 chain C regions	LAC3_HUMAN	11368.46973	6.92	4
53	14-3-3 protein beta/alpha	1433B_HUMAN	28156.81055	4.76	4
54	Ig gamma-4 chain C region	IGHG4_HUMAN	36331.75	7.18	4
55	Ig gamma-2 chain C region	IGHG2_HUMAN	36383.62109	7.66	4
56	Alpha-1-antitrypsin	A1AT_HUMAN	46844.98047	5.37	4
57	Glutathione S-transferase P	GSTP1_HUMAN	23524.9707	5.43	4
58	Keratin, type I cytoskeletal 10	K1C10_HUMAN	58975.64063	5.13	4
59	Ig mu chain C region	IGHM_HUMAN	49827.37891	6.35	4
60	UPF0762 protein C6orf58	CF058_HUMAN	38177.48047	5.78	4
61	Kallikrein-1	KLK1_HUMAN	29376.74023	4.68	4
62	Peptidyl-prolyl cis-trans isomerase A	PPIA_HUMAN	18184.83984	7.68	4
63	Desmoglein-1	DSG1_HUMAN	114503.6406	4.9	4
64	14-3-3 protein theta	1433T_HUMAN	27976.69922	4.68	4
65	L-lactate dehydrogenase A chain	LDHA_HUMAN	36895.30078	8.44	4
66	Phosphatidylethanolamine-binding protein 1	PEBP1_HUMAN	21135.64063	7.01	4
67	Aldehyde dehydrogenase, dimeric NADP-preferring	AL3A1_HUMAN	50684.76172	6.11	4
68	Protein DJ-1	PARK7_HUMAN	20016.44922	6.33	4
69	Peroxiredoxin-6	PRDX6_HUMAN	25111.16016	6	4
70	Malate dehydrogenase, cytoplasmic	MDHC_HUMAN	36586.96875	6.91	4
71	Ig kappa chain C region	IGKC_HUMAN	11739.62988	5.58	3
72	Cystatin-B	CYTB_HUMAN	11178.58008	6.96	3
73	Phosphoglycerate mutase 1	PGAM1_HUMAN	28877.81055	6.67	3
74	Ig heavy chain V-III region BRO	HV305_HUMAN	13310.37012	6.45	3
75	Beta-2-microglobulin	B2MG_HUMAN	13797.88965	6.06	3
76	Interleukin-1 receptor antagonist protein	IL1RA_HUMAN	20317.88086	5.83	3
77	Transthyretin	TTHY_HUMAN	15969.01953	5.52	3
78	Deleted in malignant brain tumors 1 protein	DMBT1_HUMAN	266593.1875	5.18	3
79	Ubiquitin-40S ribosomal protein S27a	RS27A_HUMAN	18229.41992	9.68	3
80	Glyceraldehyde-3-phosphate dehydrogenase	G3P_HUMAN	36168.35938	8.57	3
81	14-3-3 protein gamma	1433G_HUMAN	28422.88086	4.8	3
82	Alpha-actinin-4	ACTN4_HUMAN	105156.3672	5.27	3
83	Superoxide dismutase [Cu-Zn] SV=2	SODC_HUMAN	16109.84961	5.7	3
84	Protein S100-A8	S10A8_HUMAN	10873.63965	6.51	3

85	Cellular retinoic acid-binding protein 2	RABP2_HUMAN	15820.98047	5.42	3
86	Cornulin	CRNN_HUMAN	53685.58984	5.73	3
87	Cystatin-D	CYTD_HUMAN	16299.87988	6.7	2
88	Keratin, type II cytoskeletal 2 epidermal	K22E_HUMAN	65623.14844	8.07	2
89	Ig kappa chain V-I region EU	KV106_HUMAN	11872.74023	8.62	2
90	Transgelin-2	TAGL2_HUMAN	22515.14063	8.41	2
91	Desmoglein-3	DSG3_HUMAN	108569.0703	4.86	2
92	Heat shock protein beta-1	HSPB1_HUMAN	22814.48047	5.98	2
93	Ig kappa chain V-III region WOL	KV305_HUMAN	11830.87988	9.07	2
94	Rho GDP-dissociation inhibitor 2	GDIR2_HUMAN	23019.58984	5.1	2
95	Cystatin-C	CYTC_HUMAN	15973.03027	9	2
96	Phosphoglucomutase-1	PGM1_HUMAN	61640.46875	6.3	2
97	Cysteine-rich secretory protein 3	CRIS3_HUMAN	28347.81055	8.09	2
98	Ig heavy chain V-III region CAM	HV307_HUMAN	13751.15039	9.65	2
99	Catalase	CATA_HUMAN	59902.69922	6.9	2
100	Ig kappa chain V-IV region Len	KV402_HUMAN	12724.16992	7.92	2
101	Ig kappa chain V-IV region JI	KV403_HUMAN	14715.2998	6.15	2
102	Protein S100-A6	S10A6_HUMAN	10219.33008	5.33	2
103	Neutrophil gelatinase-associated lipocalin	NGAL_HUMAN	22711.67969	9.02	2
104	ERO1-like protein alpha	ERO1A_HUMAN	55047.87109	5.48	2
105	Protein S100-A2	S10A2_HUMAN	11293.30957	4.68	2
106	Alpha-actinin-1	ACTN1_HUMAN	103452.5078	5.25	2
107	Carbonic anhydrase 6	CAH6_HUMAN	35436.57031	6.51	2
108	Galectin-3-binding protein	LG3BP_HUMAN	66025.09375	5.13	2
109	Desmocollin-2	DSC2_HUMAN	101048.4297	5.19	2
110	Lactoperoxidase	PERL_HUMAN	80972.67969	8.89	2
111	Leukotriene A-4 hydrolase	LKHA4_HUMAN	69747.10156	5.8	2
112	Plastin-2	PLSL_HUMAN	70703.77344	5.29	2
113	Transcobalamin-1	TCO1_HUMAN	48589.91016	4.96	2
114	Glycogen phosphorylase, liver form	PYGL_HUMAN	97408.9375	6.71	2
115	BPI fold-containing family B member 2	BPIB2_HUMAN	49233.69922	8.82	2
116	Glycogen phosphorylase, brain form	PYGB_HUMAN	97186.36719	6.4	2

Protein identification from mechanically ventilated patient saliva:

Peptide no'	Protein Name	Accession Number	Protein MW	Protein PI	Peptide Count
1	Serum albumin	ALBU_HUMAN	70931.0625	5.92	36
2	Complement C3	CO3_HUMAN	188271.5469	6.02	29
3	Serotransferrin	TRFE_HUMAN	78853.14063	6.81	28
4	Alpha-2-macroglobulin	A2MG_HUMAN	164337.5781	6.03	20
5	Polymeric immunoglobulin	PIGR_HUMAN	84197.39063	5.58	15
6	Alpha-amylase 2B	AMY2B_HUMAN	58178.87109	6.64	14
7	Alpha-1-antitrypsin	A1AT_HUMAN	46844.98047	5.37	14
8	Haptoglobin	HPT_HUMAN	45728.42188	6.13	13
9	Fibrinogen beta chain	FIBB_HUMAN	56444.10938	8.54	13
10	Lactotransferrin	TRFL_HUMAN	79649.50781	8.5	12
11	Apolipoprotein	APOA1_HUMAN	30758.92969	5.56	11
12	Actin, cytoplasmic 2	ACTG_HUMAN	42041.71875	5.31	11
13	Zinc-alpha-2-glycoprotein	ZA2G_HUMAN	34421.05078	5.71	10
14	Ig gamma-1 chain C region	IGHA1_HUMAN	38320.44922	6.08	9
15	Ig gamma-2 chain C region	IGHM_HUMAN	49827.37891	6.35	9
16	Ig gamma-3 chain C region	IGHG3_HUMAN	42088.14063	8.23	9
17	Hemoglobin subunit beta	HBB_HUMAN	16080.26953	6.75	8
18	Alpha-1-antichymotrypsin	AACT_HUMAN	47758.5	5.33	8
19	Ig gamma-1 chain C region	IGHG1_HUMAN	36497.05859	8.46	8
20	Ig gamma-2 chain C region	IGHG2_HUMAN	36383.62109	7.66	8
21	Ceruloplasmin	CERU_HUMAN	122817.3984	5.44	8
22	Complement factor B	CFAB_HUMAN	86582.22656	6.67	8
23	Ig alpha-2 chain C region	IGHA2_HUMAN	37146.82813	5.71	7
24	Fibrinogen alpha chain	FIBA_HUMAN	95512.25	5.7	7
25	Protein S100-A8	S10A8_HUMAN	10873.63965	6.51	7
26	Myeloperoxidase	PERM_HUMAN	84596.63281	9.19	7
27	Ig gamma-4 chain C region	IGHG4_HUMAN	36331.75	7.18	6
28	Alpha-1-acid glycoprotein 1	A1AG1_HUMAN	23680.71094	4.93	6
29	Small proline-rich protein 3	SPRR3_HUMAN	18510.13086	8.86	6
30	Fibrinogen gamma chain	FIBG_HUMAN	51984.73047	5.37	6
31	Leucine-rich alpha-2-glycoprotein	A2GL_HUMAN	38338.05859	6.45	6
32	Fibronectin	FINC_HUMAN	265357.3125	5.46	6
33	Lysozyme C	LYSC_HUMAN	16894.18945	9.38	6
34	Lipocalin-1	LCN1_HUMAN	19375.7793	5.39	5
35	Cystatin-B	CYTB_HUMAN	11178.58008	6.96	5
36	Alpha-enolase	ENOA_HUMAN	47415.23828	7.01	5
37	Alpha-1B-glycoprotein	A1BG_HUMAN	54679.42188	5.56	5
38	14-3-3 protein zeta/delta	1433Z_HUMAN	27865.68945	4.73	5
39	Cystatin-S	CYTS_HUMAN	16433.90039	4.95	5
40	Protein S100-A9	S10A9_HUMAN	13279.5	5.71	5
41	Hemopexin	HEMO_HUMAN	52241.10938	6.55	5
42	Alpha-2-HS-glycoprotein	FETUA_HUMAN	39943.53906	5.43	5
43	Matrix metalloproteinase-9	MMP9_HUMAN	79282.11719	5.69	5
44	Immunoglobulin lambda-like polypeptide 5	IGLL5_HUMAN	23324.56055	9.08	4
45	Immunoglobulin J chain	IGJ_HUMAN	18454.90039	5.12	4
46	Hemoglobin subunit alpha	HBA_HUMAN	15293.91016	8.72	4
47	Transthyretin	TTHY_HUMAN	15969.01953	5.52	4
48	Leukocyte elastase inhibitor	ILEU_HUMAN	42806.67969	5.9	4
49	Transaldolase	TALDO_HUMAN	37654.42188	6.36	4
50	Antithrombin-III	ANT3_HUMAN	52936.76172	6.32	4

51	Plasma protease C1 inhibitor	IC1_HUMAN	55303.33984	6.09	4
52	Alpha-1-acid glycoprotein 2	A1AG2_HUMAN	23817.57031	5.03	4
53	6-phosphogluconate dehydrogenase	6PGD_HUMAN	53519.83984	6.8	4
54	Complement C4-B	CO4B_HUMAN	193872.2031	6.89	4
55	Keratin, type II cytoskeletal 1	K2C1_HUMAN	66136.96094	8.15	4
56	Ig lambda-3 chain C regions	LAC3_HUMAN	11368.46973	6.92	3
57	Cystatin-SN	CYTN_HUMAN	16561.28906	6.73	3
58	Triosephosphate isomerase	TPIS_HUMAN	31001.63086	5.65	3
59	Coronin-1A	COR1A_HUMAN	51545.64844	6.25	3
60	Thioredoxin	THIO_HUMAN	11959.67969	4.82	3
61	Keratin, type I cytoskeletal 10	K1C10_HUMAN	58975.64063	5.13	3
62	14-3-3 protein beta/alpha	1433B_HUMAN	28156.81055	4.76	3
63	Ig heavy chain V-III region CAM	HV307_HUMAN	13751.15039	9.65	3
64	Heat shock 70 kDa protein 1A/1B	HSP71_HUMAN	70238.96875	5.48	3
65	Prolactin-inducible protein	PIP_HUMAN	16791.73047	8.26	3
66	Kininogen-1	KNG1_HUMAN	72785.92188	6.34	3
67	Vitamin D-binding protein	VTDB_HUMAN	54216.67969	5.4	3
68	Beta-2-glycoprotein 1	APOH_HUMAN	39330.37891	8.34	3
69	Vitronectin	VTNC_HUMAN	54914.98828	5.55	3
70	Cystatin-SA	CYTT_HUMAN	16664.15039	4.85	3
71	Plastin-2	PLSL_HUMAN	70703.77344	5.29	3
72	14-3-3 protein sigma	1433S_HUMAN	27848.66992	4.68	3
73	Keratin, type II cytoskeletal 6B	K2C6B_HUMAN	60260.23047	8.09	3
74	Ig kappa chain C region	IGKC_HUMAN	11739.62988	5.58	2
75	Ig kappa chain V-I region EU	KV106_HUMAN	11872.74023	8.62	2
76	Inter-alpha-trypsin inhibitor heavy chain H4	ITIH4_HUMAN	103476.9297	6.51	2
77	Moesin	MOES_HUMAN	67869.75781	6.08	2
78	Ig heavy chain V-III region BRO	HV305_HUMAN	13310.37012	6.45	2
79	Involucrin	INVO_HUMAN	68529.17969	4.62	2
80	Protein S100-P	S100P_HUMAN	10439.20996	4.75	2
81	Histone H4	H4_HUMAN	11360.37988	11.36	2
82	Lactoperoxidase	PERL_HUMAN	80972.67969	8.89	2
83	Deleted in malignant brain tumors 1 protein	DMBT1_HUMAN	266593.1875	5.18	2
84	Transketolase	TKT_HUMAN	68386.57813	7.58	2
85	Ig kappa chain V-III region WOL	KV305_HUMAN	11830.87988	9.07	2
86	Ig heavy chain V-III region TIL	HV304_HUMAN	12440.03027	9.24	2
87	Ig heavy chain V-III region VH26	HV303_HUMAN	12712.17969	8.49	2
88	Olfactomedin-4	OLFM4_HUMAN	57473.73047	5.5	2
89	Cystatin-A	CYTA_HUMAN	10999.66016	5.38	2
90	Ig heavy chain V-III region GAL	HV320_HUMAN	12814.17969	8.7	2
91	Apolipoprotein A-II	APOA2_HUMAN	11259.87012	6.26	2
92	Carbonic anhydrase 6	CAH6_HUMAN	35436.57031	6.51	2
93	Neutrophil gelatinase-associated lipocalin	NGAL_HUMAN	22711.67969	9.02	2
94	Neutrophil defensin 3	DEF3_HUMAN	10514.08984	5.71	2
95	Interleukin-1 receptor antagonist protein	IL1RA_HUMAN	20317.88086	5.83	2
96	Brain acid soluble protein 1	BASP1_HUMAN	22680.00977	4.64	2
97	Peptidyl-prolyl cis-trans isomerase A	PPIA_HUMAN	18184.83984	7.68	2
98	Gelsolin	GELS_HUMAN	85966.10156	5.9	2

Saliva, plasma and ETT cytokine quantification:

Sample	PN	Date	Respiratory pathogen in dental plaque	Sample time point	IL-8	IL-1β	IL-6
SAL	PN002	30/07/13	x	d1	118008.2	2884.96	21780.46
SAL	PN002	02/08/13	x	d4	233.66	5569.67	13.46
SAL	PN002	05/08/13	x	d7	576716.92	15310.08	10326.68
SAL	PN004	04/08/13	x	d1	99636.88	4148.64	5465.98
SAL	PN004	09/08/13	x	d5	2589.68	491.7	58.43
SAL	PN004	11/08/13	x	d7	6747.93	755.25	517.73
SAL	PN004	23/08/13	x	WARD	49.61	29.41	0
SAL	PN004	26/08/13	x	WARD	121.82	107.46	24.92
SAL	PN004	31/08/13	x	WARD	17.56	45.31	0
SAL	PN004	04/10/13	x	WARD	18.42	46.53	0
SAL	PN004	16/05/14	x	d1*	1555.03	5394.17	46.87
SAL	PN004	22/05/14	x	d6*	2134	6365.93	368.13
SAL	PN007	20/08/13	Y	d3	42072.25	8338.68	418.51
SAL	PN007	22/09/13	Y	WARD	24.02	18.87	22.41
SAL	PN010	23/08/13	x	d1	738.07	1121.46	26.63
SAL	PN010	25/08/13	x	d3	113.13	208.93	14.84
SAL	PN010	11/09/13	x	WARD	21.7	45.92	20.34
SAL	PN012	30/08/13	Y	d5	13048.92	17168.84	38.09
SAL	PN012	02/09/13	Y	d8	5129	4003.84	173.11
SAL	PN012	09/09/13	Y	d15	42525.48	60147.49	12275.72
SAL	PN021	07/10/13	Y	d1	1336.8	4678.3	47.5
SAL	PN021	09/10/13	Y	d3	375.33	462.17	41.59
SAL	PN022	09/10/13	x	d1	20023.6	287.4	134.2
SAL	PN022	12/10/13	x	d4	17872.5	3797.3	1137.7
SAL	PN028	31/10/13	Y	d3	8863.14	2741.7	96.06
SAL	PN028	03/11/13	Y	d6	6747.93	1380.56	56
SAL	PN028	10/11/13	Y	d13	256.19	139.29	11.72
SAL	PN038	13/01/14	x	d4	236.15	141.61	19.93
SAL	PN045	15/02/14	x	d13	890.3	886.58	213.97
SAL	PN046	06/02/14	Y	d1	502664.5	75386.85	341237.95
SAL	PN046	08/02/14	Y	d3	11631.89	25218.47	3076.35
SAL	PN046	10/02/14	Y	d5	1865.44	4212.16	585.16
SAL	PN046	11/02/14	Y	d6	123297.02	59232.76	51413.34
SAL	PN046	18/02/14	Y	d13	610341.51	31131.69	8685.59
SAL	PN046	03/03/14	Y	d26	806.13	259.12	75.76
SAL	PN046	18/03/14	Y	d41	335666.18	11527.93	26681.68
SAL	PN046	28/03/14	Y	d51	397793.19	63957.67	57809.31

SAL	PN050	18/02/14	x	d1	4146.05	3507.56	101.21
SAL	PN051	19/02/14	Y	d1	13749.03	5052.41	1690.26
SAL	PN060	12/03/14	Y	d3	29605.76	5875.1	599.53
SAL	PN060	20/03/14	Y	d11	22501.02	5934.37	1616.99
SAL	PN060	27/03/14	Y	d18	2255.99	1639.81	179.67
SAL	PN062	17/03/14	Y	d1	1723.35	2274.42	110.23
SAL	PN079	21/05/14	Y	d8	7734.73	742.25	66.75
SAL	PN083	26/05/14	Y	d1	3745.01	9635.92	3810.54
SAL	PN083	29/05/14	Y	d4	1217.9	569.07	17.44
SAL	PN099	10/07/14	Y	d1	2661.64	123.75	34.72
SAL	PN099	12/07/14	Y	d3	464.4	555.5	72.91
SAL	PN099	14/07/14	Y	d5	511.23	522.78	96.06
SAL	PN099	21/07/14	Y	d12	2182.05	1037.65	44.72
SAL	PN099	04/08/14	Y	d26	1337.8	148.73	220.15
SAL	PN104	18/07/14	Y	d1	378870.73	927.61	460.05
SAL	PN106	19/07/14	Y	d1	46839.8	38079.77	530.64
SAL	PN106	15/08/14	Y	WARD	160.58	66.74	0
SAL	PN107	27/07/14	Y	d1	22032.61	3805.47	6138.83
SAL	PN107	30/07/14	Y	d4	13464.57	13451.2	636.83
SAL	PN109	02/08/14	Y	d1	13324.56	5476.11	278.72
SAL	PN109	05/08/14	Y	d4	126.97	90.83	0
SAL	PN109	08/08/14	Y	d7	54477.45	10383.36	11288.71
SAL	B1	29/04/14	Healthy control	d1	199.38	98.01	19.93
SAL	B2	01/05/14	Healthy control	d3	99.05	134.72	20.76
SAL	B3	06/05/14	Healthy control	d8	63.5	45.31	20.76
SAL	B4	07/05/14	Healthy control	d9	13.31	30.48	0
SAL	B5	14/05/14	Healthy control	d16	133.58	77.46	11.72
SAL	D1	29/04/14	Healthy control	d1	100.18	121.63	0
SAL	D2	06/05/14	Healthy control	d8	56.39	112.39	14.84
SAL	D3	07/05/14	Healthy control	d9	53.81	96.18	24.92
SAL	D4	14/05/14	Healthy control	d16	160.58	166.32	19.93
PL	PN002	30/07/13	x	d1	295.83	0	223.35
PL	PN002	01/08/13	x	d3	199.83	1.38	1528.78
PL	PN002	03/08/13	x	d5	898.65	7.32	951.05
PL	PN002	05/08/13	x	d7	486.04	2.56	292.97
PL	PN007	18/08/13	Y	d1	308.14	4.58	103.36
PL	PN007	20/08/13	Y	d3	305.41	3.37	65.63
PL	PN007	23/08/13	Y	d6	1317.57	12.78	61.43
PL	PN004	04/08/13	x	d1	74.56	3.24	405.41
PL	PN004	06/08/13	x	d3	123.98	4.31	252.13
PL	PN004	09/08/13	x	d6	223.61	3.91	78.38
PL	PN004	10/08/13	x	d7	89.86	3.51	96.93
PL	PN004	23/08/13	x	d20	280.66	6.27	37.3
PL	PN010	23/08/13	x	d1	48.58	4.04	38.46

PL	PN010	25/08/13	x	d3	45.96	3.24	9.67
PL	PN012	26/08/13	Y	d1	196.39	0	201.79
PL	PN012	28/08/13	Y	d3	74.56	3.91	63.8
PL	PN012	30/08/13	Y	d5	106.73	4.04	188.32
PL	PN012	02/09/13	Y	d8	48.05	3.64	22.62
PL	PN012	09/09/13	Y	d15	51.31	2.56	28.57
PL	PN012	16/09/13	Y	d22	94.3	39.26	31.78
PL	PN012	23/09/13	Y	d29	39.17	6.12	23.81
PL	PN019	05/10/13	x	d1	128.67	2.05	151.79
PL	PN019	07/10/13	x	d3	157.42	3.77	139.79
PL	PN019	09/10/13	x	d5	134.74	4.04	141.08
PL	PN021	07/10/13	Y	d1	65.37	17.38	350.9
PL	PN021	09/10/13	Y	d3	27.7	4.58	31.61
PL	PN021	11/10/13	Y	d5	76.82	11.24	51.22
PL	PN022	09/10/13	x	d1	99.4	3.37	196.28
PL	PN022	12/10/13	x	d3	123.98	7.48	539.12
PL	PN022	15/10/13	x	d7	131.07	3.37	166.35
PL	PN028	29/10/13	Y	d1	79.92	4.44	65.01
PL	PN028	31/10/13	Y	d3	36.93	4.04	34.01
PL	PN028	03/11/13	Y	d6	16.56	5.69	25.19
PL	PN028	10/11/13	Y	d13	38.72	5.13	34.9
PL	PN030	02/11/13	Y	d1	132.28	3.77	127.58
PL	PN030	05/11/13	Y	d4	163.24	4.31	156.02
PL	PN030	07/11/13	Y	d6	206.32	6.56	315.99
PL	PN030	14/11/13	Y	d13	556.57	7.63	310.06
PL	PN038	09/01/14	Y	d1	54.14	2.5	63.2
PL	PN038	13/01/14	Y	d4	54.14	5.34	72.75
PL	PN038	19/01/14	Y	d10	84.77	4.31	136.64
PL	PN038	26/01/14	Y	d17	44.95	4.04	33.66
PL	PN045	03/02/14	x	d1	30.71	4.51	84.41
PL	PN045	05/02/14	x	d3	33.11	3.91	29.98
PL	PN045	08/02/14	x	d6	92.5	10.68	137.26
PL	PN045	15/02/14	x	d13	53	4.44	15.19
PL	PN045	22/02/14	x	d20	44.44	3.51	15.59
PL	PN046	06/02/14	Y	d1	12614.68	25.22	6748.59
PL	PN046	08/02/14	Y	d3	83.13	3.91	158.9
PL	PN046	10/02/14	Y	d5	100.83	3.44	247.47
PL	PN046	11/02/14	Y	d6	175.49	4.04	716.16
PL	PN046	18/02/14	Y	d13	531.94	7.63	549.99
PL	PN046	25/02/14	Y	d20	105.73	4.04	47.85
PL	PN046	03/03/14	Y	d26	108.77	4.17	139.79
PL	PN046	11/03/14	Y	d34	72.35	4.31	57.47
PL	PN049	09/02/14	Y	d1	248.88	3.51	128.75
PL	PN049	11/02/14	Y	d3	145.01	3.24	110.21

PL	PN050	18/02/14	x	d1	43.7	4.04	86.78
PL	PN050	21/02/14	x	d3	115.07	2.84	298.55
PL	PN050	24/02/14	x	d6	101.79	2.91	10.97
PL	PN050	03/03/14	x	d13	47	1.97	40.04
PL	PN051	19/02/14	Y	d1	97.99	3.37	219.3
PL	PN051	22/02/14	Y	d4	86.44	4.31	51.22
PL	PN051	25/02/14	Y	d7	100.83	4.04	70.08
PL	PN051	03/03/14	Y	d13	114	3.84	22.49
PL	PN056	05/03/14	x	d1	210.04	4.58	94.29
PL	PN056	07/03/14	x	d3	92.5	5.27	174.16
PL	PN056	10/03/14	x	d6	920.03	16.1	47.85
PL	PN056	17/03/14	x	d13	69.49	5.76	48.32
PL	PN060	10/03/14	Y	d1	89.86	2.42	61.43
PL	PN060	12/03/14	Y	d3	239.52	4.52	119.21
PL	PN060	20/03/14	Y	d11	244.53	0	102.19
PL	PN060	27/03/14	Y	d18	79.75	1.51	154.04
PL	PN062	17/03/14	Y	d1	104.7	1.71	191.27
PL	PN062	20/03/14	Y	d4	252.23	3.36	566.92
PL	PN062	23/03/14	Y	d7	99.96	1.23	147.78
PL	PN079	14/05/14	Y	d1	140.43	2.5	205.47
PL	PN079	21/05/14	Y	d8	209.13	2.05	256.95
PL	PN004	16/05/14	x	d1	129.92	3.22	319.14
PL	PN004	18/05/14	x	d3	555.68	0	278.58
PL	PN004	20/05/14	x	d5	42.79	0	78.81
PL	PN083	26/05/14	Y	d1	148.37	0	455.3
PL	PN083	29/05/14	Y	d4	103.5	0	36.22
PL	PN083	01/06/14	Y	d7	484.51	3.08	11.58
PL	PN083	06/06/14	Y	d12	99.38	0	15.31
PL	PN099	10/07/14	x	d1	129.92	0	19.01
PL	PN099	12/07/14	x	d3	75.03	0	31.02
PL	PN099	14/07/14	x	d5	70.53	0	19.82
PL	PN099	21/07/14	x	d12	196.3	0	93.78
PL	PN099	27/07/14	x	d18	247.08	4.82	83.24
PL	PN099	04/08/14	x	d26	104.7	2.79	72.16
PL	PN104	18/07/14	Y	d1	409.65	5.28	1861.79
PL	PN104	20/07/14	Y	d3	257.48	0	92.78
PL	PN104	23/07/14	Y	d6	260.16	0	66.01
PL	PN104	30/07/14	Y	d13	479.77	1.88	126.33
PL	PN106	19/07/14	Y	d1	184.18	1.71	134.53
PL	PN106	21/07/14	Y	d3	202.63	3.36	83.24
PL	PN106	23/07/14	Y	d5	109.64	0	86
PL	PN106	01/08/14	Y	d13	88.86	0	103.29
PL	PN107	27/07/14	Y	d1	110.9	0	32.62
PL	PN107	30/07/14	Y	d4	102.31	1.88	95.82

PL	PN107	01/08/14	Y	d6	41.6	1.51	32.21
PL	PN109	02/08/14	Y	d1	61.78	14.3	36
PL	PN109	05/08/14	Y	d4	544.93	28.63	75.42
PL	PN109	07/08/14	Y	d6	86.77	22.89	34.27
ETT	PN002	07/08/13	x	d9	28098.81	100.49	30.15
ETT	PN007	20/08/13	Y	d6	22722.69	1187.11	2.64
ETT	PN010	27/08/13	x	d5	3432.2	193.02	26.27
ETT	PN012	27/08/13	Y	d2	3000.32	17.15	23.18
ETT	PN012	09/09/13	Y	d13	18916.02	18188.25	671.44
ETT	PN021	10/10/13	Y	d4	27800.64	13027.32	15.54
ETT	PN022	15/10/13	x	d8	14685.34	1079.76	6.44
ETT	PN028	04/11/13	Y	d33	349.21	2.79	2.36
ETT	PN030	19/11/13	Y	d17	34673.58	1842.13	7.19
ETT	PN038	11/01/14	Y	d3	1878.55	17.15	14.2
ETT	PN038	23/01/14	Y	d14	29646.68	1673.36	664.8
ETT	PN045	11/02/14	x	d8	4411.53	34.43	2.27
ETT	PN045	18/02/14	x	d15	9596.89	243.86	122.4
ETT	PN046	11/02/14	Y	d5	38039.51	11064.98	684.94
ETT	PN046	11/03/14	Y	d34	20889.85	765.03	125
ETT	PN046	23/03/14	Y	d47	39310.19	3654.07	23.65
ETT	PN046	24/03/14	Y	d48	32315.17	1789.71	12.95
ETT	PN049	14/02/14	Y	d6	22016.03	661.1	420.35
ETT	PN050	20/02/14	x	d3	27214.29	8380.95	6.91
ETT	PN050	06/03/14	x	d15	11046.76	444.92	292.98
ETT	PN051	05/03/14	Y	d14	8100.58	203.71	493.11
ETT	PN056	13/03/14	x	d14	18050.96	305.69	1116.09
ETT	PN056	19/03/14	x	d20	9501.3	436.58	503.04

	No detection
	Near out of detection limits
	In acceptable range
	Over detection limit >than

Sample	PN	Date	Respiratory pathogen in dental plaque	Sample time point	IL-10	TNFα	IL-12p70
SAL	PN002	30/07/13	x	d1	44.13	986.11	0
SAL	PN002	02/08/13	x	d4	9.12	7.21	8.77
SAL	PN002	05/08/13	x	d7	27.24	872.78	0
SAL	PN004	04/08/13	x	d1	25.19	0	39.81
SAL	PN004	09/08/13	x	d5	0	0	27.3
SAL	PN004	11/08/13	x	d7	0	15.58	18.34
SAL	PN004	23/08/13	x	WARD	11.57	0	31.18

SAL	PN004	26/08/13	x	WARD	0	0	32.81
SAL	PN004	31/08/13	x	WARD	0	0	0
SAL	PN004	04/10/13	x	WARD	0	0	20.29
SAL	PN004	16/05/14	x	d1*	0	0	19.68
SAL	PN004	22/05/14	x	d6*	0	0	0
SAL	PN007	20/08/13	Y	d3	0	0	15.23
SAL	PN007	22/09/13	Y	WARD	0	0	0
SAL	PN010	23/08/13	x	d1	0	0	0
SAL	PN010	25/08/13	x	d3	0	0	15.23
SAL	PN010	11/09/13	x	WARD	0	0	23.68
SAL	PN012	30/08/13	Y	d5	13.06	0	27.3
SAL	PN012	02/09/13	Y	d8	11.13	0	0
SAL	PN012	09/09/13	Y	d15	18.44	24.59	23.68
SAL	PN021	07/10/13	Y	d1	0	0	14.1
SAL	PN021	09/10/13	Y	d3	0	0	20.29
SAL	PN022	09/10/13	x	d1	0	13.7	11.4
SAL	PN022	12/10/13	x	d4	0	0	0
SAL	PN028	31/10/13	Y	d3	18.13	11.79	11.44
SAL	PN028	03/11/13	Y	d6	0	0	37.97
SAL	PN028	10/11/13	Y	d13	0	0	11.44
SAL	PN038	13/01/14	x	d4	0	0	23.68
SAL	PN045	15/02/14	x	d13	0	0	18.34
SAL	PN046	06/02/14	Y	d1	142.22	6074.83	0
SAL	PN046	08/02/14	Y	d3	0	14.96	17.08
SAL	PN046	10/02/14	Y	d5	0	0	16.16
SAL	PN046	11/02/14	Y	d6	0	14.61	20.29
SAL	PN046	18/02/14	Y	d13	20	11.75	51.81
SAL	PN046	03/03/14	Y	d26	0	0	15.23
SAL	PN046	18/03/14	Y	d41	29.71	40.24	36.21
SAL	PN046	28/03/14	Y	d51	119.39	605.96	0
SAL	PN050	18/02/14	x	d1	11.57	0	29.23
SAL	PN051	19/02/14	Y	d1	46.4	10.9	54.01
SAL	PN060	12/03/14	Y	d3	14.38	0	22.99
SAL	PN060	20/03/14	Y	d11	0	0	17.08
SAL	PN060	27/03/14	Y	d18	0	0	0
SAL	PN062	17/03/14	Y	d1	0	0	20.29
SAL	PN079	21/05/14	Y	d8	0	25.5	11.44
SAL	PN083	26/05/14	Y	d1	0	0	18.34
SAL	PN083	29/05/14	Y	d4	0	14.61	0
SAL	PN099	10/07/14	Y	d1	0	0	0
SAL	PN099	12/07/14	Y	d3	0	0	15.23
SAL	PN099	14/07/14	Y	d5	0	0	27.3
SAL	PN099	21/07/14	Y	d12	0	0	0
SAL	PN099	04/08/14	Y	d26	0	0	22.3

SAL	PN104	18/07/14	Y	d1	0	25.04	0
SAL	PN106	19/07/14	Y	d1	14.38	24.59	20.29
SAL	PN106	15/08/14	Y	WARD	0	0	0
SAL	PN107	27/07/14	Y	d1	0	0	0
SAL	PN107	30/07/14	Y	d4	0	17.43	17.08
SAL	PN109	02/08/14	Y	d1	0	20.12	21.97
SAL	PN109	05/08/14	Y	d4	0	21.9	0
SAL	PN109	08/08/14	Y	d7	13.74	0	0
SAL	B1	29/04/14	Healthy control	d1	11.13	0	13.4
SAL	B2	01/05/14	Healthy control	d3	0	14.61	0
SAL	B3	06/05/14	Healthy control	d8	0	0	16.16
SAL	B4	07/05/14	Healthy control	d9	0	0	0
SAL	B5	14/05/14	Healthy control	d16	0	18.34	17.71
SAL	D1	29/04/14	Healthy control	d1	0	0	13.38
SAL	D2	06/05/14	Healthy control	d8	0	0	0
SAL	D3	07/05/14	Healthy control	d9	18.75	0	20.29
SAL	D4	14/05/14	Healthy control	d16	0	0	29.23
PL	PN002	30/07/13	x	d1	11.03	0	1.99
PL	PN002	01/08/13	x	d3	6.62	0	0
PL	PN002	03/08/13	x	d5	17.32	2.98	4.15
PL	PN002	05/08/13	x	d7	30.74	0	0
PL	PN007	18/08/13	Y	d1	5.54	0	0
PL	PN007	20/08/13	Y	d3	5.29	0	0
PL	PN007	23/08/13	Y	d6	4.32	0	2.99
PL	PN004	04/08/13	x	d1	4.92	0	0
PL	PN004	06/08/13	x	d3	12.26	0	2.54
PL	PN004	09/08/13	x	d6	6.56	0	4.44
PL	PN004	10/08/13	x	d7	6.82	0	4.15
PL	PN004	23/08/13	x	d20	5.17	0	3.93
PL	PN010	23/08/13	x	d1	2.28	0	0
PL	PN010	25/08/13	x	d3	3.62	0	3.43
PL	PN012	26/08/13	Y	d1	6.95	0	0
PL	PN012	28/08/13	Y	d3	5.41	0	0
PL	PN012	30/08/13	Y	d5	8.62	0	0
PL	PN012	02/09/13	Y	d8	6.69	0	0
PL	PN012	09/09/13	Y	d15	3.97	0	0
PL	PN012	16/09/13	Y	d22	8.91	0	2.54
PL	PN012	23/09/13	Y	d29	3.97	0	2.99
PL	PN019	05/10/13	x	d1	3.62	3.62	2.54
PL	PN019	07/10/13	x	d3	3.38	0	2.22
PL	PN019	09/10/13	x	d5	4.32	0	5.77
PL	PN021	07/10/13	Y	d1	10.76	2.77	4.29
PL	PN021	09/10/13	Y	d3	3.32	0	2.99
PL	PN021	11/10/13	Y	d5	4.56	3.62	5.32

PL	PN022	09/10/13	x	d1	3.62	0	2.22
PL	PN022	12/10/13	x	d3	5.66	5.73	0
PL	PN022	15/10/13	x	d7	4.09	0	0
PL	PN028	29/10/13	Y	d1	3.03	0	0
PL	PN028	31/10/13	Y	d3	0	0	3.43
PL	PN028	03/11/13	Y	d6	2.54	0	2.99
PL	PN028	10/11/13	Y	d13	4.32	0	2.54
PL	PN030	02/11/13	Y	d1	5.05	0	0
PL	PN030	05/11/13	Y	d4	5.41	0	0
PL	PN030	07/11/13	Y	d6	25.7	0	2.54
PL	PN030	14/11/13	Y	d13	15.78	0	5.47
PL	PN038	09/01/14	Y	d1	2.54	0	0
PL	PN038	13/01/14	Y	d4	4.09	0	0
PL	PN038	19/01/14	Y	d10	2.15	0	2.22
PL	PN038	26/01/14	Y	d17	0	0	4.73
PL	PN045	03/02/14	x	d1	2.28	0	2.54
PL	PN045	05/02/14	x	d3	3.03	0	2.99
PL	PN045	08/02/14	x	d6	8.48	8.7	10.26
PL	PN045	15/02/14	x	d13	4.32	0	0
PL	PN045	22/02/14	x	d20	5.66	0	0
PL	PN046	06/02/14	Y	d1	45.73	0	4.73
PL	PN046	08/02/14	Y	d3	0	0	0
PL	PN046	10/02/14	Y	d5	5.41	0	0
PL	PN046	11/02/14	Y	d6	8.48	0	1.97
PL	PN046	18/02/14	Y	d13	13.49	0	0
PL	PN046	25/02/14	Y	d20	4.8	0	0
PL	PN046	03/03/14	Y	d26	6.95	0	0
PL	PN046	11/03/14	Y	d34	3.97	0	2.54
PL	PN049	09/02/14	Y	d1	18.51	0	0
PL	PN049	11/02/14	Y	d3	15.48	0	0
PL	PN050	18/02/14	x	d1	2.66	0	0
PL	PN050	21/02/14	x	d3	4.44	0	0
PL	PN050	24/02/14	x	d6	2.6	0	0
PL	PN050	03/03/14	x	d13	2.54	0	5.77
PL	PN051	19/02/14	Y	d1	5.29	4.46	3.71
PL	PN051	22/02/14	Y	d4	5.66	0	3.71
PL	PN051	25/02/14	Y	d7	4.92	2.98	0
PL	PN051	03/03/14	Y	d13	14.23	0	4.44
PL	PN056	05/03/14	x	d1	4.86	0	3.43
PL	PN056	07/03/14	x	d3	8.62	0	4.44
PL	PN056	10/03/14	x	d6	7.91	0	0
PL	PN056	17/03/14	x	d13	0	3.62	0
PL	PN060	10/03/14	Y	d1	3.26	0	0
PL	PN060	12/03/14	Y	d3	6.51	0	0

PL	PN060	20/03/14	Y	d11	3.48	0	2.09
PL	PN060	27/03/14	Y	d18	1.64	0	3.61
PL	PN062	17/03/14	Y	d1	2.11	0	0
PL	PN062	20/03/14	Y	d4	2.98	0	3.61
PL	PN062	23/03/14	Y	d7	2.74	0	1.44
PL	PN079	14/05/14	Y	d1	3.14	0	1.78
PL	PN079	21/05/14	Y	d8	5.18	0	0
PL	PN004	16/05/14	x	d1	40.4	0	10.81
PL	PN004	18/05/14	x	d3	66.28	0	8.76
PL	PN004	20/05/14	x	d5	33.61	0	9.11
PL	PN083	26/05/14	Y	d1	3.92	0	1.78
PL	PN083	29/05/14	Y	d4	1.8	0	2.09
PL	PN083	01/06/14	Y	d7	2.74	0	3.61
PL	PN083	06/06/14	Y	d12	2.58	0	2.09
PL	PN099	10/07/14	x	d1	3.66	0	0
PL	PN099	12/07/14	x	d3	3.31	0	0
PL	PN099	14/07/14	x	d5	3.35	0	1.78
PL	PN099	21/07/14	x	d12	4.11	0	0
PL	PN099	27/07/14	x	d18	4.58	0	1.78
PL	PN099	04/08/14	x	d26	8.84	0	1.61
PL	PN104	18/07/14	Y	d1	50.52	0	3.05
PL	PN104	20/07/14	Y	d3	5.08	0	3.84
PL	PN104	23/07/14	Y	d6	12.9	2.26	1.78
PL	PN104	30/07/14	Y	d13	23.72	0	1.44
PL	PN106	19/07/14	Y	d1	3.83	0	0
PL	PN106	21/07/14	Y	d3	3.66	0	0
PL	PN106	23/07/14	Y	d5	5.5	0	2.09
PL	PN106	01/08/14	Y	d13	3.14	0	2.62
PL	PN107	27/07/14	Y	d1	3.92	0	2.94
PL	PN107	30/07/14	Y	d4	3.66	0	1.44
PL	PN107	01/08/14	Y	d6	2.34	0	0
PL	PN109	02/08/14	Y	d1	3.14	16.84	3.05
PL	PN109	05/08/14	Y	d4	3.7	29.25	0
PL	PN109	07/08/14	Y	d6	3.66	25.71	1.44
ETT	PN002	07/08/13	x	d9	0	5.18	0
ETT	PN007	20/08/13	Y	d6	0	2.42	0
ETT	PN010	27/08/13	x	d5	0	3.16	0
ETT	PN012	27/08/13	Y	d2	1.8	0	2.09
ETT	PN012	09/09/13	Y	d13	1.38	2184.73	0
ETT	PN021	10/10/13	Y	d4	1.17	5.32	0
ETT	PN022	15/10/13	x	d8	0	2.42	2.09
ETT	PN028	04/11/13	Y	d33	2.27	0	3.38
ETT	PN030	19/11/13	Y	d17	2.9	11.71	3.61
ETT	PN038	11/01/14	Y	d3	1.29	0	0

ETT	PN038	23/01/14	Y	d14	0	70.83	4.02
ETT	PN045	11/02/14	x	d8	0	0	0
ETT	PN045	18/02/14	x	d15	0	0	1.44
ETT	PN046	11/02/14	Y	d5	1.88	25.33	0
ETT	PN046	11/03/14	Y	d34	0	1.76	0
ETT	PN046	23/03/14	Y	d47	1.56	11.26	0
ETT	PN046	24/03/14	Y	d48	2.11	4.76	2.09
ETT	PN049	14/02/14	Y	d6	0	13.63	1.44
ETT	PN050	20/02/14	x	d3	0	24.23	0
ETT	PN050	06/03/14	x	d15	0	0	2.09
ETT	PN051	05/03/14	Y	d14	0	2.72	4.2
ETT	PN056	13/03/14	x	d14	1.64	2.72	0
ETT	PN056	19/03/14	x	d20	1.17	2.42	0

Appendix IV

In vitro biofilm development analysis

- Ethical approval documents for dental plaque collection from healthy volunteers
- ETT biofilm enumeration (CFU/cm^2) for single and mixed species
- Mixed species MIC and MBIC tabulated data:
- Mixed species enumeration post CHX exposure
- CFU/ml counts for aerobic and anaerobic species, and *C. albicans* after exposure to CHX for 30 min, 1 h and 12 h (relation to figure 5.13, section 5.3.5.1)
- CFU/ml counts for aerobic and anaerobic species, and *C. albicans* after exposure to salivary pHs (relation to figure 5.16, section 5.3.6.1)
- CFU/ml counts of *S. aureus* inoculated CDFF biofilms after exposure to different salivary pHs
- CFU/ml counts of *P. aeruginosa* inoculated CDFF biofilms after exposure to different salivary pHs

Ethical approval documents for dental plaque collection from healthy volunteers:

(Version 3, 30th January 2015)

CONSENT FORM

EVALUATING THE EFFECTS OF RESPIRATORY PATHOGENS WITHIN ORAL BIOFILMS

Name of Researchers: Kirsty M Sands, Professor David W Williams, Dr Melanie J Wilson, Dr Matthew P Wise and Professor Michael AO Lewis

Sandsk1@cardiff.ac.uk

Please initial

A. I confirm that I have read and understood the study information sheet (Version 3, 30th January) and have had an opportunity to ask any questions and had sufficient time to come to my decision. _____

B. I give permission that the dental plaque be collected and stored within the Cardiff School of Dentistry only for microbial biofilm analysis _____

C. I understand that my participation is voluntary and that I am free to withdraw at any time prior to my samples being taken in the study without giving any reason, without my medical care or legal rights being affected. _____

D. I understand that results from this study may be used in medical/scientific journals and/or publications, but donated plaque will not be identifiable. _____

E. I understand that the sample is given as a "gift" and that I will have no right to a share of any profits which might arise from research using it (e.g. as part of a new medical treatment or test). _____

F. I understand that during the collection of dental plaque there will be inadvertent collection of human tissue, and furthermore the storage of human tissue. Any tissue collected will not be used in this study and will be destroyed in the experimental process. _____

_____	_____	_____
Name of Cardiff Uni volunteer	Date	Signature
_____	_____	_____
Name of Person taking consent	Date	Signature

Version 3, 30th January 2015

PARTICIPANT INFORMATION SHEET
Cardiff School of Dentistry

EVALUATING THE EFFECTS OF RESPIRATORY PATHOGENS WITHIN ORAL BIOFILMS

You are being invited to take part in a research study. Before you decide it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. *Thank you for reading this.*

1. What is the purpose of the study?

Dental plaque is an oral biofilm, containing aggregates of several microbial species including both bacteria and fungi. To date over 800 bacterial species have been identified within the oral cavity, representing one of the largest human microbiome' alongside the gastrointestinal tract. Studies have suggested that the dental plaque community can change to include respiratory pathogens (not usually present in the plaque of healthy individuals) once a patient undergoes mechanical ventilation in critical care (to assist breathing). Using an *in vitro* model to recreate the microbial shift determined from the patient study (introducing respiratory pathogens into the oral biofilm) allows the investigation into reasons behind this significant microbial shift, and also to explore the effects of antimicrobial mouthwashes such as Chlorhexidine.

This study will involve using pooled dental plaque (a type of mixed species or polymicrobial biofilm) from 10 volunteers to develop oral biofilms using a constant depth film fermenter (an *in vitro* biofilm model). The aim of this study is to first create complex oral biofilms using healthy samples of dental plaque. Once biofilms have been developed a small inoculum of respiratory pathogens (*P. aeruginosa)* will be added to represent the changes that occur within the oral cavity within mechanically ventilated patients. Once the analysis and cell counting of *P. aeruginosa* and oral bacteria is complete, the experiment will be repeated changing parameters each time, including pH change, protein conditioning, adapting protein composition within artificial saliva and the use of a clinically relevant mouthwash (Chlorhexidine). The overall aim of this study is to evaluate the effects of certain conditions on the numbers of respiratory pathogens within the oral biofilm. The duration of the study is 3 months. The research has been considered and accepted by the Research Ethics Committee within the School of Dentistry. The inclusion criteria to be followed are: >18 years of age, and the volunteer must not be taking any antibiotics.

2. Why have I been chosen?

You have been chosen as you meet the required inclusion/exclusion criteria (outlined above) and are deemed able to readily donate a sample of dental plaque. A total of 10 individuals will contribute to this study.

3. Do I have to take part?

It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw without giving a reason any time before the sample is collected. After the samples have been collected, they will be pooled and anonymous and the volunteer can no longer withdraw their sample so it must remain in the study. Deciding not to take part or withdrawing will not affect any current or future treatment. Samples for 10 volunteers will (ideally) be collected within the same session to prevent repeated sample freeze-thaw cycles and immediately pooled for storage.

4. **What will happen to me if I take part?**

One sample of dental plaque will be collected, which will take no longer than 5 minutes of your time. Dental instruments will be used to collect the sample, performed by a dentist. This procedure will be similar to the scraping performed in a routine dental exam.

5. **What about confidentiality?**

Samples collected from a total of 10 volunteers will be pooled together. No personal identification data will be collected or used within this study. After the study any remaining aliquots will be destroyed via autoclaving at 121C for 15 min followed by sample incineration. During dental plaque collection it is likely the plaque sample will be contaminated with blood from the gums and some gingival crevicular fluid, therefore human tissue will be inadvertently collected. Any human tissue inadvertently collected as 'contaminant' in this study will be stored with the pooled dental plaque samples but will not be used within the study. The pooled plaque will be collected via centrifugation and the supernatant autoclaved. Only microbial species will have adequate nutrients for growth and therefore upon use of dental plaque any human tissue will be destroyed. No personal identification will be recorded; the dental plaque is serving as a reservoir to be inserted into liquid media to grow microbial biofilms. Results of the study will be available at the end of the study for brief discussion.

6. **What do I have to do?**

The participant will be required to donate a sample of dental plaque in the morning of sample collection (Date to be confirmed) before lunchtime. Dental plaque from individuals will be pooled together and further divided into aliquots allowing for multiple repetitions of experiments. To comply with HTA guidelines aliquots of dental plaque will be stored in the -80C freezer, 4th Floor, room 404 freezer 2.

7. **Are there any risks?**

There are no obvious risks to you in taking part in this study, however you may experience some minor bleeding and pain/discomfort of the gums around the collection site. This should however cease within a few minutes of collection

8. **What will happen to the results of the research study?**

Results from this study may form part of a publication (to be published at a later stage) A copy of the publication, if successful will be available within the School of Dentistry. No identification of individuals will occur within any written results from this study. Samples will be used for biofilm growth and autoclaved after cell counting and biofilm imaging using both scanning electron microscopy (SEM) and Confocal Laser Scanning Microscopy (CLSM) coupled with molecular fluorescent probes for species identification of respiratory pathogens.

9. **Who is organising and funding the research?**

This study is organised within the School of Dentistry, with Professor David Williams and Dr Melanie Wilson as leading supervisors. The research is funded by The School of Dentistry, Cardiff University.

10. **Contact for Further Information**

Miss Kirsty Sands
Sandsk1@cardiff.ac.uk
Mobile: 07703467561

Any complaints can be made via contact with Dr Fiona Gagg – the Laboratory Manager (WhiteFs@cf.ac.uk 02920742546).

ETT biofilm enumeration (CFU/cm^2) for single species:

Single species biofilm development on ETTs:

	S. aureus	*S. mutans*	*P. aeruginosa*	*C. albicans*
1	1.75E+07	1.75E+07	3.26E+07	1.86E+07
2	4.78E+06	1.63E+07	1.86E+07	2.56E+07
3	3.96E+06	4.78E+06	2.10E+07	1.52E+07
4	1.98E+07	2.33E+06	2.10E+07	1.98E+07
5	1.17E+06	2.91E+07	1.63E+07	1.75E+07
6	4.55E+06	6.41E+06	2.68E+07	1.86E+07
7	4.43E+06	2.21E+07	3.03E+07	2.21E+07
8	5.22E+06	1.12E+07	3.56E+07	1.88E+07
9	1.17E+07	2.68E+07	1.86E+07	2.68E+07
Mean	**8.12E+06**	**1.52E+07**	**2.45E+07**	**2.03E+07**
SD	**6.60E+06**	**9.69E+06**	**6.97E+06**	**3.82E+06**
SEM	**2.20E+06**	**3.23E+06**	**2.32E+06**	**1.27E+06**

ETT biofilm enumeration (CFU/cm^2) for mixed species:

S. aureus and *S. mutans* mixed biofilms:

	S. aureus	*S. mutans*
1	7.69E+06	1.52E+06
2	3.26E+07	1.63E+06
3	5.48E+06	1.52E+06
4	2.68E+07	8.28E+06
5	2.91E+07	1.63E+07
6	8.51E+06	1.86E+07
7	2.80E+06	7.93E+05
8	8.28E+07	1.86E+07
9	8.98E+07	1.45E+07
Mean	**3.17E+07**	**9.09E+06**
SD	**3.29E+07**	**7.94E+06**
SEM	**1.10E+07**	**2.65E+06**

P. aeruginosa and *S. mutans* mixed biofilms:

	S. mutans	*P.aeruginosa*
1	2.10E+06	4.31E+06
2	2.10E+06	1.63E+07
3	1.52E+06	2.68E+07
4	8.28E+06	1.17E+06
5	1.63E+07	3.61E+07
6	1.86E+07	3.15E+07
7	1.96E+06	4.90E+06
8	1.86E+07	3.96E+07
9	1.45E+07	2.10E+07
Mean	**9.34E+06**	**2.02E+07**
SD	**7.67E+06**	**1.44E+07**
SEM	**2.56E+06**	**4.81E+06**

S. aureus and *C. albicans* mixed biofilms:

	S.aureus	*C.albicans*
1	3.38E+06	3.85E+06
2	9.09E+05	3.26E+06
3	1.03E+06	4.78E+06
4	8.39E+06	2.10E+06
5	2.56E+06	2.80E+06
6	2.80E+06	3.50E+06
7	3.85E+06	7.58E+05
8	2.10E+06	5.59E+06
9	8.65E+06	1.43E+06
Mean	**3.74E+06**	**3.12E+06**
SD	**2.88E+06**	**1.55E+06**
SEM	**9.59E+05**	**5.16E+05**

P. aeruginosa and *C. albicans* mixed biofilms:

	P. aeruginosa	*C. albicans*
1	3.38E+06	2.33E+06
2	5.36E+06	1.52E+07
3	7.58E+07	4.78E+06
4	7.23E+06	6.99E+06
5	2.80E+07	1.75E+07
6	3.61E+07	3.15E+07
7	2.68E+07	2.56E+07
8	2.21E+07	7.32E+06
9	8.51E+06	6.76E+06
Mean	**2.37E+07**	**1.31E+07**
SD	**2.28E+07**	**1.01E+07**
SEM	**7.59E+06**	**3.36E+06**

Mixed species MIC and MBIC tabulated data:

	S. aureus & S. mutans		*P. aeruginosa & S. mutans*	
	MIC	MBIC	MIC	MBIC
1	0.0000586	0.0026	0.0008	0.006
2	0.0000586	0.0014	0.00074	0.009
3	0.0000586	0.007	0.00078	0.0086
Mean	**0.0000586**	**0.003666667**	**0.000773333**	**0.007866667**
SD	**8.29919E-21**	**0.002948446**	**3.05505E-05**	**0.001628906**
SEM	**4.79154E-21**	**0.001702286**	**1.76383E-05**	**0.000940449**

	S. aureus & C. albicans		*P. aeruginosa & C. albicans*	
	MIC	MBIC	MIC	MBIC
1	0.0000586	0.0125	0.0007	0.009
2	0.0000586	0.0115	0.00075	0.011
3	0.0000586	0.012	0.00082	0.012
Mean	**0.0000586**	**0.012**	**0.000756667**	**0.010666667**
SD	**8.29919E-21**	**0.0005**	**6.02771E-05**	**0.001527525**
SEM	**4.79154E-21**	**0.000288675**	**3.4801E-05**	**0.000881917**

Mixed species planktonic (CFU/ml) counts following CHX exposure at sub-MIC level in microtitre wells:

Mixed species planktonic counts

	S. aureus	*S. mutans*
1	1.30E+04	2.30E+03
2	2.60E+04	3.20E+03
3	1.90E+03	1.90E+03
Mean	**1.36E+04**	**2.47E+03**
SD	**1.21E+04**	**6.66E+02**

	P. aeruginosa	*S. mutans*
1	8.80E+04	0.00E+00
2	8.80E+04	0.00E+00
3	8.80E+04	0.00E+00
Mean	**8.80E+04**	**0.00E+00**
SD	**0.00E+00**	**0.00E+00**

	S. aureus	*C. albicans*
1	6.60E+04	1.70E+04
2	3.80E+04	2.10E+04
3	5.10E+04	3.20E+04
Mean	**5.17E+04**	**2.33E+04**
SD	**1.40E+04**	**7.77E+03**

	P. aeruginosa	*C. albicans*
1	3.80E+04	0.00E+00
2	3.80E+04	0.00E+00
3	3.80E+04	0.00E+00
Mean	**3.80E+04**	**0.00E+00**
SD	**0.00E+00**	**0.00E+00**

Mixed species biofilm (CFU/ml) counts following CHX exposure at sub-MIC level in microtitre wells:

Mixed species biofilm counts

	S. aureus	*S. mutans*
1	3.90E+04	1.99E+02
2	3.90E+04	1.09E+02
3	4.20E+04	1.30E+02
Mean	**4.00E+04**	**1.46E+02**
SD	**1.73E+03**	**4.71E+01**

	P. aeruginosa	*S. mutans*
1	5.10E+05	0.00E+00
2	4.90E+05	0.00E+00
3	2.80E+05	0.00E+00
Mean	**4.27E+05**	**0.00E+00**
SD	**1.27E+05**	**0.00E+00**

	S. aureus	*C. albicans*
1	3.20E+04	1.30E+04
2	3.90E+04	2.90E+03
3	2.90E+04	9.90E+03
Mean	**3.33E+04**	**3.93E+00**
SD	**5.13E+03**	**5.17E+03**

	P. aeruginosa	*C. albicans*
1	4.50E+05	0.00E+00
2	7.88E+05	0.00E+00
3	2.90E+05	0.00E+00
Mean	**5.09E+05**	**0.00E+00**
SD	**2.54E+05**	**0.00E+00**

Total CFU/ml counts for aerobic and anaerobic species, and *C. albicans* after exposure to CHX for 30 min, 1 h and 12 h (relation to figure 5.13, section 5.3.5.1):

	30 min CHX exposure		
	Aerobic spp.	Anaerobic spp.	*C. albicans*
1	4.61	4.79	2.68
2	4.70	4.89	2.89
3	4.88	4.95	3.04
Mean	**4.73**	**4.88**	**2.87**
SD	**0.14**	**0.08**	**0.18**
SEM	**0.08**	**0.05**	**0.10**

	1 h CHX exposure		
	Aerobic spp.	Anaerobic spp.	*C. albicans*
1	4.58	4.71	2.60
2	4.70	4.00	2.83
3	4.69	4.95	2.94
Mean	**4.66**	**4.55**	**2.79**
SD	**0.07**	**0.50**	**0.17**
SEM	**0.04**	**0.29**	**0.10**

	12 h CHX exposure		
	Aerobic spp.	Anaerobic spp.	*C. albicans*
1	3.28	3.48	2.60
2	3.26	3.65	2.45
3	3.48	3.08	2.48
Mean	**3.34**	**3.40**	**2.51**
SD	**0.12**	**0.29**	**0.08**
SEM	**0.07**	**0.17**	**0.05**

Total CFU/ml counts of CDFF biofilms after exposure to different salivary pHs (relation to figure 5.16, section 5.3.6.1):

	pH 4		
	Aerobic spp.	Anaerobic spp.	*C. albicans*
1	5.30E+04	3.00E+04	8.00E+02
2	2.70E+04	5.10E+04	5.00E+02
3	3.50E+04	8.10E+04	6.20E+02
Mean	**3.83E+04**	**5.40E+04**	**6.40E+02**
SD	**1.33E+04**	**2.56E+04**	**1.51E+02**
SEM	**7.70E+03**	**1.48E+04**	**8.73E+01**

	pH 5		
	Aerobic spp.	Anaerobic spp.	*C. albicans*
1	2.50E+04	3.80E+04	1.20E+03
2	3.00E+04	3.90E+04	8.20E+02
3	4.90E+04	3.40E+04	7.90E+02
Mean	**3.47E+04**	**3.70E+04**	**9.37E+02**
SD	**1.27E+04**	**2.65E+03**	**2.29E+02**
SEM	**7.32E+03**	**1.53E+03**	**1.32E+02**

	pH 6.5		
	Aerobic spp.	Anaerobic spp.	*C. albicans*
1	2.30E+04	7.70E+04	4.00E+02
2	1.30E+04	7.90E+04	4.20E+02
3	1.90E+04	6.10E+03	4.00E+02
Mean	**1.83E+04**	**5.40E+04**	**4.07E+02**
SD	**5.03E+03**	**4.15E+04**	**1.15E+01**
SEM	**2.91E+03**	**2.40E+04**	**6.67E+00**

CFU/ml counts of *S. aureus* inoculated CDFF biofilms after exposure to different salivary pHs:

pH 4		*S. aureus*	*C. albicans*	Aerobic sp.	Anaerobic sp.
	1	0.00E+00	9.10E+07	1.30E+08	8.00E+07
	2	0.00E+00	1.10E+08	8.90E+07	8.90E+07
	3	0.00E+00	1.20E+08	9.90E+07	9.00E+07
	4	0.00E+00	1.30E+08	1.40E+08	9.90E+07
	5	0.00E+00	9.60E+07	1.50E+08	1.30E+08
	6	0.00E+00	9.80E+07	1.20E+08	1.40E+08
	Mean	**0.00E+00**	**107500000**	**121333333.3**	**104666666.7**
	SD	**0.00E+00**	**15254507.53**	**23627667.4**	**24459490.32**
	SEM	**0.00E+00**	**6.23E+06**	**9.65E+06**	**9.99E+06**

pH 5		*S. aureus*	*C. albicans*	Aerobic sp.	Anaerobic sp.
	1	3.00E+07	7.30E+07	1.20E+08	1.40E+08
	2	3.30E+07	6.10E+07	9.80E+07	9.00E+07
	3	3.00E+07	7.70E+07	8.10E+07	1.10E+08
	4	4.50E+07	7.00E+07	9.00E+07	8.90E+07
	5	3.30E+07	4.90E+07	1.10E+08	9.00E+07
	6	3.00E+07	5.00E+07	1.10E+08	1.30E+08
	Mean	**3.35E+07**	**6.33E+07**	**1.02E+08**	**1.08E+08**
	SD	**5.82E+06**	**1.19E+07**	**1.45E+07**	**2.25E+07**
	SEM	**2.38E+06**	**4.88E+06**	**5.92E+06**	**9.17E+06**

pH 6.5		*S. aureus*	*C. albicans*	Aerobic sp.	Anaerobic sp.
	1	3.30E+09	3.00E+07	1.10E+10	1.90E+10
	2	3.60E+09	4.70E+07	1.20E+10	1.10E+10
	3	2.90E+09	5.90E+08	9.90E+09	1.20E+10
	4	3.40E+09	5.00E+06	1.10E+10	2.00E+10
	5	3.00E+09	3.80E+08	1.00E+09	1.10E+10
	6	3.10E+09	4.00E+08	8.90E+09	1.20E+10
	Mean	**3.22E+09**	**2.42E+08**	**8.97E+09**	**1.42E+10**
	SD	**2.64E+08**	**2.47E+08**	**4.05E+09**	**4.17E+09**
	SEM	**1.08E+08**	**1.01E+08**	**1.65E+09**	**1.70E+09**

CFU/ml counts of *P. aeruginosa* inoculated CDFF biofilms after exposure to different salivary pHs:

pH 4		*P. aeruginosa*	*C. albicans*	Aerobic spp.	Anaerobic spp.
	1	8.77E+00	7.48E+00	9.28E+00	9.94E+00
	2	8.80E+00	7.48E+00	9.11E+00	9.94E+00
	3	8.90E+00	7.48E+00	9.18E+00	9.98E+00
	4	8.78E+00	7.52E+00	9.26E+00	1.00E+01
	5	8.79E+00	7.54E+00	8.96E+00	1.01E+01
	6	8.76E+00	7.71E+00	9.11E+00	1.00E+01
	Mean	**8.80E+00**	**7.53E+00**	**9.15E+00**	**1.00E+01**
	SD	**4.98E-02**	**8.96E-02**	**1.15E-01**	**7.65E-02**
	SEM	**2.03E-02**	**3.66E-02**	**4.68E-02**	**3.12E-02**

pH 5		*P. aeruginosa*	*C. albicans*	Aerobic spp.	Anaerobic spp.
	1	0.00E+00	7.48E+00	7.91E+00	7.63E+00
	2	0.00E+00	7.57E+00	8.00E+00	8.11E+00
	3	0.00E+00	7.85E+00	8.00E+00	8.08E+00
	4	0.00E+00	7.56E+00	8.00E+00	8.00E+00
	5	0.00E+00	7.89E+00	8.59E+00	8.08E+00
	6	0.00E+00	7.89E+00	8.00E+00	7.83E+00
	Mean	**0.00E+00**	**7.70E+00**	**8.08E+00**	**7.95E+00**
	SD	**0.00E+00**	**1.89E-01**	**2.52E-01**	**1.89E-01**
	SEM	**0.00E+00**	**7.71E-02**	**1.03E-01**	**7.70E-02**

pH 6.5		*P. aeruginosa*	*C. albicans*	Aerobic spp.	Anaerobic spp.
	1	8.77E+00	7.48E+00	9.28E+00	9.94E+00
	2	8.80E+00	7.48E+00	9.11E+00	9.94E+00
	3	8.90E+00	7.48E+00	9.18E+00	9.98E+00
	4	8.78E+00	7.52E+00	9.26E+00	1.00E+01
	5	8.79E+00	7.54E+00	8.96E+00	1.01E+01
	6	8.76E+00	7.71E+00	9.11E+00	1.00E+01
	Mean	**8.80E+00**	**7.53E+00**	**9.15E+00**	**1.00E+01**
	SD	**4.98E-02**	**8.96E-02**	**1.15E-01**	**7.65E-02**
	SEM	**2.03E-02**	**3.66E-02**	**4.68E-02**	**3.12E-02**